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Chromosomal gains measured in cytology samples from women with abnormal cervical cancer screening results

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HIGHLIGHTS

- Chromosomal gains at multiple loci are associated with cervical disease severity.
- FISH was used to evaluate gains of four genomic loci (3q26, 5p15, 20q13 and cen7) simultaneously.
- FISH signal enumeration was achieved through an automated scanning process.

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ABSTRACT

Objective. Chromosomal gains at 3q26, 5p15 and 20q13 have been described in cervical precancer and cancer. We evaluated a novel fluorescence in situ hybridization (FISH) assay that detects gains at these three loci simultaneously as a possible biomarker for detecting cervical precancer.

Methods. Chromosomal copy numbers at 3q26, 5p15, 20q13 and the centromere of chromosome7 (cen7) in liquid-based cytology specimens from 168 women enrolled in the Biopsy Study were determined by FISH. The number of cells with ≥3 or ≥4 signals for a genomic locus was enumerated and diagnostic test performance measures were calculated using receiver operating characteristic (ROC) analyses. Sensitivity and specificity values were determined for the detection of CIN2+ and/or HSIL.

Results. The median number of cells with ≥3 signals increased with the severity of cervical lesion for each genomic locus (p-trend < 0.02 for each locus). ROC analysis for the number of cells with ≥3 signals resulted in area under the curve values of 0.70 (95% CI: 0.54–0.86), 0.67 (0.52–0.83), 0.67 (0.51–0.83) and 0.78 (0.64–0.92) for 3q26, 5p15, 20q13 and cen7, respectively, for the detection of CIN2+ and/or HSIL. Positivity for gains at multiple loci resulted in only slightly better test performance measures than those for the individual probes for four distinct combinations of probes.

Conclusions. Chromosomal gains at 3q26, 5p15, 20q13 and cen7 are associated with severity of cervical lesions. Further studies are required to quantify risk stratification of FISH assays for cervical cancer screening.

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Introduction

The implementation of cytology-based screening programs, or Pap smears, has greatly reduced the incidence and mortality of cervical cancer in the United States and other developed countries [1]. However, population-based screening programs have changed since the identification of carcinogenic types of human papillomavirus (HPV) as the necessary cause of cervical cancer. Several HPV-based tests have been developed that are more sensitive than Pap smears for identifying women with high-grade cervical disease [2,3] and therefore are superior to Pap tests in ruling out disease over extended follow-up periods [4]. However, because many HPV-positive women will clear their infection without developing cancer, HPV-based tests have limited specificity and a secondary test is needed to determine women who are HPV positive and at increased risk of progressing to high-grade disease.

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Chromosomal abnormalities such as gains, losses and rearrangements of whole or parts of chromosomes are a hallmark of cancer and have been described in many tumor types, including cervical cancer [5–10]. The most commonly described abnormality in cervical cancer is gain of the q26 region of chromosome 3 (3q26) where two genes important for carcinogenesis are located, the RNA component of human telomerase (*TERC*), and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*). Genomic gain of the 3q26 locus has been associated with the progression from high-grade cervical disease to cancer [11–13]. In addition, copy number gains of several genomic regions have been detected in earlier stages of cervical carcinogenesis and have been shown to increase with increasing severity of cervical lesion, making them potentially useful biomarkers [10,14,15]. Specifically, gains at 3q26 have been suggested to have clinical utility in identifying women who are likely to progress to high-grade cervical disease [16–19].

In addition to 3q26, gains at 5p15 have also been frequently described in cervical cancer ([5–8,20] and https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). Within this region, *TERC*, the other functional component of the telomerase enzyme is thought to be one potential oncogene. Another commonly reported chromosomal gain in cervical cancer is 20q13, where *EYA2* has been suggested to be the target oncogene ([5,20–22] and https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). Furthermore, gains of 20q13 are detectable in precancerous lesions, suggesting potential utility in screening for cervical cancer [23]. Including multiple markers may improve the diagnostic measures of an assay, however, data on these and other chromosomal markers and marker combinations are very limited.

