Research Article

Cancer Prevention Research

A Pilot Study of Low-Cost, High-Resolution Microendoscopy as a Tool for Identifying Women with Cervical Precancer

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Abstract

Cervical cancer remains one of the leading causes of death among women in developing countries. Without resources to support Pap smear cytology and colposcopy, cost-effective approaches which enable single-visit "see-and-treat" protocols offer the potential to reduce morbidity and mortality due to this preventable disease. We carried out a pilot clinical study in Shanxi province, China, to evaluate a low-cost, high-resolution microendoscope (HRME) imaging system which enables evaluation of epithelial cell morphology *in vivo*. HRME images were obtained at discrete sites on the cervix in 174 women, in addition to visual inspection with acetic acid (VIA) and colposcopic examination. Of 69 sites appearing abnormal on colposcopy, only 12 showed high-grade disease (CIN2+) on pathology. Quantification of the nuclear-to-cytoplasm ratio by HRME enabled an *ad hoc* threshold to be defined, which correctly classified all 12 sites as abnormal, whilst classifying 38 of the remaining 57 pathology normal sites as normal. All patients with biopsy confirmed high-grade disease also tested positive for high-risk human papilloma virus (HPV) DNA and were classified as abnormal by HRME. Among the remaining patients who tested positive for HPV but were either normal by colposcopy or showed <CIN2 on pathology, only 6 of 32 (18.8%) were classified as abnormal by HRME.

Visual examination techniques for cervical cancer screening may overestimate the prevalence of precancerous lesions, leading to unnecessary treatment, expense, and patient stress. The results of this study suggest that evaluation of suspicious lesions by HRME may assist in ruling out immediate cryotherapy, thus increasing the efficiency of current see-and-treat programs. *Cancer Prev Res; 1–7. 2012 AACR.*

Introduction

Cervical cancer is the third most common cancer amongst women worldwide; an estimated 530,000 new cases occurred and 275,000 women died from this treatable disease in 2008 (1). More than 80% of these cases occur in developing countries (2), which lack the resources and expertise required to maintain the regular screening programs used in industrialized nations. In low-resource settings, techniques such as visual inspection with acetic acid (VIA) or with Lugol's iodine (VILI) have been proposed as cost-effective alternatives to traditional Pap/cytology programs for cervical cancer screening. In several large clinical studies, VIA has shown clinical sensitivity ranging from 41% to 92%, approaching that of standard colposcopy (3–5). Such methods have enabled "see-and-treat" programs to be implemented, using cryotherapy for immediate ablation of any lesion appearing abnormal by VIA. The ability to deliver diagnostic and therapeutic services in a single clinic visit is a key factor in reducing patient loss to follow-up after a positive screening test, which can amount to 15% of patients or more when a multi-visit screening approach is required (6).

While the sensitivity of VIA/VILI is quite good, some studies have reported specificity figures as low as 49% (7). Poor specificity, along with the potential for loss to follow-up, has raised concerns that see-and-treat programs using VIA/VILI may lead to overtreatment of many benign conditions which do not represent significant cervical cancer risk and will resolve without intervention. Overtreatment raises the expense of these programs and may cause unnecessary concern for the patient. In the absence of colposcopically guided biopsy collection with histopathology processing and review, new approaches are required to identify those patients who genuinely require treatment. Optical imaging and spectroscopy techniques have been

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shown to detect alterations in tissue morphology and biochemistry within epithelial and stromal tissue components, associated with the onset and progression of cervical neoplasia (8–10). Macroscopic optical imaging (similar to standard colposcopy) examines an entire organ surface under white light, narrow-band illumination, and/or under conditions required for fluorescence excitation. In contrast, microscopic optical imaging involves placement of the tip of a small fiber optic probe directly onto the cervical epithelium, enabling individual cells to be visualized *in vivo* (11). By using exogenous contrast agents such as acetic acid or acriflavine/proflavine, morphologic features used by pathologists such as nuclear crowding, pleomorphism, and nuclear-to-cytoplasm ratio can be assessed *in vivo* and in real-time (12, 13).

