Cancer Biomarkers 27 (2020) 303–312
DOI 10.3233/CBM-182232
IOS Press

FHIT and C-MYC expression in cervical histology and cytology as biomarkers for detecting high-grade intraepithelial neoplasia in human papillomavirus-positive women

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Abstract.
BACKGROUND: The current cervical cancer screening strategies based on Papanicolaou (Pap) and Human papillomavirus (HPV) tests receive great achievement but still exhibit many limitations in clinical practice. Exploring new biomarkers as stratified management method in HPV primary screening is becoming the tendency of current research.
METHODS: Immunocytochemistry (ICC) of FHIT and C-MYC were performed on exfoliated cervical cells from 197 eligible high-risk HPV positive women. Mann-Whitney U test, Pearson Chi-Square test, logistic regression analysis and receiver operating characteristic (ROC) curves were used to assess the diagnostic efficiency.
RESULTS: ICC staining intensity of FHIT and C-MYC in high-grade cervical intraepithelial neoplasia (CIN) specimens was significantly different from low-grade CIN and normal specimens. Compared with Pap test, ROC analysis of ICC in detecting high-grade CIN resulted in a larger area under the curve (AUC) (0.805 and 0.814 vs 0.723, $p < 0.001$). FHIT achieved higher sensitivity than Pap test (79.41% vs 66.67%, $p = 0.04$). Logistic regression analysis of the combination of two biomarkers led to higher AUC value, specificity and PPV than any single biomarker.
CONCLUSIONS: The utility of FHIT and C-MYC ICC analysis in cervical exfoliated cells of HPV-positive women displayed superior diagnostic potential and may improve clinical performance of cervical cancer screening.

Keywords: CIN; Biomarker; FHIT; C-MYC; Immunocytochemistry

1. Introduction

Due to screening and high-risk human papillomavirus (HR-HPV) vaccination, cervical cancer dropped from the ninth to the tenth leading cause of cancer deaths worldwide in the last decade. However, cervical cancer, a preventable and treatable disease, still causes approximately 526000 new diagnoses and 239000 deaths annually [1], with approximately 85% of the worldwide incidence occurring in developing countries [2]. According to statistics, in low-sociodemographic index (SDI) countries, 1 in 24 women develop cervical cancer during their lifetime, whereas only 1 in 115 do so in high-SDI countries [1]. The high incidence of this disease in low-SDI countries and the large gap between developed and developing countries indicate that current screening strategies have some limitations. In particular, Papanicolaou (Pap) cytology is limited by its low sensitivity, high subjec-

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ISSN 1574-0153/20/$35.00

tivity and requirement for well-trained cytologists [3]. Therefore, there is a clear need to develop new screening strategies to reduce the incidence and mortality of cervical cancer.

The discovery of etiologic association between HR-HPV infection and cervical cancer led to the application of the HR-HPV test for cervical cancer screening. Evidence has shown that compared to Pap cytology, the HR-HPV test provides superior protection against high-grade cervical intraepithelial neoplasia (CIN) [4]. In some European countries and America, HR-HPV test has been recommended as the preferred strategy for primary cervical cancer screening [5–7]. However, while most HPV infections are transient, HPV test does not discriminate between transient and persistent infection, and this makes it suboptimal as a stand-alone primary screening method. Therefore, it is very important to apply additional stratified management of HPV-positive women in primary cervical cancer screening. Pap cytology and HPV 16/18 genotyping have been recommended as stratified screening triage approach for HPV-positive women [8,9]. However, Pap cytology lacks sensitivity, while HPV 16/18 genotyping lacks specificity. There is a substantial demand for a method of identifying novel biomarkers that will improve specificity without compromising sensitivity in stratified screening of high-risk HPV positive women. Researchers have identified several novel biomarkers related to molecular alterations caused by HPV; these include p16/ki67, ProexC, gene promoter methylation and microRNAs [10–13]. However, none of these biomarkers has been widely accepted in clinical practice.