In the present analysis, we examined the feasibility of using an automated scanning method to enumerate signals from a fluorescence in situ hybridization (FISH) assay that simultaneously detects gains at 3q26, 5p15 and 20q13 in cytology specimens from women referred to colposcopy following an abnormal screening result. We evaluated the frequency of chromosomal abnormalities in disease categories and explored the diagnostic accuracy of genomic gains for the detection of high-grade cervical disease.

Materials and methods

Study population

Samples from 168 women enrolled in the Biopsy Study between 2009 and 2010 were used in the current analysis. Women in the Biopsy Study were referred to colposcopy at the University of Oklahoma Health Sciences Center (OUHSC) following an abnormal cervical cancer screening result. Details regarding the study design and inclusion/exclusion criteria have been reported elsewhere [24]. Briefly, women were excluded if they were less than 18 years of age, pregnant at the time of their visit, or previously treated with chemotherapy or radiation for any cancer or for cervical intraepithelial neoplasia (CIN). Written informed consent was obtained from all women enrolled in the study. Institutional Review Board approval was provided by OUHSC and the US National Cancer Institute.

Colposcopy and specimen collection

Details regarding sample collection have been previously described [24]. Prior to colposcopy, a cervical cytology sample was obtained using a Papette broom device and transferred directly into PreservCyt solution (Cytyc Corp). The cytology specimen was used for ThinPrep liquid-based cytology and HPV DNA testing using the Linear Array HPV Genotyping Test (Roche Molecular Diagnostics). Up to four biopsies were taken from distinct acetowhite areas or large heterogeneous lesions extending over two quadrants. As per standard practice, all CIN3 and most CIN2 were treated by loop electrosurgical excision procedure (LEEP) of the transformation zone. Analyses presented here were based on the worst histologic diagnosis including biopsy diagnoses and LEEP outcomes.

Fluorescence in situ hybridization (FISH)

Upon receipt of specimens (0.5–1.0 ml per specimen) at Cancer Genetics Inc., cell pellets were resuspended in Carnoy's fixative (methanol: acetic acid, 3:1), and aliquots dropped onto 12 mm diameter circles on slides. Slides were aged (2 h at 37 °C) and treated with pepsin (Sigma-Aldrich, 0.5 mg/ml, Cat# P7012) for 10 min at 37 °C. After a 1× phosphate-buffered saline (PBS) wash, cells were fixed in 1% formaldehyde and washed again in 1× PBS, all at room temperature (RT). Slides were dehydrated (70%, 85%, and 100% alcohol) for 1 min each at RT and air dried. A four-color DNA-FISH probe [3q26 (904 kbp, red), 5p15 (601 kbp, green), 20q13 (493 kbp, gold), and cen7 (70 kbp, aqua), Cancer Genetics, Inc.] was applied (3 μl) to each circle, followed by co-denaturation for 3 min at 80 °C and hybridization for 48 h at 37 °C. Slides were then washed briefly in 2× saline sodium citrate (SSC) (pH 7.0), two times in 2× SSC/0.1% Tween-20 at 45 °C for 5 min each, and finally rinsed in distilled water. After air drying, DAPI/antifade (1:10, Vector) was applied to the hybridized area.

Signal enumeration

Calibration of the instrument software (Metafer4, Metasystems) for automated scanning was done using an independent set of 10 specimens with a variety of cellular and hybridization characteristics to ensure that each nuclei was counted and proper signal enumeration for each FISH probe was achieved. Initially, we evaluated the feasibility of using the multi-colored FISH assay on cervical cytology specimens, enumerating signal counts in each cell manually as previously described [13,16,17]. We then used an automated scanning method to enumerate signals, as manual scoring of FISH signals would not be feasible on a large scale. Due to the previous manual scoring of samples, volumes were limited for a number of samples for the automated scanning process, leading to sample dropout. All signal counts for nuclei with greater than 2 signals were manually confirmed. All nuclei within the 12 mm diameter circle were scanned, imaged and signals enumerated.