We describe here the results of a pilot clinical study using a recently developed low-cost microscopic imaging system, termed the high-resolution microendoscope (HRME; ref. 14), for evaluation of cervical lesions appearing abnormal under VIA or colposcopy. Our long-term hypothesis is that HRME imaging can improve the specificity of early detection of cervical cancer and its precursors by ruling out many of the visually or colposcopically apparent lesions that are actually benign. Here, we set out to establish whether HRME can identify cervical lesions which do not require treatment in patients initially screened by VIA or human papilloma virus (HPV) testing. Reducing the numbers of lesions treated unnecessarily following visual examination or colposcopy would clearly benefit the patient while also lowering the overall costs of see-and-treat programs in the settings where their impact is greatest.

Materials and Methods

Study population

Institutional Review Boards at each of the clinical sites and academic institutions involved, including Johns Hopkins University (Baltimore, MD), the Chinese Academy of Medical Sciences (Beijing, China), and Rice University (Houston, TX), approved the study. A total of 2,500 women older than 18 years living in Xiangyuan County, China, underwent initial VIA/VILI examination as part of their involvement in the national cancer screening program. On the basis of prior data from the program, it was estimated that 4% of this cohort would receive a positive VIA/VILI test result (Fig. 1A). As a nested pilot study to evaluate HRME, patients with an abnormal VIA/VILI examination ($n = 63$) were invited to participate in this study, along with a random selection of patients with a normal VIA/VILI examination ($n = 111$). The imaging portion of the study was completed in August 2010. The mean, median, interquartile range, and total range of age for the 174 subjects were 41, 41, 36–45, and 29–58 years, respectively.

Study procedures

The 174 participants were offered transportation from their local villages to the Xiangyuan County Maternal and Child Health Hospital of Shanxi, where the study was conducted (Fig. 1B). Each participant had an initial one-on-one interview with a trained health worker, where basic demographic information (age, education), past medical/gynecologic history, family history, HPV knowledge assessment, and other behavioral factors (transportation method, access to medical care) were collected. The interview was conducted in the local Chinese dialect.

After the initial interview, a clinician collected a cervical exfoliated cellular (Pap) sample for HPV testing using a Qiagen cervical sampler brush (Qiagen) and a Whatman indicating FTA elute cartridge (GE Healthcare). Next, a second VIA examination was conducted, this time by the study clinician, and the location of any abnormal appearing lesion was recorded. Each patient then immediately underwent a standard colposcopic examination, conducted by the

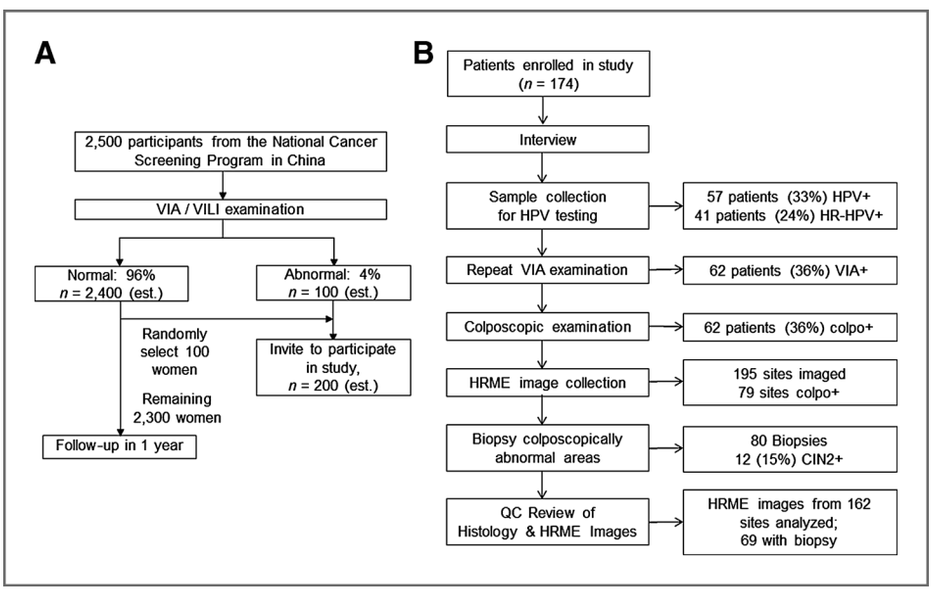

Figure 1. A, design of the prescreening phase of the study. The study was designed to accrue an estimated 100 patients with cervical precancerous lesions and 100 normal patients. B, design of the HRME evaluation study. A total of 174 patients were recruited to the study from the prescreening phase.

