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Original Article

An special epithelial staining agents: folic acid receptor-mediated diagnosis (FRD) effectively and conveniently screen patients with cervical cancer

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Abstract: High-quality screening with cytology has markedly reduced mortality from cervical cancer. However, it needs experienced pathologists to review and make the final decisions. We have developed folic acid receptor-mediated diagnosis (FRD) kits to effectively and conveniently screen patients with cervical cancer. We conduct present study aim to assess clinical significances of FRD in screening cervical cancer. A total of 169 patients were enrolled at Chinese People's liberation Army (PLA) general hospital. We compared diagnostic significances of FRD with thinprep cytology test (TCT). Meanwhile, colposcopy was also performed to confirm any lesion suspicious for cervical cancer. The sensitivity and specificity of FRD were 71.93% and 66.07% in diagnosis cervical cancer, respectively. Meanwhile, the positive predictive values (PPV), negative predictive values (NPV), Youden index were 51.90%, 82.22%, 0.38, respectively. On the other hand, the sensitivity and specificity of TCT in diagnosis cervical cancer were 73.68% and 61.61% respectively. PPV, NPV and Youden index for TCT were 49.41%, 82.14% and 0.35 respectively. Overall, FRD have high values of sensitivity, specificity and Youden index. However, this difference failed to statistical significance. FRD have comparable diagnostic significance with TCT. Therefore, FRD might serve as one effective method to screen cervical cancer. Especially for those patients living in remote regions of China, where cytology was unavailable.

Keywords: Cervical lesion, folic acid receptor-mediated diagnosis, folate receptor

Introduction

Cervical cancer is estimated to be the secondly leading causes of cancer among women worldwide, with approximately 530,000 new cases and 275,000 deaths each year [1]. An estimated 85% of the world's cervical cancer cases occur in developing countries, especially in China. Cervical cancer is a paradigm of global healthy disparity, disproportionately affecting young women from the poorest countries and the most disadvantaged populations [2]. As dispread of high risk human papillomavirus (hrHPV) infection recognized most significant risk factor in its aetiology. Currently, clinical management of pre-invasive cervical cancer largely relies on histological examination to confirm cervical intraepithelial neoplasia (CIN) [3]. Cytology-based cervical screening has been regarded the most valuable way to reduce the incidence of cervical cancer [4]. Therefore, the lowest incidence and mortality rates are recorded in countries, especially in western countries, where screening is available to women [5]. However, cytology-based cervical screening also has some limitations. The major problem is that low sensitivity of single smear fails to detect high-grade precursor lesions [6]. In addition, cytology has low reproducibility, leading false positives increases substantially over time [7]. For instance, there are multiple benign mimics of neoplastic cells, such as atypia of repair, atrophy, radiation change, effect of intrauterine device, and metaplasia [8]. Besides, most women lived in remote area are inconvenient adopt to cytology-based cervical screening, because of limited device and economic burden. Thus, there are urgent need to develop an effectively and costly screening algorithm.

Recently, folate receptor (FR) conjugated with other agents, such as fluorescent and nanoparticles, have been used to detect ovarian can-

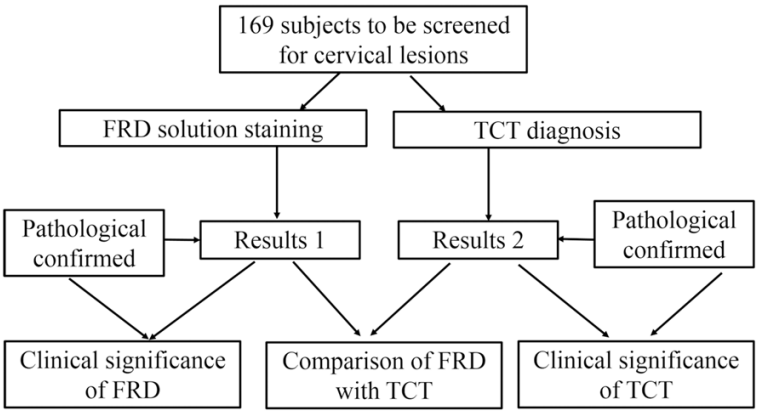

Figure 1. The diagram of presents study. FRD, folic acid receptor; TCT, thinprep cytology test.

cers [9-11]. FRs are highly expressed in a variety of cancers, especially in ovarian and lung cancers, whereas most normal tissues express low to negligible levels [12]. Therefore, FR may be a potential target for capturing cancer cells. A novel solution, named folic acid receptor-mediated diagnosis (FRD), which is a specific epithelial cells staining agent target to cervical cancer cells. FRD can reflect cell pathological changes by oxidation. At present study, we explore the clinical significance of FRD to diagnosis patients with cervical lesions. More importantly, the purpose of this study is primary explore the potential prospect of FRD as one of effective and convenient screening algorithm to identify patients with cervical cancer.