Final analytic population

Samples were excluded (n = 34) from the final analysis based on the following criteria: low cellularity (<100 cells in the sample; n = 21), poor sample quality (i.e., excessive cellular debris; n = 2) and weak or uneven signals for all probe colors (n = 11), leaving a final analytic population of 134 samples. Of these, 7 had uneven signals for one FISH color which was excluded from analyses (aqua/cen7, n = 6; red/3q26, n = 1). Three samples had two consecutive enumerated slides which were combined for the final analysis.

Disease categories were based on a composite endpoint using histology and cytology results, as previously described [25]: a) <CIN2 histology and normal cytology (NILM) (n = 32); b) <CIN2 histology and LSIL or ASC-US cytology (n = 45); c) CIN2 histology or <CIN2 histology with HSIL or ASC-H cytology (n = 34); and d) CIN3+ (CIN3, adenocarcinoma in situ (AIS) or cancer, regardless of cytology; n = 23). Two women were missing cytology results and were categorized based on available histology diagnoses. For analyses with dichotomized outcomes, we combined groups a) and b) from above into <CIN2 and <HSIL and c) and d) into CIN2+ and/or HSIL. Similar results were observed for endpoints based on histology or cytology alone.

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Statistical analyses

Differences in baseline characteristics were determined by one-way analysis of variance (ANOVA) and chi-square statistics where appropriate. Analyses were based on the number of cells with gain of a locus, independent of the other loci. Our main analysis defined gains as the number of nuclei that contained 3 or more (≥3) signals of a color, as previously described [17]; however, secondary analyses considered the number of nuclei with ≥4 signals. Since tetraploid cells may represent normal dividing cells rather than dysplastic cells and their significance remains unclear [17], we conducted sensitivity analyses excluding tetraploid cells from the abnormal cell counts. To account for potential misclassification we used two sets of patterns to define tetraploid cells. One definition was more stringent and only one probe was allowed to deviate from the strict tetraploid definition (i.e., n = 4 for all loci; patterns of 4-4-4-4, 4-4-4-3, or 4-4-4-5 were considered tetraploid in this definition). The second definition was slightly more liberal and included the previous patterns as well as 4-4-3-3. Analyses that excluded these sets of cells, using either definition, did not alter our results. Additionally, we used signals from the cen7 probe to account for ploidy of cells in two ways. In the first approach, we enumerated the number of cells with relative gains compared with the cen7 probe, as previously described [17]. The second approach enumerated the number of signals for the 3q26, 5p15 and 20q13 probes in cells with <3 signals for cen7 (i.e. diploid cells). Differences in distributions and trends in the number of cells with chromosomal abnormalities across disease categories were determined using Kruskal–Wallis tests and nptrend, respectively. Receiver operating characteristic (ROC) curve analyses were performed using number of cells with ≥3 or ≥4 signals of each locus, comparing low-grade (<CIN2 and <HSIL) to high-grade (CIN2+ and/or HSIL) disease. The maximum Youden's Index (sensitivity + specificity-1) was calculated from the ROC curves to identify a cut point for each locus. Sensitivity and specificity values were calculated based on these cut points for individual and each combination of probes, using CIN2+ and/or HSIL as the outcome. For comparison, we evaluated p16/Ki-67 cytology as a marker for high-grade disease based on previously described methods [24]. All statistical analyses were performed using STATA version 11.2 (STATA Corporation, College Station, TX).