same clinician, again with the location of any abnormal appearing lesions recorded. Proflavine solution was then topically applied to the cervix (Fig. 2B). HRME imaging immediately followed, with gentle placement of the fiber optic probe tip directly onto the site of interest (Fig. 2C). All colposcopically abnormal lesions were imaged with HRME, in addition to one colposcopically normal site per patient. Figure 2D shows the HRME image obtained with the probe placed at the site indicated in Fig. 2C. The imaged field-of-view is a 720-μm wide *en face* view, corresponding to the area of tissue beneath the diameter of the fiber optic probe tip. A biopsy was taken at each colposcopically abnormal site and immediately placed in fixative for standard histopathology processing. Colposcopic identification of lesions, HRME probe placement and imaging, and biopsy collection were all conducted within a single examination, by the same clinician, in an attempt to co-register measurement sites. Slides with hematoxylin and eosin (H&E)-stained tissue sections were prepared at the Xiangyuan County Women's and Children's Hospital, China, and read by the study pathologist. The entire imaging portion of the study was typically completed in 5 minutes.

HPV testing

Cervical specimens were also tested for 37 HPV genotypes using the Roche HPV linear array test (Roche Diagnostics) at Johns Hopkins University, as previously described (15). Patients were considered positive for high-risk HPV (HR-HPV) if their test was positive for any of the high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and/or 68).

Colposcopy

Biopsies were obtained from all lesions which were visually apparent under colposcopy. Specimens were processed for histopathology and graded by the study pathologist as normal, cervical intraepithelial neoplasia grade 1 (CIN1), grade 2 (CIN2), grade 3 (CIN3), or cancer [squamous cell carcinoma (SCC)].

High-resolution microendoscopy

Immediately before imaging with the HRME, topical proflavine solution (0.01% w/v in sterile PBS) was applied to the cervix with a Q-tip, similar to application of acetic acid in standard colposcopy. Proflavine is a fluorescent contrast agent which selectively stains cell nuclei. The dye strongly absorbs blue light with an optical absorption peak at a wavelength of 445 nm, producing green fluorescence emission with a peak wavelength of 515 nm. Once the dye was applied, patients immediately underwent HRME imaging; no additional incubation period was necessary. Technical details on the HRME design and assembly have been described previously by Pierce and colleagues (14). Briefly, the system operates as a compact, battery-powered fluorescence microscope, coupled to a flexible fiber optic imaging probe, 1 mm in diameter (Fig. 2A). Blue light provided by a light-emitting diode (LED) at a wavelength of 455 nm is delivered from the HRME unit, through the fiber optic