The integration of HR-HPV DNA into the host genome results in overexpression of the viral oncoproteins E6 and E7, which bind to and inactivate p53 and pRb, respectively. The inactivation of p53 and pRb leads to cell cycle disruption and consequently tocervical cell malignant transformation [14]. However, this does not explain the entire etiological process of cervical carcinogenesis. An increasing number of studies confirm that the genetic or epigenetic alterations HPV integration causes in key oncogenes also play important roles in the development of cervical cancer. In a previous study, researchers used whole-genome sequencing and high-throughput viral integration detection to identify several high-frequency HPV integration breakpoints in cervical cancer tissues and cell lines. Among these hot-spot HPV integration breakpoints, FHIT and C-MYC were two well-known tumor-related genes. Researchers also confirmed that HPV integration into FHIT introns reduced FHIT protein expression, while HPV integration into the flanking regions of C-MYC increased C-MYC protein expression [15]. The FHIT gene is located at a common fragile site, FRA3B, and is frequently expressed at lower levels in preneoplasias and cancers. Many tumor-related biological functions are altered as a result of FHIT loss; these include apoptosis, epithelial-mesenchymal transition (EMT), genotoxic resistance and genome instability [16]. The C-MYC gene encodes a helix-loop-helix transcription factor that regulates many cellular functions, including cell growth, cell cycle progression, cell biosynthesis and apoptosis [17]. The HPV-induced dysregulation of FHIT and C-MYC expression indicates their potential value as biomarkers for cervical cancer screening.

The aim of this study is to search out new stratified screening triages for HPV-positive women. In order to implement this aim, we evaluate the clinical diagnosis value of FHIT and C-MYC ICC in detecting high-grade CIN among HPV-positive women and reveal their potential application as biomarkers for cervical cancer screening.

2. Materials and methods

2.1. Subject recruitment and sample collection

Women who were test for both Pap cytology and HPV in the gynecologic outpatient clinic of our hospital from September 2015 to September 2017 were prospectively recruited. The study population ($n = 197$) consisted of women who obtained HR-HPV positive results and agreed to undergo colposcopy. The exclusion criteria were as follows: aged younger than 30 years old or older than 65 years old; previously diagnosed with CIN, cervical cancer or other malignancies; pregnancy or refusal to consent/participate. All eligible women underwent colposcopy, and their residual cytology samples were collected for subsequent experiments. Cytology-negative (NILM) and colposcopy-negative women were regarded as histologically normal and received no biopsy. Those cytology-positive (ASCUS or worse) and colposcopy-negative women received routine cervical biopsies at the 3, 6, 9, and 12 o'clock positions. Colposcopy-positive women underwent biopsies in suspicious areas and random areas. The procedures used for sample collection and to obtain biopsy results are shown in Fig. 1. All colposcopy procedures were performed

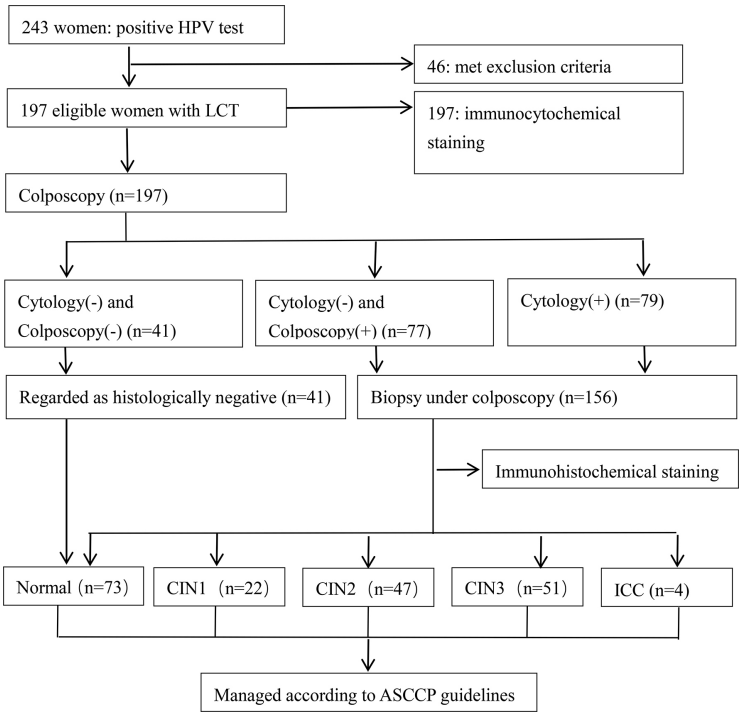

Fig. 1. Procedure of sample collection and results of biopsies. LCT: liquid-based cytology test; CIN: cervical intraepithelial neoplasia; ASCUS: abnormal squamous cells of uncertain significance; ASCCP: American Society for Colposcopy and Cervical Pathology. Cytology (-) indicates a negative Pap test result. Cytology (+) indicates a positive Pap test result (ASCUS or worse).