Results

Study population

Basic demographics for the study population are presented in Table 1. The average age for women diagnosed with <CIN2 and NILM, <CIN2 and LSIL, CIN2 and/or HSIL and CIN3+ was 29.4, 28.6, 25.2 and 26.9 years, respectively, and did not differ significantly between groups (p = 0.08). The proportion of samples with different levels of cellularity, defined as the number of cells counted, did not differ by disease category (p = 0.95, p = 0.60, p = 0.50, for samples with ≥200, ≥500, and ≥1000 cells, respectively).

Chromosomal abnormalities by cervical disease category

The distribution of the number of cells with ≥3 or ≥4 signals of a locus within each disease category is presented in Fig. 1. The cellularity of cytology samples is an important factor to consider when determining the level of chromosomal abnormalities, as these would generally be rare events and, depending on the lesion size, may be difficult to detect when small numbers of cells are evaluated. When we compared the distribution of the number of cells with gains of an individual locus in the overall study population (Fig. 1A and C) with the distribution in samples with high cellularity (≥1000 cells counted; Fig. 1B and D) we noted that trends for increasing numbers of cells with abnormalities could be detected only in samples with more than 1000 cells counted. Similar patterns were seen for the number of cells with ≥3 and ≥4 signals of an individual locus. Therefore, subsequent analyses were restricted to samples with ≥1000 cells (n = 48, 36% of all specimens tested).

The median number of cells with ≥3 signals of each locus increased with increasing severity of cervical lesion (Table 2). The median number of cells with ≥3 signals of 3q26 increased from 25 in <CIN2 and NILM to 47 in CIN3+ samples (p-trend = 0.02; Table 2). Similar patterns were seen for 5p15 (p-trend = 0.01), 20q13 (p-trend = 0.01) and cen7 (p-trend = 0.01) and for the number of cells with ≥4 signals (Table 2). Among women with <CIN2 and NILM, we observed reduced numbers of cells with chromosomal gains in HPV-negative women compared with HPV-positive women for all loci, although the differences were not statistically significant (data not shown).

Detection of CIN2+ and/or HSIL by individual and combinations of probes

ROC curve analysis was used to evaluate the ability of the number of cells with ≥3 or ≥4 signals to distinguish between women with high-grade disease (CIN2+ and/or HSIL) and those with low grade disease (<CIN2 and <HSIL) for each genomic locus. The number of cells with ≥3 signals for cen7 had the greatest area under the curve (AUC = 0.78, [95% CI: 0.64–0.92]), followed by 3q26 (0.70 [0.54–0.86]), 5p15 (0.67 [0.52–0.83]) and 20q13 (0.67 [0.51–0.83]; Table 3). ROC plots for the number of cells with ≥3 signals for individual probes are shown in Fig. 2 (lines). The AUC values for cen7 and 3q26 remained

Table 1
Demographics of study population.

	<CIN2 and NILM (n = 32)	<CIN2 and LSIL (n = 45)[a]	CIN2 and/or HSIL (n = 34)	CIN3+ (n = 23)
Age, mean (SD)	29.4 (8.7)	28.6 (7.3)	25.2 (5.6)	26.9 (6.2)
HPV status, n (%)				
HPV16+[b]	3 (11.1)	4 (10.8)	11 (34.4)	12 (52.2)
Other HR-HPV+[c]	16 (59.3)	30 (81.1)	20 (62.5)	11 (47.8)
LR-HPV+[d]	5 (15.6)	8 (17.8)	2 (5.9)	0 (0)
Negative	8 (29.6)	3 (8.1)	1 (3.1)	0 (0)
Number of p16 + samples, n (%)	6 (18.8)	19 (42.2)	27 (79.4)	21 (91.3)
Number of cells counted, n (%)				
≥100	32 (100)	45 (100)	34 (100)	23 (100)
≥200	29 (90.6)	41 (91.1)	31 (91.2)	20 (87.0)
≥500	21 (65.6)	27 (60.0)	21 (61.8)	11 (47.8)
≥1000	11 (34.4)	20 (44.4)	10 (29.4)	7 (30.4)

CIN: cervical intraepithelial neoplasia. NILM: negative for intraepithelial lesion or malignancy. LSIL: low-grade squamous intraepithelial lesion. HSIL: high-grade squamous intraepithelial lesion.