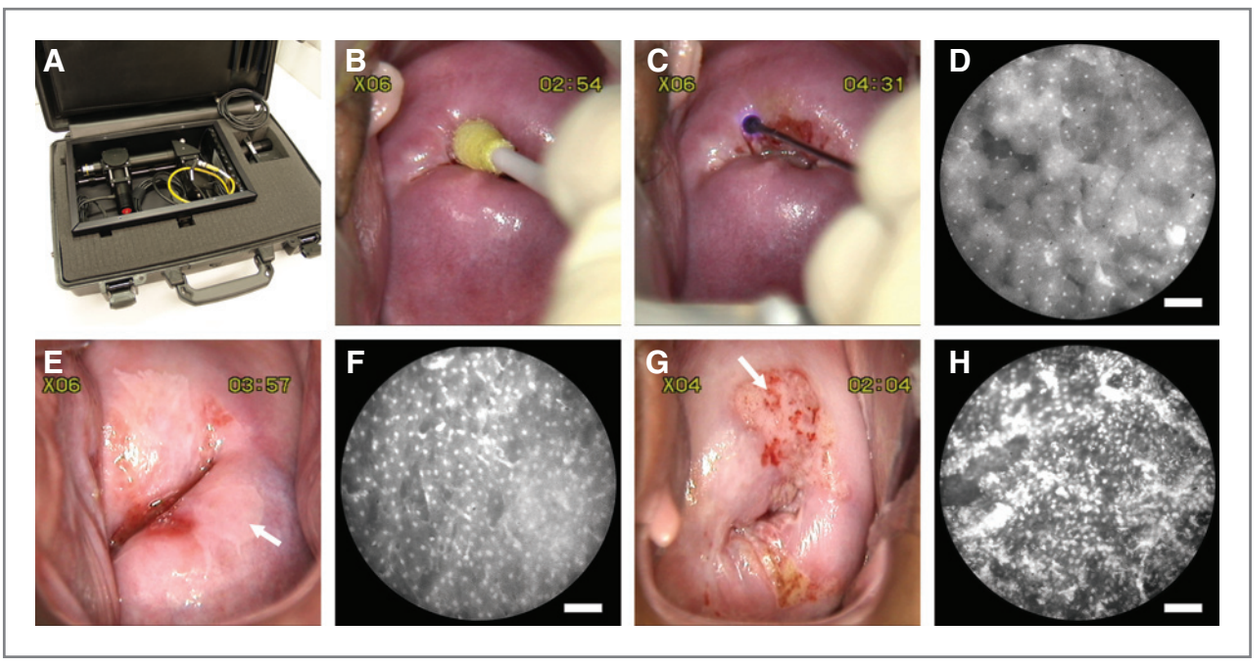

Figure 2. A, photograph of the HRME system. B–D, demonstration of the imaging procedure. B, proflavine is applied using a cotton-tipped swab. C, the fiber optic probe is placed in gentle contact with the cervix. D, a high-resolution image is displayed on a laptop computer in real-time. E, colposcopic view of an acetowhite cervical lesion at 5 o'clock (arrow). F, resulting HRME image. Histologic diagnosis of this site was normal, consistent with the HRME image which shows small, evenly spaced nuclei. G, colposcopic view of another acetowhite cervical lesion at 12 o'clock (arrow). H, resulting HRME image. Histologic diagnosis of this site was CIN3, consistent with the HRME image which shows large, crowded, pleomorphic nuclei. HRME image scale bars = 100 μm.

probe, to the tissue surface. Fluorescence from proflavine-stained epithelium is transmitted back through the same probe to the HRME unit and imaged onto a CCD camera. Images are displayed on a laptop computer screen in real-time at 12 frames per second. The fiber optic probe used in the current study provides a 0.72-mm diameter field-of-view with 4.4-μm resolution. After imaging each patient, the fiber optic probe was disinfected with Cidex OPA, according to the manufacturer's instructions (Johnson & Johnson).

Data analysis

HRME images of proflavine-stained tissue primarily reveal cell nuclei as discrete bright dots on a dark background. To quantify parameters related to nuclear morphology, image analysis software was written (Matlab, R2010b) to automatically identify nuclei, based on their characteristic size, shape, and brightness in HRME images. Raw grayscale images were subject to an adaptive histogram equalization algorithm to optimize contrast across the entire field-of-view, followed by a 2-dimensional median filter to reduce the appearance of the fiber optic probe's internal structure. A binary image was then generated by applying a single user-defined intensity threshold to a user-selected region of interest in each image, leaving pixels with original values above the threshold as 1, and pixels below the threshold as 0. Morphologic processing then removed small objects (noise) and large objects (clumps) before labeling each group of connected "1" pixels as unique objects and documenting their properties (location, size, outline). Each object was considered to be an individual cell nucleus. The average nuclear-to-cytoplasm ("N/C") ratio for each image was calculated by dividing the total number of image pixels identified as nuclei, by the total number of pixels (minus nuclei and eliminated clumps) within the region of interest. Examples of this image processing procedure are shown in Supplementary Fig. S1.

Before quantitative image analysis, each HRME image underwent a quality control (QC) review by one of the study investigators (M.C. Pierce), which removed images from the data pool if any of the following criteria were met: (i) The focused portion of the HRME image occupied less than half of the available field of view, (ii) there was excessive loose tissue or debris in the field of view, or (iii) there was cellular material/debris visibly adhered to the fiber tip.

Results

Visually apparent lesions were noted both by VIA and colposcopy in 62 patients, whereas VIA and colposcopic examinations were negative in 111 patients. No VIA or colposcopic impression was recorded for one patient and the HRME data obtained from this patient were not analyzed further. One hundred and ninety-five unique cervical sites were imaged in the remaining 173 patients. Seventy-nine of these sites were at colposcopically abnormal lesions in 62 patients. The remaining sites were at colposcopically normal locations in the remaining 111 patients. Figure 2 presents the colposcopic appearance and HRME images from abnormal appearing sites in 2 different patients. In Fig. 2E, the acetowhite region at 5 o'clock was considered abnormal by the colposcopist. Following placement of the fiber optic probe onto this site, HRME imaging revealed nuclei appearing as discrete dots, sparsely and evenly distributed throughout the field-of-view, characteristic of normal squamous epithelium (Fig. 2F). Following biopsy, the pathology diagnosis for this site was non-neoplastic. A second patient with a lesion considered abnormal under standard colposcopy at the 12 o'clock location (Fig. 2G) also underwent HRME imaging. In the HRME image (Fig. 2H), nuclei appear more crowded and unevenly spaced, with some loose debris and mucus within the field-of-view. The histopathologic diagnosis at this site was CIN3.