by skilled, high-level gynecologists who were blinded to any cytology results. All diagnostic and treatment procedures were performed according to the American Society for Colposcopy and Cervical Pathology (ASCCP) 2017 guidelines and had no effect on the results of subsequent experiments. Informed consent forms were signed by all women participating in the study before collection of their cervical cell samples or any other clinical procedures, and the study was approved by the Ethics Committee of the hospital.

2.2. *Pap cytology [liquid-based cytology (LBC)] and HPV cotest*

Pap cytology specimens were collected by cytobrush and stored in BD SurePath Liquid-Based Cytology preservative fluid (BD Diagnostics, Sparks, MD). Thin-layer LBC was performed with a ThinPrep 2000 processor (Cytyc Corp, Boxborough, MA), and each cytological diagnosis was determined by two pathologists according to the Bethesda System (TBS, 2001). HPV tests were conducted with a Hybrid Capture 2 (HC2) assay (Digene, Gaithersburg, MD) to detect 13 types of HR-HPV, and more than 1 pg/mL HPV DNA in the specimen tested was regarded as positive result.

2.3. *Construction of tissue micro-array (TMA) blocks*

Tissue specimens obtained from biopsies were formalin-fixed and paraffin-embedded. Hematoxylin and eosin-stained slides were processed and evaluated by pathologists to locate representative lesion areas. For each specimen, a 1 mm^2 piece of the selected area was inserted into recipient TMA blocks, and three sections with a thickness of 0.4 mm were collected from each block for subsequent analysis.

2.4. *Immunohistochemistry (IHC)*

IHC assays were performed on TMA slides after they were deparaffinized with xylene and rehydrated with a descending ethanol series. Antigen retrieval was conducted by high-pressure boiling in a citrate pretreatment solution to inhibit nonspecific antibody binding. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and nonspecific antibody binding was blocked with goat serum. The TMA slides were then incubated overnight in a humidified chamber at 4°C with the following primary antibodies: a monoclonal antibody against FHIT (1:200, ProteinTech) or a monoclonal antibody against C-MYC (1:200, Pro-

teinTech). An HRP Detection System was used to label reagents via incubation with a secondary antibody for 30 minutes at room temperature and incubation with DAB for two minutes. Finally, the slides were counterstained with hematoxylin, dehydrated through an ascending ethanol series, cleared with xylene, and mounted. Staining intensity was classified as negative, weak, moderate, or strong.

2.5. Cell line analyses

Human cervical carcinoma HeLa cells and human embryonic kidney (HEK) 293 cells were chosen to verify the FHIT and C-MYC protein expression levels in cells. The HeLa cells and HEK 293 cells were generously gifted by the State Key Laboratory of Oncology in South China and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA), 100 U/ml penicillin in a 5% CO_2-humidified atmosphere at 37°C. The cultured cells were fixed on slides in ice-cold acetone for 15 minutes and then dehumidified in the open air for 5 minutes. Then, the slides were pretreated with 0.5% Triton X-100 (Solarbio, Beijing, China) for 15 minutes to increase cytomembrane permeability. The subsequent staining procedures included endogenous peroxidase blocking, antibody incubation, HRP detection, DAB incubation and hematoxylin counterstaining, similar to the IHC procedures described above, and are therefore not elaborated in this section.

2.6. Immunocytochemistry (ICC)

After HPV tests and LBC tests were performed, two additional cytology slides were produced from each residual specimen using a ThinPrep 2000 processor (Cytyc Corp, Boxborough, MA, USA). The ICC staining procedures also included fixation, desiccation, antigen retrieval, endogenous peroxidase blocking, antibody incubation, HRP detection, DAB incubation and hematoxylin counterstaining. After ICC staining, the slides were analyzed and scored independently by two experienced pathologists blinded to the Pap test results and histological diagnosis. All cells were observed and evaluated in three random fields, and each cell was scored based on the intensity of immune staining. Staining intensity was classified as negative, weak, moderate, or strong, with negative scored as 0 points, weak as 1 point, moderate as 2 points, and strong as 3 points. The scores for each cell in one field were added together and divided by the total cell number in this field to produce the staining score of the corresponding field. The average score of three random fields from each slide was calculated as the "staining score" used to represent the staining intensity of the corresponding slide.