[a] Includes atypical cells of undetermined significance (ASC-US).

[b] Positive for HPV16.

[c] Positive for high-risk (HR)-HPV types other than HPV16 (HPV18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59).

[d] Positive for low-risk (LR)-HPV types only (includes all other HPV types on the Linear Array not listed previously).

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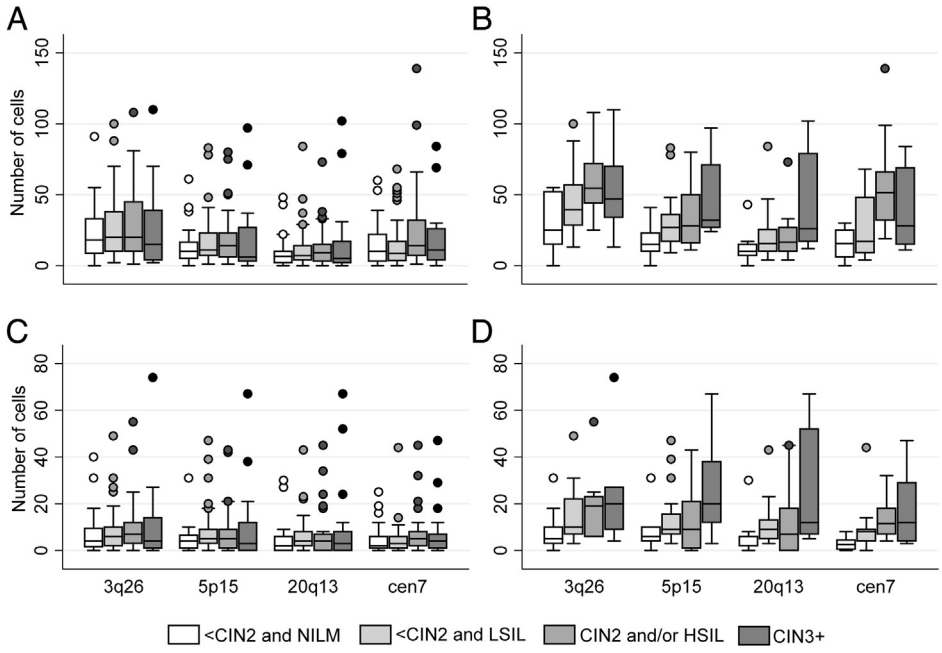

Fig. 1. Distribution of number of cells with 3 or more signals of individual FISH probes in each disease category for (A) all samples (n = 134), (B) samples with more than 1000 cells (n = 48) and the distribution of number of cells with 4 or more signals of individual FISH probes in each disease category for (C) all samples (n = 134) and (D) samples with more than 1000 cells (n = 48).

statistically significant for the number of cells with ≥4 signals, whereas 5p15 and 20q13 did not (Table 3). In addition, we observed similar results when using the cen7 probe to define ploidy of a cell (Table 3). This was true for the number of cells with relative gains compared to the cen7 probe as well as the number of cells with gains in diploid cells (defined by the presence of <3 signals for the cen7 probe).