Material and methods

Subjects

A total of 169 women who presenting for routine cervical lesion screening were enrolled at Chinese People's liberation army (PLA) general hospital, from August 2012 to March 2013. All enrolled cases were non-pregnant and not in menstrual period. The study protocol was proved by the institutional review ethics boards of PLA general hospital. All women provided written informed consent before undergoing any study procedures. The flow chart of present study is described in **Figure 1**.

Cervical TCT

The patients had no application of drugs in the vagina, washing, sexual life, vagina and cervical performance three days before examination. A specified cytobrush was placed into the cervical canal about one centimeter and rotated five cycles clockwise. And then it was taken out and put into the specified container. Finally, the liquid was sent to exam after the brush and washed 10 times. ThinPrep automatic pelleter was adopted to make smears, automatic cell meter to disperse and the filtrate samples, and microscope is used to observe and diagnose by two independently experienced pathologists.

Cervical cells FRD staining

Major components of FRD (Shanxi Gaoyuan Medical Device Ltd) were folic acid compound, methylene blue and acetic acid. Subjects enrolled in this study as mentioned above were subsequently subjected with FRD staining. A gynecological cotton swab was applied on the cervix entrance about 1 cm and rotated five laps, and then pressed for 5 seconds. After that, the swab were immediately removed and put in solution of FRD staining (less than 10 seconds). The swab was immediately to observe the color change and compare it with the reference cards provided by FRD kit in two minutes.

Cytologic diagnoses

Cytologic diagnoses and specimen were classified according to Bethesda System (TBS 2001) for reporting cervicovaginal cytology [13]. The cytological results were as following description. The patients were diagnosed as negative for those with negative for intraepithelial lesion or malignancy (NILM). The patients recognized as abnormal squamous cells of uncertain significance (ASC-US) were included atypical squamsus cells-cannot exclude HSIL (ASC-H), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC). Besides, the patients with abnormal glandular epithelium were those with atypical glandular cells (AGC), adenocarcinoma in situ (AIS), and adenocarcinoma (AC). Positive cytology was defined as positive in cytologic diagnosis ASC-US or AGC.