3. Statistical analysis

In this study, FHIT and C-MYC immunocytochemical staining scores of two groups were compared by the nonparametric Mann-Whitney U test. ROC curves were used to assess the diagnostic efficiency of immunocytochemical staining in detecting high-grade intraepithelial neoplasia (CIN2+). The optimal cut-off points of the FHIT and C-MYC staining scores were determined by the maximal Youden index. ASCUS+ was considered the cut-off point for the Pap test. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each test were calculated and compared using the Pearson chi-square test. In all cases, $P < 0.05$ was considered statistically significant.

4. Results

The results of Pap test and colposcopies and histological diagnoses are shown in Fig. 1. Among the 197 HR-HPV-positive eligible women who underwent colposcopy, 156 underwent a directed biopsy under colposcopy for abnormal cytology results ($n = 79$) or normal cytology but had positive colposcopy findings ($n = 77$). The other 41 women with normal cytology results (NILM) and negative colposcopy findings were regarded as histologically negative and did not receive biopsies. Of the patients who underwent biopsies, the histologic diagnosis was normal in 32, CIN1 in 22, CIN2 in 47, CIN3 in 51 and invasive cancer in 4.

First, IHC staining was performed to analyze the corresponding protein expression in cervical lesions at different stages. Representative FHIT and C-MYC staining patterns are shown in Fig. 2. In IHC staining of different stages of cervical lesions, the FHIT expression-positive rates in normal cervical tissue, CIN1, CIN2, CIN3 and invasive cancer were 84.4%, 68.2%, 47.0%, 27.4% and 0%, respectively, and there was a tendency for positive rates to be correlated with the grade of cervical lesion ($p < 0.01$). The C-MYC expression-positive rates in normal cervical

Table 1
Frequencies of each level of FHIT staining intensity in different cervical lesions

Subgroup	FHIT IHC staining intensity, n (%)				
	Negative	Weak	Moderate	Strong	Positive rate, %
Normal ($n = 32$)	1 (3.1)	4 (12.5)	16 (50.0)	11 (34.4)	84.4
CIN1 ($n = 22$)	2 (9.1)	5 (22.7)	10 (45.5)	5 (22.7)	68.2
CIN2 ($n = 47$)	4 (8.5)	21 (44.7)	19 (40.4)	3 (6.4)	47.0
CIN3 ($n = 51$)	10 (19.6)	27 (52.9)	12 (23.5)	2 (3.9)	27.4
ICC ($n = 4$)	1 (25.0)	3 (75.0)	0	0	0

Table 2
Frequencies of each level of MYC staining intensity in different cervical lesions

Subgroup	MYC IHC staining intensity, n (%)				
	Negative	Weak	Moderate	Strong	Positive rate, %
Normal ($n = 32$)	15 (46.9)	14 (43.8)	3 (9.4)	0	9.4
CIN1 ($n = 22$)	7 (31.8)	12 (54.5)	3 (13.6)	0	13.6
CIN2 ($n = 47$)	4 (8.5)	8 (17.0)	21 (44.7)	14 (29.8)	74.5
CIN3 ($n = 51$)	3 (5.9)	7 (13.7)	25 (49.0)	16 (31.4)	90.4
ICC ($n = 4$)	0	0	2 (50.0)	2 (50.0)	100