For each probe, a cut point for the number of cells with ≥3 signals was chosen based on the maximum Youden's Index (YI) and the sensitivity and specificity of each individual probe and combinations of probes for the detection of CIN2+ and/or HSIL were determined. The cut points for each probe were ≥39 cells for 3q26, ≥24 cells for 5p15, ≥15 cells for 20q13, and ≥23 cells for cen7. Using these cut points, 3q26 had the highest sensitivity (82%) for the detection of CIN2+ and/or HSIL, whereas 5p15, 20q13 and cen7 had a sensitivity of 77% (Table 4). The corresponding specificity estimates were 53%, 57%, 57%, and 65%, respectively. The sensitivity and specificity values for each combination of probes are overlaid onto the ROC curve for individual probes in Fig. 2 (circles). Four combinations showed better diagnostic performance measures compared to the individual probes (i.e., they were located north-west of the ROC lines for the individual probes): 5p15 OR cen7; 20q13 AND cen7; 5p15 AND cen7; and 3q26 AND 20q13 AND cen7. The sensitivity and specificity of these combinations are presented in Table 4; estimates for all probe combinations are shown in Supplemental Table 1. p16/Ki-67 cytology results are shown in Table 4 and Fig. 2 (square) as a benchmark for comparison. In this population, the YI for p16/Ki-67 was higher than those of all individual FISH probes and their combinations.

Discussion

We evaluated the feasibility of using a four-color FISH assay to detect genomic gains in cytology samples from women referred to colposcopy due to an abnormal Pap screening result. Specifically, we determined the diagnostic accuracy of the number of cells with

Table 2
Chromosomal abnormalities by disease category.

	<CIN2 and NLIM (n = 11)	<CIN2 and LSIL (n = 20)[a]	CIN2 and/or HSIL (n = 10)	CIN3+ (n = 7)	p-Trend
	Median (range)				
Number of cells counted	1555 (1086–2503)	2261 (1011–9285)	1559 (1045–4240)	1436 (1000–7874)	–
Tetraploid (8)[b]	1 (0–4)	2 (0–21)	3 (0–30)	3 (0–27)	0.40
Tetraploid (14)[c]	1 (0–4)	2 (0–12)	3 (0–27)	3 (0–23)	0.49
≥3 signals					
3q26	25 (0–55)	40 (13–100)	55 (25–108)	47 (13–110)	0.02
5p15	15 (0–41)	27 (9–83)	28 (11–80)	32 (24–97)	0.01
20q13	10 (0–43)	16 (4–84)	17 (4–73)	26 (12–102)	0.01
cen7	16 (0–30)	17 (4–68)	52 (19–139)	28 (11–84)	0.01
≥4 signals					
3q26	5 (0–31)	10 (3–49)	19 (6–55)	20 (4–74)	0.01
5p15	6 (0–31)	9 (3–47)	9 (0–43)	20 (3–67)	0.02
20q13	6 (0–30)	9 (0–43)	7 (0–45)	12 (5–67)	0.03
cen7	3 (0–8)	8 (0–44)	12 (4–32)	12 (3–47)	0.001

CIN: cervical intraepithelial neoplasia. NILM: negative for intraepithelial lesion or malignancy. LSIL: low-grade squamous intraepithelial lesion. HSIL: high-grade squamous intraepithelial lesion. Data presented are based on samples with ≥1000 cells counted.

[a] Includes atypical cells of undetermined significance (ASC-US).

[b] Tetraploidy was defined by 8 classifiers fitting the patterns of 4-4-4-4, 4-4-4-3 or 4-4-4-5.

[c] Tetraploidy was defined by 14 classifiers fitting the patterns of 4-4-4-4, 4-4-4-3, 4-4-4-5, or 4-4-3-3.

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Table 3
Receiver operating characteristics (ROC) for individual probes.

	Overall	Gains relative to cen7[a]	Gains in diploid cells[b]
	AUC (95% CI)		
≥3 signals			
3q26	0.70 (0.54–0.86)	0.70 (0.54–0.85)	0.69 (0.53–0.84)
5p15	0.67 (0.52–0.83)	0.69 (0.54–0.85)	0.70 (0.55–0.84)
20q13	0.67 (0.51–0.83)	0.71 (0.55–0.86)	0.71 (0.56–0.86)
cen7	0.78 (0.64–0.92)	–	–
≥4 signals			
3q26	0.67 (0.51–0.83)	0.67 (0.52–0.83)	0.65 (0.48–0.81)
5p15	0.62 (0.43–0.81)	0.59 (0.48–0.81)	0.60 (0.42–0.78)
20q13	0.59 (0.40–0.78)	0.57 (0.38–0.75)	0.56 (0.38–0.75)
cen7	0.76 (0.61–0.91)	–	–

AUC: area under the curve. CI: confidence interval. Outcome: CIN2+ and/or HSIL vs. <CIN2 and <HSIL.
[a] Gains were defined as signal counts for FISH probes that were greater than signal counts for cen7 in the same cell.
[b] Gains were determined only in cells with <3 signals for the cen7 probe.