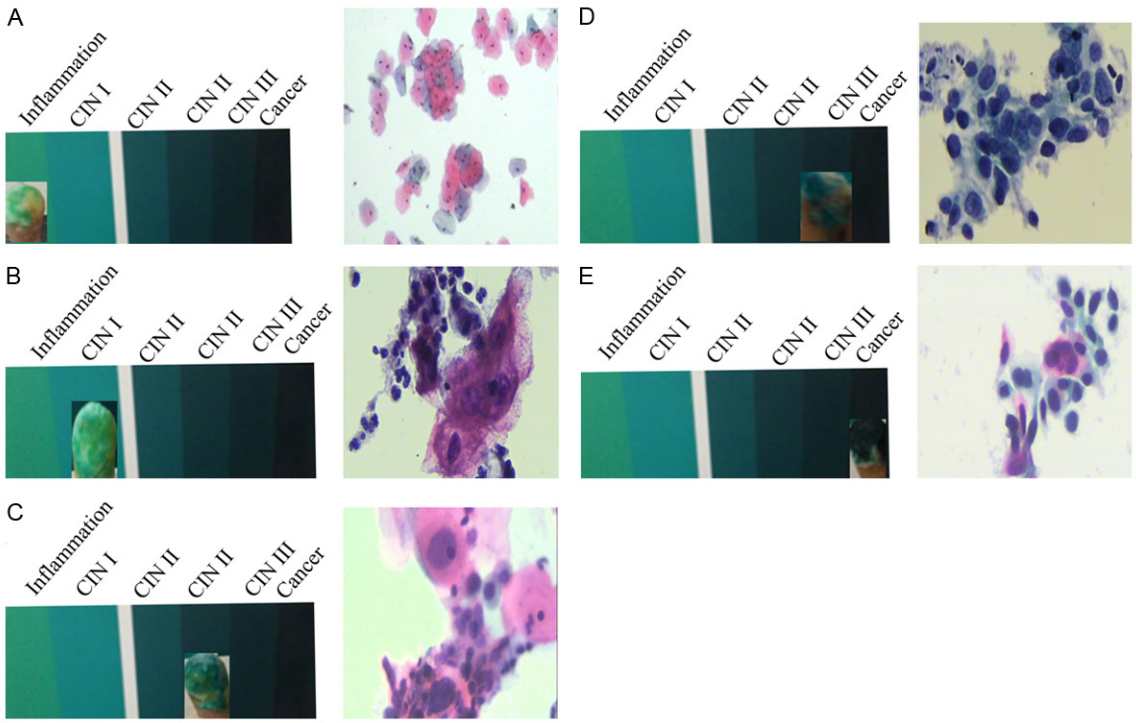

Figure 2. Results of five representative FRD (left) and cytology diagnoses (right) shows with swabs, FRD reference cards and cytological images. A. Light brown colors, the pathology indicated this patients with inflammation. B. Light blue-green implied that the patient with cervical CIN I lesion. C. Dark blue-green or brown green indicated that patients suffer from CIN II cervical lesions. D. Dark green represent that patients have a period of CIN III cervical lesions. E. Purple-black means the patients have developed malignant tumor cervical lesions. FRD, folic acid receptor; CIN, cervical intraepithelial neoplasia.

FRD staining diagnoses

The FRD cervical specific stainings were performed by specially trained gynecologists following the instructions. The diagnoses for FRD staining were based on the following criteria. The subjects diagnosed as negative were stained with the color including: light brown (Inflammation), light blue-green (CIN I). The subjects were diagnosed as positive with staining color following: dark blue-green (CIN II), brown green (CIN II), dark green (CIN III), and purple-black (Cancer). Those indicated cervical lesions. The cotton swabs which were saturated, supersaturated, or contaminated with blood would be excluded. Those swabs with black-purple resulted in undistinguished comparison.

Statistical analysis

Receiver operating characteristic (ROC) analysis was used to assess possible cut-points of the cutoff ratio to define positive findings of the FRD for cervical patients. To choose an optimum cut-off ratio for FRD using cervical, Youden's index [Y= sensitivity-(1-specificity)] was calculated [14]. The sensitivity and specificity for FRD and TBS were also analyzed. The correlation between the TCT and FRD staining were examined by Spearman's rank correlation. All *P* values less than 0.05 (two-sided) were considered statistically significant. Analyses were done in SPSS for Windows (version 19.0, SPSS Inc, Chicago, IL, USA).

Results

Baseline characteristics of the enrolled subjects

A total of 169 women aged from 19 to 68 with a mean ± SD age of 41. 61±10.29 years were enrolled into the study. Besides, the mean pregnancy and birth times were 2.99±1.77 and 1.32±1.05 years, respectively. Among of them, there were 71 (41.89%) subjects with non-subjective symptom, 75 (44.59%) cases with leucorrhea increases, 42 (25.00%) subjects with cervical contractive bleeding, 19 (11.49%)

Table 1. Diagnostic significance of FRD in screening cervical cancer patients

FRD solution	The patients number of pathological diagnoses		Total
	Positive	Negative	
Positive	41	38	79
Negative	16	74	90
Total	57	112	169

FRD, folic acid receptor.

Table 2. Diagnostic significance of TCT in screening cervical cancer patients

TCT	The patients number of pathological diagnoses		Total
	Positive	Negative	
Positive	42	43	85
Negative	15	69	84
Total	57	112	169

TCT, Thinprep cytology test.

Table 3. The comparison of FRD solution and TCT in diagnosis patients with cervical cancer

	TCT	FRD	χ^2	*P*
Sensitivity	73.68%	71.93%	0.04	>0.05
Specificity	61.61%	66.07%	0.48	>0.05
missed diagnosis	26.32%	28.07%	0.04	>0.05
misdiagnosis rate	30.39%	33.93%	0.48	>0.05
accuracy	65.68%	68.05%	0.12	>0.05
PPV	49.41%	51.90%	0.10	>0.05
NPV	82.14%	82.22%	0.00	>0.05

FRD, folic acid receptor-mediated diagnosis; TCT, Thinprep cytology test; PPV, Positive predictive value; NPV, Negative predictive value.

Table 4. The consistency of FRD and TCT

	The patients number of pathological diagnoses		Total
	Positive	Negative	
FRD	41	74	115
TCT	42	69	111
Total	83	143	226
	χ^2=0.12, *P*>0.05		

FRD, folic acid receptor-mediated diagnosis; TCT, Thinprep cytology tes

cases with unscheduled bleeding, 12 (0.68%) subjects with vaginal fluids, and 12 (0.68%) with vaginal pain. Pathological findings shows that 88 (52.07%) patients were diagnosed as inflammation, 24 (14.20%) subjects as CIN I, 15 (8.88%) as CIN II, 34 (20.12%) subjects as CIN III, and 8 (4.73%) subjects as invasive cervical squamous cell carcinoma.