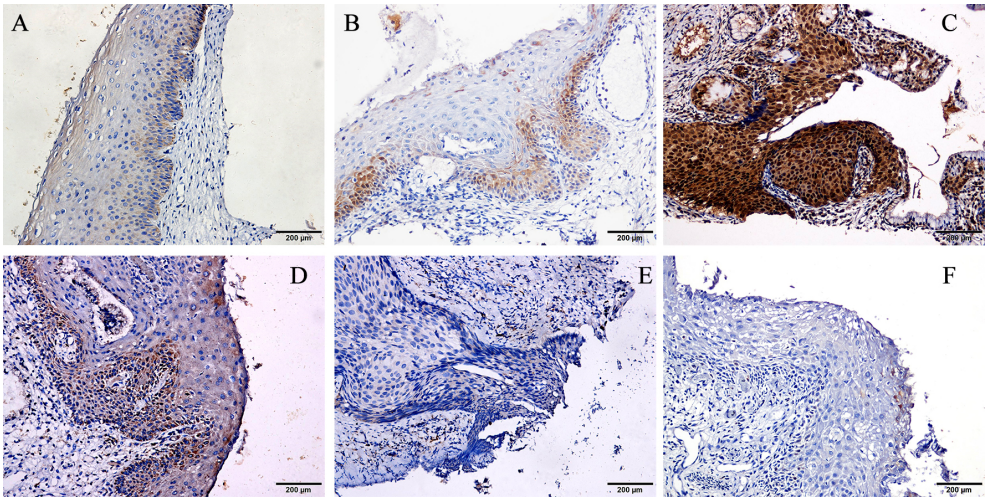

Fig. 2. Representative images of immunohistochemistry (IHC) of cervical samples obtained from normal women and patients with cervical intraepithelial neoplasia (CIN). (A) FHIT expression in CIN3 tissue. (B) FHIT expression in CIN1tissue. (C) FHIT expression in normal tissue. (D) C-MYC expression in CIN3 tissue. (E) C-MYC expression in CIN1 tissue. (F) C-MYC expression in normal tissue. Original magnification $\times$ 200.

tissue, CIN1, CIN2, CIN3 and invasive cancer were 9.4%, 13.6%, 74.5%, 90.4% and 100%, respectively, and there was a tendency for positive rates to be correlated with the grade of cervical lesion ($p < 0.01$). The frequencies and proportions of different staining intensities for FHIT and C-MYC in different cervical lesions are shown in Tables 1 and 2.

Next, ICC was performed to evaluate FHIT and C-MYC expression in HeLa and HEK293 cells. As shown in Fig. 3, strong dark brown positive staining for FHIT was observed in the cytoplasm of HEK293 cells, while only light brown staining was observed in HeLa cells. For C-MYC, strong dark brown positive staining was observed in the nuclei of HeLa cells, while only light brown staining was observed in HEK293 cells. These figures demonstrate that FHIT and C-MYC expression levels are different between normal human cells and cervical carcinoma cells.

Furthermore, we performed ICC on cytology slides obtained from eligible patient samples (see representative images in Fig. 4). This study included 95 patients with CIN1$-$ disease (CIN1 or better) and 102 patients with CIN2+ disease (CIN2 or worse). Their median ages were 40.0 and 42.5 years old, respectively, and age was not significantly different between the groups ($P = 0.483$). The protein expression levels in each

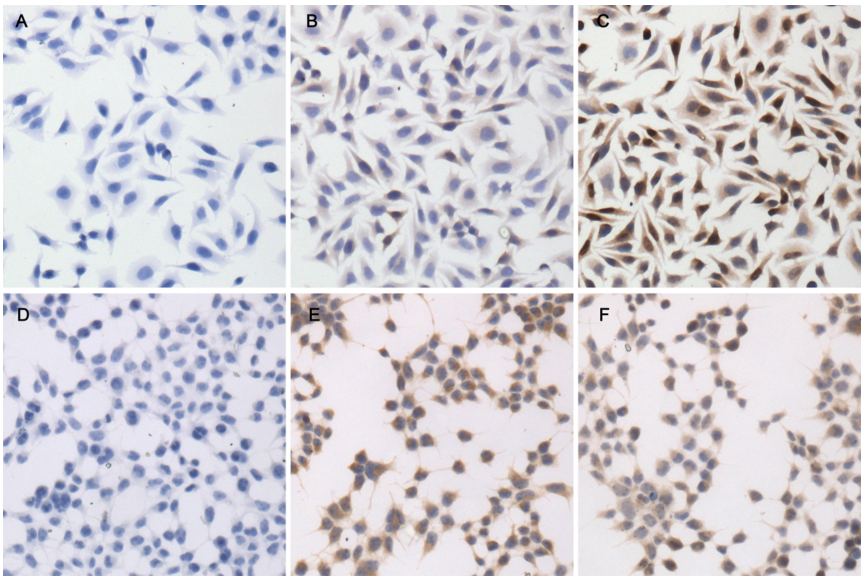

Fig. 3. Representative images of ICC in HeLa and HEK293 cells. (A) Blank control in HeLa cells. (B) FHIT expression in HeLa cells. (C) MYC expression in HeLa cells. (D) Blank control in HEK293 cells. (E) FHIT expression in HEK293 cells. (F) MYC expression in HEK293 cells. Original magnification × 200.