We calculated N/C ratio values for each of the 69 colposcopically abnormal sites which passed the quality control review (10 sites were eliminated by QC review). These sites were biopsied on the basis of colposcopic appearance, therefore enabling comparison of HRME-derived N/C ratio values against the histopathologic diagnosis. We also established the N/C ratio at one imaged site in each of 95 colposcopically normal patients (images from 16 of these 111 patients were eliminated by QC). These sites were not biopsied and

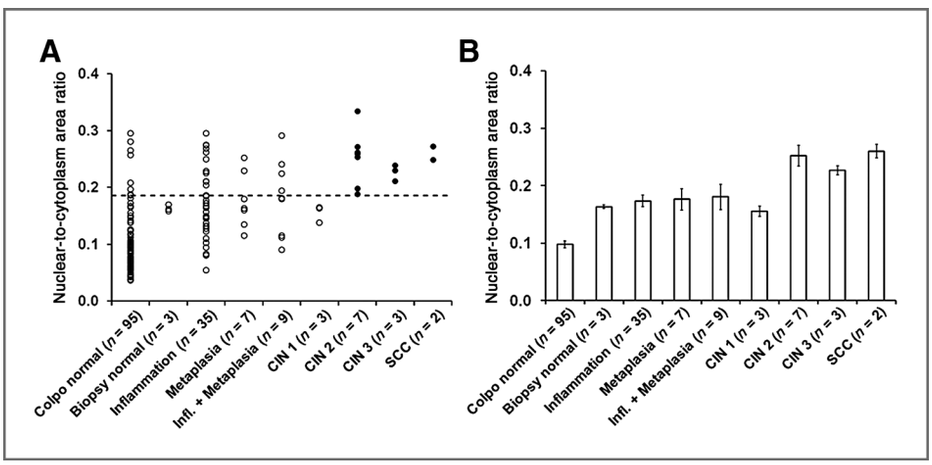

Figure 3. A, individual N/C ratio values measured at each of the 69 sites with a pathology diagnosis, as well as the 95 colposcopically normal sites imaged. The dashed line represents a *post hoc* threshold at the lowest value which correctly classified all 12 CIN2+ sites as "neoplastic." B, mean SE values for N/C ratios at each pathology grade.

therefore only permit comparison of N/C ratio to colposcopic appearance. Figure 3A shows the individual N/C ratio values measured at each of the 69 sites with a pathology diagnosis as well as the 95 colposcopically normal sites imaged. Figure 3B shows the mean SE values for N/C ratios at each pathology grade. The 12 lesions diagnosed as CIN2/3/SCC had higher mean and median N/C ratios than each of the lower grade categories. Combining all diagnoses less severe than CIN2 into a single category (median N/C = 0.164) and comparing with the CIN2 or more severe diagnosis (median N/C = 0.251) indicated that the mean ranks of the N/C ratio values are significantly different for these 2 groups (Kruskal–Wallis, $P = 1.3601 \quad 10^{-4}$).

Figure 3A also shows a horizontal dashed line representing a *post hoc* N/C ratio threshold value of 0.185, which was the lowest value that correctly classified all 12 CIN2+ sites as "neoplastic," Using this threshold, 38 of 57 sites considered abnormal by colposcopy (and therefore biopsied) were correctly classified as non-neoplastic by HRME, based on their histopathologic diagnoses (open markers below the threshold in Fig. 3A). Nineteen of these 57 sites with a pathology grade of normal or CIN1 were incorrectly classified by HRME as abnormal (open markers above the N/C = 0.185 threshold in Fig. 3A). When the same threshold was applied to the colposcopically normal (and not biopsied) sites (Fig. 3A), only 8 of the 95 sites exhibited an N/C figure above 0.185.

Table 1 summarizes the fraction of sites classified as neoplastic by HRME versus histologic diagnosis, also stratified by colposcopic impression. Only 8.4% of sites with a normal colposcopic impression were classified as neoplastic by HRME. All histologically neoplastic sites (CIN2+) were identified correctly by HRME. Of the 54 colposcopically abnormal sites with benign histology (false positive by colposcopy), only 35% were classified as neoplastic by HRME.