Table 4
Sensitivity and specificity of individual probes and selected probe combinations for the detection of CIN2+ and/or HSIL.

	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Youden's Index[a]
Individual probes			
3q26	82.4 (56.6, 96.2)	53.3 (34.3, 71.7)	0.357
5p15	76.5 (50.1, 93.2)	56.7 (37.4, 74.5)	0.332
20q13	76.5 (50.1, 93.2)	56.7 (37.4, 74.5)	0.332
cen7	76.5 (50.1, 93.2)	65.4 (44.3, 82.8)	0.419
Combinations			
5p15 OR cen7	94.1 (71.3, 99.9)	40.0 (22.7, 59.4)	0.341
20q13 AND cen7	58.8 (32.9, 81.6)	92.6 (75.7, 99.1)	0.514
5p15 AND cen7	53.0 (27.8, 77.0)	83.3 (65.3, 94.4)	0.363
3q26 AND 20q13 AND cen7	47.1 (23.0, 72.2)	86.7 (69.3, 96.2)	0.338
Positive for any probe	100.0 (80.5, 100.0)	23.3 (9.90, 42.3)	0.233
Positive for all probes	29.4 (10.3, 56.0)	90.0 (73.5, 97.9)	0.194
p16/Ki-67	82.4 (56.6, 96.2)	67.7 (48.6, 83.3)	0.501

CI: confidence interval. Cut points were determined by Youden's Index for the number of cells with ≥3 signals: 3q26: ≥39 cells; 5p15: ≥24 cells; 20q13: ≥15 cells; and cen7: ≥23 cells. Outcome: CIN2+ and/or HSIL vs. <CIN2 and <HSIL.
[a] Youden's Index = sensitivity + specificity-1.

gains at 3q26, 5p15, 20q13 and cen7 for detecting high-grade cervical disease. As a first important observation, we noted that at least 1000 cells need to be evaluated in a specimen to allow for discrimination between cervical disease categories. In this subset of samples, we observed an association between the number of cells with gains (≥3 or ≥4 signals for a locus) and severity of cervical disease for each of the genomic loci examined, suggesting the number of cells with gains at an individual locus may be able to distinguish between women with high-grade (CIN2+ and/or HSIL) and low-grade disease (<CIN2 and <HSIL). Considering gains at more than one locus showed slightly, but not significantly, improved performance for four combinations.

Previous studies of genomic imbalance during cervical carcinogenesis have focused on gains of 3q26 with reported sensitivity values from 52% for the detection of HSIL to 78% for the detection of CIN2+, with corresponding specificity values of 97% and 84%, respectively [16,26]. Although it is difficult to compare results across cytogenetic studies due to differences in cut points and outcomes, our data are generally consistent with previous results. A cut point of ≥39 cells with ≥3 signals of 3q26 resulted in a sensitivity of 82% and specificity of 53% for the detection of CIN2+ and/or HSIL in samples where at least 1000 cells could be evaluated, consistent with another study where automated scanning was also employed [18,26]. Further studies are required to determine the optimal sample volume and cellularity for this type of assay.