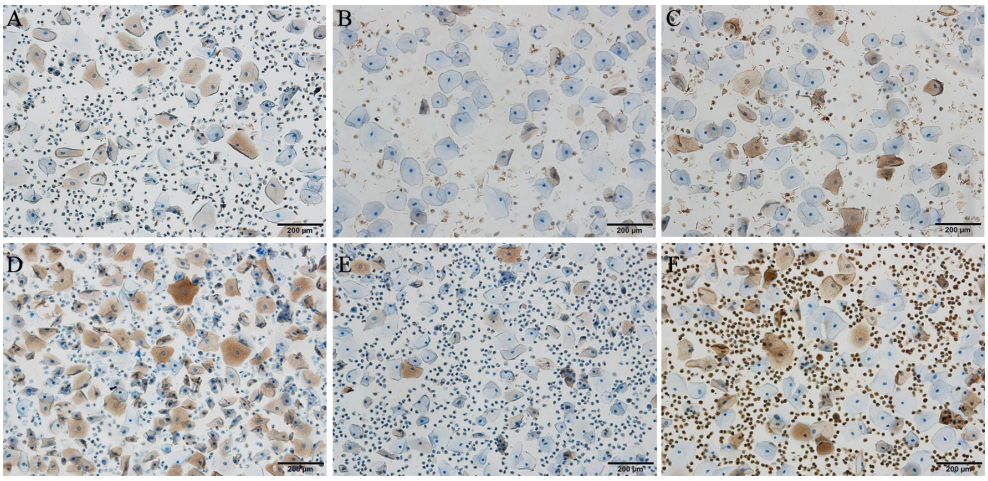

Fig. 4. Representative images of ICC in cervical exfoliated cells obtained from normal women and patients with cervical intraepithelial neoplasia (CIN). (A) FHIT expression in exfoliated cells obtained from normal tissue. (B) FHIT expression in exfoliated cells obtained from CIN1 tissue. (C) FHIT expression in exfoliated cells obtained from CIN3 tissue. (D) C-MYC expression in exfoliated cells obtained from normal tissue. (E) C-MYC expression in exfoliated cells obtained from CIN1 tissue. (F) C-MYC expression in exfoliated cells obtained from CIN3 tissue. Original magnification × 400.

sample are presented as the average staining scores of three random fields. The staining scores were calculated based on the number of stained cells and the staining intensity, as previously described. As shown in Table 3, the protein expression level (staining score) of FHIT was significantly lower in the CIN2+ group than in the CIN1− group, and the protein expression level (staining score) of C-MYC was significantly lower in the CIN2+ group than in the CIN1− group (all $P < 0.001$).

To evaluate the diagnostic efficiency of FHIT and C-MYC ICC staining in cervical exfoliated cells as a strategy for detecting CIN2+, ROC curves were obtained for ICC staining and Pap cytology (LBC) tests (Fig. 5). ICC staining for both FHIT and C-MYC showed advantages over Pap tests with higher AUCs

Table 3
Comparison of FHIT and c-MYC immunocytochemical staining scores in cervical exfoliated cells obtained from patients with CIN1− and CIN2+

Variable	CIN1− ($n = 95$) median (IQR)	CIN2+ ($n = 102$) median (IQR)	P
Age, y	40.0 (35.0–46.0)	42.5 (32.5–47.25)	0.483
FHIT	0.750 (0.500–0.890)	0.310 (0.208–0.510)	$< 0.001^{a}$
C-MYC	0.350 (0.230–0.540)	1.005 (0.578–1.290)	$< 0.001^{a}$

FHIT: fragile histidine triad; CIN: cervical intraepithelial neoplasia; CIN1−: CIN1 or better; CIN2+: CIN2 or worse; IQR: interquartile range. P^{a} value from the nonparametric Mann-Whitney U test.