Table 2 summarizes the fraction of patients classified as neoplastic by HRME versus histologic diagnosis, this time stratified by whether the patient tested positive for high-risk HPV. Nine patients had histologically confirmed disease (CIN2+); all 9 tested positive for HR-HPV and also had an N/C ratio above the 0.185 threshold on HRME. Among women who were colposcopically normal or had a pathology diagnosis of <CIN2, there was no difference in the percentage that had a HRME N/C ratio above 0.185 for those who were HR-HPV negative (19 of 133 = 14.3%) and those who were HR-HPV positive (6 of 32 = 18.8%; $P = 0.6$, Fisher exact test).

Discussion

Through improvements in our knowledge of the pathogenesis of cervical cancer, the disease is now mostly preventable but still disproportionately affects women living in developing countries. Screening and treatment approaches based on cervical cytology have been successful in reducing the burden of cervical cancer where effective programs have been established. However, attempts to establish such programs in lower resource settings, which experience more than 80% of the burden of cervical cancer incidence and mortality, have been largely unsuccessful. Several new tools have emerged that may help to address these disparities, including HPV vaccination, lower cost HPV DNA testing, visual inspection methods, and ablative treatment (16). The use of VIA with cryotherapy has enabled see-and-treat programs to be implemented in several countries, providing women with the opportunity for cervical screening and treatment to be completed in a single clinic visit. However, there remains concern about the real possibility of significant overtreatment based on VIA-positive results, with a similar concern for management of HPV-positive women participating in HPV test–based screening. This study evaluated a recently developed low-cost imaging device which provides real-time information on cervical cell morphology *in vivo*. Such information may prove complementary to existing and emerging tools for diagnosis of cervical cancer and precancerous lesions in low-resource settings. Our primary goal in this study was to evaluate whether HRME imaging could potentially be used to improve the specificity of visual inspection using either colposcopy or VIA.

Visually apparent lesions with a pathology diagnosis of CIN2 or higher exhibited more crowded nuclei, often with greater variation in nuclear size and separation than at sites graded as CIN1 or normal/benign. A *post hoc* single threshold value of N/C area ratio discriminated between sites with non-neoplastic and neoplastic pathology with 100% sensitivity (12 of 12 with CIN2+) and 67% specificity (38 of 57 with colposcopically positive lesions, but <CIN2 on

Table 1. Fraction of sites classified positive by HRME image analysis versus colposcopic impression and histologic diagnosis

Colposcopic impression	Histologic diagnosis	No. of sites measured	No. of sites HRME positive	% Sites HRME positive
Normal	No biopsy	95	8	8.4
Abnormal	Normal/benign	54	19	35
	CIN1	3	0	0
	CIN2	7	7	100
	CIN3	3	3	100
	SCC	2	2	100

Table 2. Fraction of sites classified positive by HRME image analysis versus high-risk HPV test status and histologic diagnosis

High-risk HPV status	Histologic diagnosis	No. of patients measured	No. of patients HRME positive	% Patients HRME positive
HR HPV	No biopsy	90	5	5.6
	Normal/benign	43	14	32.6
HR HPV$^+$	No biopsy	21	2	9.5
	Normal/benign	9	4	44.4
	CIN1	2	0	0
	CIN2	6	6	100
	CIN3	2	2	100
	SCC	1	1	100

NOTE: No patients who were negative for high-risk HPV types had a biopsy with a diagnosis of CIN (any grade) or SCC.

pathology). Significantly, the 57 pathologically non-neoplastic sites were all deemed sufficiently abnormal in appearance under VIA/colposcopy to warrant biopsy collection, a false-positive rate of $57/69 = 83\%$. More than two thirds of those unnecessary biopsies were identified as non-neoplastic by HRME imaging. Of the 19 colposcopically abnormal sites which were incorrectly classified by HRME as abnormal against a gold-standard of pathology, the majority of these false-positive sites (17 of 19) showed chronic inflammation, either alone or with metaplasia (Fig. 3A). Given the generally high prevalence of inflammation in patients in low-resource settings, such conditions may impact the accuracy of HRME in these populations. However, the results reported here from China suggest that HRME may improve specificity over VIA alone while emphasizing the need for further evaluation in populations with even higher prevalence of inflammation.