The assessment of FRD

All enrolled patients accepted FRD solution testing. The cotton swab was used to take cervical cells and dip into FRD solution. Then, swab was taken out and compared color changes with standardized reference card (**Figure 2**). The reference card contained six difference colors, which were light brown, light blue-green, dark blue-green, brown green, dark green, and purple-black. Five representative diagnoses are shown in **Figure 2** with cytology image, the swabs presents light brown colors, the pathology indicated this patients with inflammation (**Figure 2A**). The swabs presents light blue-green, while pathology implied that the patient with cervical CIN I lesion (**Figure 2B**). The patients inflammation and CIN I lesions were regarded as negative. However, when the colors of swabs are dark blue-green or brown green, it indicated that patients suffer from CIN II cervical lesions (**Figure 2C**). On the other hands, when the colors of swabs presents with dark green, which represent that patients have a period of CIN III cervical lesions (**Figure 2D**). Besides, once the colors of swabs show the colors of purple-black, it means that patients mostly likely have developed malignant tumor cervical lesions (**Figure 2E**).

FRD screening

FRD and pathological identification were performed in 169 women, and results are summarized in **Table 1**. Of these women, 79 (46.75%) women were positive and 90 (53.25%) were negative. To assess the clinical significance of FRD, the ROC curve was used to analyze FRD as the potential tools to screen cervical cancer. As a result, the sensitivity and specificity of FRD were 71.93% and 66.07% respectively, with an accuracy of 68.05%. The values of PPV and NPV were 51.90% and 82.22% respectively. Youden's index of FRD was 0.38. Odds ratio and positive likelihood ratio of FRD were 4.99 and 2.12 respectively.

TCT screening

Furthermore, TCT was also performed in 169 women, and results are summarized in **Table 2**.

Table 5. The diagnostic significance of combining FRD and TCT and both methods with positive results

Combination of FRD and TCT	The patients number of pathological diagnoses		Total
	Positive	Negative	
Positive	33	14	47
Negative	24	98	122
Total	57	112	169

FRD, folic acid receptor-mediated diagnosis; TCT, Thinprep cytology test.

Table 6. The diagnostic significance of combining FRD and TCT and one of them with positive results

Combination of FRD and TCT	The patients number of pathological diagnoses		Total
	Positive	Negative	
Positive	50	67	117
Negative	7	45	52
Total	57	112	169

FRD, folic acid receptor-mediated diagnosis; TCT, Thinprep cytology test.

Of these women, 85 (50.30%) women were positive and 84 (49.70%) were negative. The sensitivity and specificity of TCT were 49.41% and 61.61% respectively, with an accuracy of 65.68%. The values of PPV and NPV were 51.90% and 82.14% respectively. Youden's index of FRD was 0.35. Odds ratio and positive likelihood ratio of FRD were 4.49 and 1.91 respectively.

The comparison FRD with TCT

We next determined whether FRD could instead of TCT as primary screening method to diagnosis patients with cervical cancer. We compared the diagnostic significance of FRD and TCT. The results were shown in **Tables 3** and **4**, the sensitivity of FRD solution was lower than TCT. On the other hand, specificity, misdiagnosis rate, accuracy, PPV, and NPV were slightly higher than TCT. However, all of those discrepancy failed to reach statistically significance.

The diagnostic significance in the combination TCT with FRD

Furthermore, we also determined diagnostic significance in combination TCT with FRD to screening patients with cervical cancer (**Table 5**). When both methods were shown the patients with abnormal lesion. The sensitivity and specificity were 57.89% and 87.50% respectively, with an accuracy of 77.51%. Youden's index was 0.45. Odds ratio and positive likelihood ratio were 9.63 and 4.63 respectively.

When combined FRD with TCT, we concluded positive conclusion in the condition that any one of them with positive results (**Table 6**). The sensitivity and specificity were 87.72% and 40.18%, with accuracy of 56.21%. PPV and NPV values were 42.74% and 86.54%. Youden's index was 0.28, and odds ratio and positive likelihood ratio were 9.63 and 4.63 respectively.