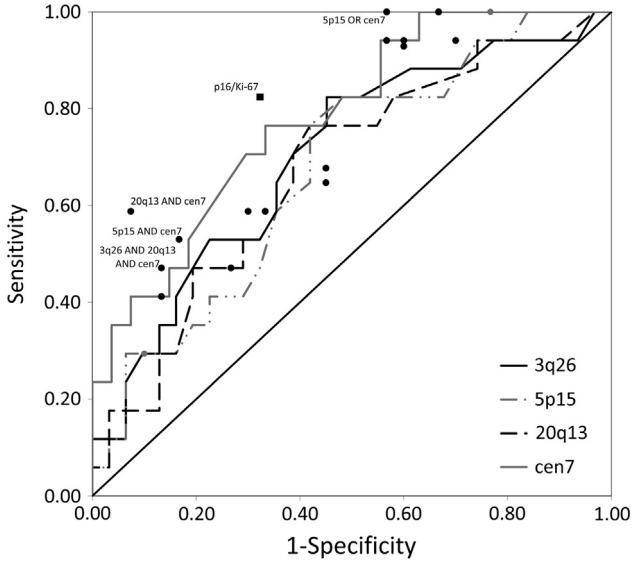

Fig. 2. Receiver operating characteristic (ROC) analysis of the number of cells with ≥3 signals for each of the individual probes are shown by solid or dashed lines (3q26: solid black; 5p15: dashed grey; 20q13: dashed black and cen7: solid black). The sensitivity and 1-specificity of each combination of probes and for any or all probes are shown by black and gray circles, respectively and p16 is indicated by the black square. Combinations that are outside the ROC lines for individual probes are labeled.

The number of cells with gain at one locus was highly correlated with the number of cells with gain at any of the other loci, with correlation coefficients for two loci that ranged from 0.42 to 0.78 for ≥3 signals and from 0.78 to 0.86 for ≥4 signals. The two probes with the lowest correlation coefficient were cen7 and 20q13 ($r = 0.42$). This combination showed the greatest improvement of clinical performance compared to the individual probes, supporting the notion that increased clinical utility only occurs when markers are less correlated. Continued evaluation of these markers is needed to identify optimal probe combinations with the best discriminatory power.

The results obtained in the current study for the cen7 probe suggest that gains at this locus are associated with cervical disease stage. Some array-comparative genomic hybridization (aCGH) analyses of cervical cancer and precancerous tissues have shown gains of the cen7 region ([23,27] and https://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). In addition, evidence from functional studies suggest that expression of E6/E7 can lead to centrosome duplication errors at precancerous stages, which is a contributing factor to general genomic instability and can be detected early in carcinogenesis [28–30]. Together with the data from this study, this suggests that the cen7 region may not be an appropriate control for FISH assays to detect HPV-related disease.

The distribution of number of cells with gains at the four loci studied showed differences in the stage of carcinogenesis where the gains occurred (Fig. 1). Gains of the 3q26, cen7 or 5p15 loci appeared at earlier stages of cervical disease. Specifically, differences in the number of cells with gains were observed at the transition from <CIN2 and <HSIL to CIN2+ and/or HSIL and the frequency remained high in CIN3+. In contrast, gains at the 20q13 locus became apparent only in more severe diagnoses (CIN3+). This suggests potential differences in biological roles for the amplification of these loci during cervical carcinogenesis; however, this requires further evaluation. In addition, differences in chromosomal abnormalities at specific loci based on HPV-type infection have been suggested [31], however, we did not have sufficient sample size to evaluate this in our study.

In conclusion, we evaluated a FISH assay that detects chromosomal abnormalities at four genomic loci simultaneously in cervical cytology specimens. Our data suggest that gains at 3q26, 5p15, and 20q13 are associated with severity of cervical disease. In addition, we observed

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associations of gains at cen7 with high-grade disease, as has been suggested in some aCGH studies. Furthermore, we demonstrated the ability to use an automated scanning method to enumerate FISH signals in cervical cytology specimens. Additional studies are required to evaluate the value of FISH markers as a potential triage test for HPV-positive women.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2013.06.005.

Conflict of interest statement

JH and LC are employees of Cancer Genetics, Inc.

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