When patients were initially stratified based on a positive high-risk HPV DNA test, HRME image analysis correctly identified 100% of patients with CIN2 or more severe disease (9 of 9 patients). Of the 30 patients with a positive high-risk HPV test but no histologic (11 patients < CIN2) or colposcopic (21 patients) evidence of disease, only 6 patients (18.8%) were identified as neoplastic by HRME imaging. These data support the potential for HMRE imaging to be used as an adjunct diagnostic tool in settings where HPV testing provides the initial screening result.

While reducing the amount of unnecessary biopsies can reduce program costs, the HRME system used in this study requires an upfront investment of around $3,000, the majority of this cost being allocated to the imaging camera. We have also evaluated lower cost consumer-grade cameras that retail for around $300 and have confirmed their suitability for use in HRME system (17). HRME is not the first *in vivo* cellular-level imaging technique to be evaluated for detecting cervical neoplasia. The use of confocal microscopy, in reflectance and fluorescence modes, has been reported previously with promising results. The study by Tan and colleagues (13) showed the ability of confocal fluorescence microscopy to also visualize nuclear morphology in the cervical epithelium following topical application of acriflavine dye (proflavine, the dye used in the study described here, is the fluorescent component of acriflavine). The authors developed a set of qualitative criteria which readers could apply to each image to assist in reaching a diagnostic decision. In a prospective study of 15 tissue sites, each with an independent pathology diagnosis, readers achieved 97% sensitivity and 93% specificity in identifying sites classified by pathology as CIN2 or higher. We note that the descriptive criteria developed by Tan and colleagues were entirely based on features related to nuclear morphology and could be directly applied to images generated by the HRME system in real-time. HRME images also display an area of tissue 2.5 times larger at a frame rate 6 times faster than that of the confocal platform used by Tan and colleagues, which may also improve diagnostic performance and ease of use. An assessment of training methods and learning curve for users of HRME was not directly included within this study, although it may be noted that all image data were acquired by clinical staff with no prior experience of HRME imaging. We have found that HRME images can be accurately classified as neoplastic/non-neoplastic by clinicians using qualitative criteria in other organ sites (18, 19). However, we showed here the ability to objectively classify images using quantitative analysis of features such as N/C ratio, thereby reducing the degree of subjective interpretation required by the user. Future studies will more thoroughly assess these important questions which will impact uptake of this technology.

While the HPV vaccine has immense potential to positively impact cancer prevention programs, particularly in low-resource settings, early detection techniques will still have key roles to play. The cost of the vaccine is declining but may remain unacceptably high in some regions. Current HPV vaccines target the 2 types, HPV16 and 18, which are only responsible for 70% of cervical cancers. Even when a suitably priced vaccine becomes widely available and is given to adolescents, it will take decades for the impact of vaccination to become clear. Women who have already been exposed to HPV and will not benefit from HPV

vaccination, and those who are have not been vaccinated will still require screening, ideally at between 30 and 45 years of age (20, 21).

The limitations of this study include a design which did not exactly reflect the intended use of the HRME in practice (i.e., as an adjunctive diagnostic tool for use in triaging VIA^+ or HPV^+ cases). However, this pilot study was carried out to permit evaluation of a relatively large VIA and HPV population that will certainly be encountered in the field. These data will require validation in a larger, well-powered, prospective trial that permits objective evaluation and establishment of HRME image features, including the optimum N/C ratio threshold for classifying tissue as neoplastic.

Disclosure of Potential Conflicts of Interest

R. Richards-Kortum serves as an unpaid scientific advisor to Remicalm LLC, holds patents related to optical diagnostic technologies that have been licensed to Remicalm LLC, and holds minority ownership in Remicalm LLC. P. Castle has been compensated by Merck for serving on a Data and Safety Monitoring Board for HPV vaccines and has received HPV tests and testing for research at a reduced rate or no cost from Qiagen and Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: M.C. Pierce, R. Richards-Kortum

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.Y. Guan, W.-H. Zhang, Y.-L. Qiao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.C. Pierce, M.K. Quinn, P. Castle, R. Richards-Kortum

Writing, review, and/or revision of the manuscript: M.C. Pierce, Y.Y. Guan, M.K. Quinn, P. Castle, R. Richards-Kortum

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y.Y. Guan, X. Zhang, Y.-L. Qiao

Study supervision: Y.Y. Guan, R. Richards-Kortum

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