Table 4
Results of FHIT and c-MYC immunocytochemical staining and LCT in different cervical lesions

Subgroup	Result	FHIT, no (%)	C-MYC, no (%)	LCT, no (%)
Normal ($n = 73$)	+	17 (23.29)	14 (19.18)	13 (17.81)
	−	56 (76.71)	59 (80.82)	60 (82.19)
CIN1 ($n = 22$)	+	7 (31.82)	5 (22.73)	8 (36.36)
	−	15 (68.18)	17 (77.27)	14 (63.64)
CIN2 ($n = 47$)	+	33 (70.21)	30 (63.83)	26 (55.32)
	−	14 (29.79)	17 (36.17)	21 (44.68)
CIN3 and ICC ($n = 55$)	+	48 (87.27)	45 (81.82)	44 (80.00)
	−	7 (12.73)	10 (18.18)	11 (20.00)

FHIT: fragile histidine triad; LCT: liquid-based cytology test; CIN: cervical intraepithelial neoplasia; ICC: invasive cervical cancer.

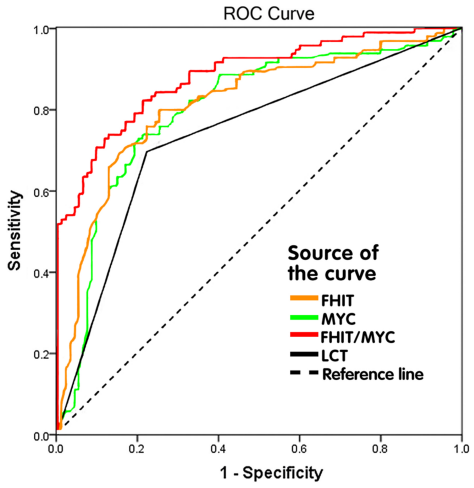

Fig. 5. The receiver operating characteristic (ROC) curves of immunocytochemistry (ICC) staining and a liquid-based cytology test (LCT) for detecting high-grade cervical intraepithelial neoplasia (CIN).

(0.805 and 0.814 vs 0.723, $p < 0.001$). Based on the maximum Youden index (YI), optimal cut-off points for each testing method were chosen as follows: 0.525 for FHIT staining and 0.625 for C-MYC. The results of FHIT and C-MYC ICC staining and Pap tests performed in women in different histological groups (normal, CIN1, CIN2, CIN3, and invasive cancer) are shown in Table 4. Using these cut-off points, the sensitivity was higher for FHIT than the Pap test (79.41% vs 66.67%, $p = 0.04$). Specificity (74.74% vs 76.40%, $p = 0.61$), PPV (77.14% vs 76.40%, $p = 0.90$) and NPV (77.17% vs 68.52%, $p = 0.17$) were not significantly different between FHIT expression and the Pap test. The sensitivity (73.53% vs 66.67%, $p = 0.28$), specificity (80.00% vs 77.89%, $p = 0.72$), PPV (79.79% vs 76.40%, $p = 0.58$) and NPV (73.79% vs 68.52%, $p = 0.40$) were not significantly different between C-MYC expression and the Pap test. Furthermore, Pearson correlation analysis showed that there was a negative correlation between the staining scores obtained for FHIT and C-MYC. We used logistic regression analysis to combine the two biomarkers into one multimarker: FHIT/C-MYC. This combination of these two biomarkers produced a higher AUC value, specificity and PPV than either single biomarker test. Compared to the Pap test, the combined FHIT/C-MYC test had a significantly higher AUC value (0.875 vs 0.723, $p < 0.001$), specificity (89.50% vs 77.89%, $p = 0.03$) and PPV (88.24% vs 76.40%, $p = 0.04$) (Table 5).

5. Discussion

In recent years, the preferred strategy for primary cervical cancer screening has gradually shifted from Pap cytology to the HPV test. Compared to cytology, the HPV test is a highly sensitive and objective test with little interobserver variation. HPV primary screening has been recommended as the pre-

Table 5
Comparison of the diagnostic efficiency of FHIT and c-MYC expression and LCT for detecting high-grade CIN