Discussion

Folate is the essential vitamin that refers to process of one-carbon transfer during nucleic acid synthesis in eukaryotic cells. The cell survival and proliferation are dependent on folic acid intake to a certain extent [15]. Previously studies show that FR expressed in several kinds of human cancer. However, the expression of FRs are not found in normal tissues except of choroid plexus and placenta [16]. Folate conjugating its receptors and multiple complexes are transferred to inner of cells by endocytosis [15]. Thus, it should be one of potential strategy to distinguish lesions and normal tissues based on the expression of FR. At present, FR have been served as the target of fluorescent paramagnetic bimodal liposomes and applied to tumor imaging [17]. Besides, folic acid modified gelatine coated quantum dots could be as potential reagents for cancer diagnostics [18]. And folate-modified self-microemulsifying have potential as the target for curing colon cancer [19]. In this study, we explored FRD as one of convenient way to screening patients with cervical cancer. The specifically epithelial cells staining solution are comprised with folic acid complex, methylene blue, and acetic acid. Once cancerous cells are contacted this solutions, folate and its complexes will conjugate with folic acid receptors. After that, the complexes combined with reductive form of methylene blue enter to intracellular of tumor cells. Oxidative stress and increased reactive oxygen (ROS) are found in most of tumor cells [20, 21]. The reductive methylene blue will be oxidized. Then, those

huge biological molecular bring with hyperostosis in tumor cells. The oxidative methylene blue secretes from tumor cells and stains cotton crab with different colors. Meanwhile, the acetic acid in FRD could react with the reagents in tissues with lesion and appears white. So, we proposed a potential primary method that using FRD to screen patients with cervical lesion based on above mentioned principle. Those patients with potential lesions will be further determined by other diagnostic methods.

Here, we prove when FRD use to screen patients with cervical lesions, including sensitivity, specificity and accuracy, have similar diagnostic significance with TCT. More importantly, in the combination FRD with TCT, we can screen patients with cervical lesions in the condition that when any of FRD or TCT indicated positive diagnoses. The specificity were significantly improved and reached 87.50% without decreases of sensitivity. At same time, the PPV and odd ratios also have been significantly improved. Although the sensitivity was substantially promoted (87.5%) when both of FRD and TCT were positive, the specificity significantly reduced and Yodden's index didn't be improved. Thus, we did not recommend the methods that combination FRD and TCT to screen patients with cervical lesions. Based on our experience in using FDR to discern cervical lesion, we concluded several advantages of FRD. At first, the FDR solutions have the ability to specifically react with oxidative-reductive molecular locating in cervical cancer cells. Secondly, the reactions of FRD with biological molecular were rapid so as to make decision as soon as possible. As a result, this diagnostic method would effectively identified those potential lesion in cervical cells. Besides, the period between patients enrolled and final conclusion made would be significantly shorten for this convenient diagnostic way. Meanwhile, the results were easily recognized without experience pathological clinicians and patients also had good compliance. At last, the FRD economic burden was extremely lower than TCT that could make this methods widely used to primary screen potential patients in rural area. However, we could not ignore the disadvantages of FRD solution. The results were mostly influenced by bleeding during the operating procedure. Also, the accuracy of FRD could be improved with increases of experience. Until to now, we still lack the standard process to guide the users to make proper results.

In our study, cervical scrapings were collected by using the standard brushing procedure to avoid effecting the accuracy of vaginoscopy. The cervical exfoliative cells were then mixed with staining solution. In clinical, FRD could be recognized as one of assistant methods to improve colposcopy. However, we still lack quantified indexes so as to quantitatively analyze results of FRD in this study. Thus, it will be necessary to incorporate related parts into future research. To some extent, the activity of exfolliative cells from cervix were suppressed, as a result that reduced accuracy of FRD diagnosis. Furthermore, the living cells were reduced among those exfolative cells. Hence, we propose that FRD could directly apply in the lesion tissues to promote accuracy. But this hypothesis need further identified in future study.

At present study, we show that applying FRD solution to screening patients with CIN II cervical lesions have similar clinical significance with TCT. However, it is impossible for FRD instead of classic TCT as the main screening system to prevent this malignant disease. Initially, we aimed to determine whether FRD have potential to be one of effectively and conveniently programs. That could be used in potential patients with cervical lesions who lived remote district, where TCT or other advanced techniques were unavailable. FRD might contribute to effectively lead distribute of patients and selected appropriate person whom should have a priority for be examined further. On the other hand, once combined FRD with TCT could improve diagnostic accuracy of cervical cancer.

However, we conducted this study in a relative small cohort. It is thus necessary to verify those findings within a larger-scale samples with multiple centers in future research. In addition, it was unclear whether testing results of FRD solution could be affect by pregnancy or fetus. Therefore, this reason might limit FRD solution used to screening whom pregnant with cervical lesion. On the other hand, the effects that could reduce bleeding contamination or pregnancy stimulation, would substantial decrease negative effects of FRD diagnoses.

Disclosure of conflict of interest

None.

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