Test	AUC (95%CI)	P	Sensitivity, %	P	Specificity, %	P	PPV, %	P	NPV, %	P
FHIT	0.805 (0.743–0.867)	< 0.001	79.41	0.04	74,74	0.61	77.14	0.90	77.17	0.17
C-MYC	0.814 (0.752–0.876)	< 0.001	73.53	0.28	80.00	0.72	79.79	0.58	73.79	0.40
FHIT/C-MYC	0.875 (0.825–0.925)	< 0.001	73.50	0.58	89.50	0.03	88.24	0.04	75.89	0.31
LCT	0.723 (0.651–0.795)		66.67		77.89		76.40		68.52	

FHIT: fragile histidine triad; LCT: liquid-based cytology test; CIN: cervical intraepithelial neoplasia; AUC: area under curve; PPV: positive predictive value; NPV: negative predictive value; P value from Pearson's chi-squared test.

ferred strategy for national cervical cancer screening programs in the U.S. and some European countries. According to the 3-year prospective ATHENA study conducted in the U.S., HPV primary screening is as effective as the HPV and Pap cotest screening strategy in detecting CIN3+ and avoids both the complexities and resource expenditure associated with a cotesting strategy [18]. However, the HPV test also has limitations, such as poor positive predictive value and high colposcopy referral rate, which leads to unnecessary invasive procedures and patient anxiety. While cytology triage could act as a recommended stratified option for HPV-positive women to overcome the limitations of the HPV primary test, cytology is flawed because of its poor reproducibility and reliance on well-trained cytologists. Therefore, it is important to explore a new additional stratified screening strategy for HPV-positive women.

ICC staining is ideal as a tool for stratified screening triage in HPV-positive women. ICC staining is conducted using liquid-based cytology slides prepared from cervical exfoliated cells, which are easy to obtain via a minimally invasive procedure and relatively inexpensive to process. Furthermore, the evaluation of ICC avoids the need to interpret cell morphological changes and could be automated by computer, thereby reducing interobserver discrepancies and dependence on cytologists. A number of ICC molecular biomarkers have been proposed for cervical cancer screening. However, a qualified biomarker should reflect the integration status of HR-HPV as well as the level of cellular atypia, rather than the presence of HR-HPV. P16 and Ki-67 immunostaining of cervical cytology specimens has become one of the most promising triage techniques being used in cervical cancer screening in recent years. Coexpression of the anti-proliferative p16 protein and the proliferation marker Ki-67 indicates high-risk HPV DNA expression and HPV-induced cell cycle deregulation [19]. Many studies have confirmed that p16/Ki-67 dual-stained cytological specimens have high specificity for CIN3+ [20,21]. Currently, p16/Ki-67 dual-staining cytology is limited to being used as a supplementary test for Pap cytology when pathologists require diagnostic confirmation, and this approach is not yet good enough to replace Pap cytology as a stratified management option for HPV-positive women.

FHIT and C-MYC are two hot spot genes shown to be located in high frequency loci for HPV integration in previous study. FHIT, which is located at 3p14.2, works as a tumor suppressor gene and is frequently reduced in expression in many types of malignancies, such as lung cancer [22], esophageal cancer [23], and oropharyngeal squamous cell carcinoma [24]. FHIT gene inactivation was found to be strongly correlated with 5'-CpG island hypermethylation, and reduced FHIT expression was significantly correlated with the transition of CIN to cervical cancer [25]. C-MYC, which located at 8q24, encodes a member of the helix-loop-helix/leucine zipper oncogenic transcription factor family. The transcription factor C-MYC regulates a variety of cellular processes associated with immortalization and transformation, such as the cell cycle, cell differentiation, metabolism, angiogenesis, and genomic instability [26–29]. Previous reports showed that C-MYC was upregulated in CIN and cervical cancer specimens, and that the C-MYC amplification rate increased with the CIN grade [30]. These findings imply that variation in FHIT and C-MYC expression is associated with the severity of cervical lesions and plays an important role in CIN evolution to cervical cancer. As far as we know, this is the first study to show that ICC for FHIT and C-MYC could be used for cervical cancer screening. We used ROC curve analysis to explore the potential of FHIT and C-MYC as diagnostic biomarkers to differentiate low-grade CIN from high-grade CIN. Compared to Pap cytology, FHIT detection had a higher AUC and significantly higher sensitivity in identifying high-grade CIN, whereas C-MYC showed no such significant difference in diagnostic performance parameters except for in AUC values. The combination of these biomarkers further improved the AUC, specificity and PPV further without decreasing sensitivity and NPV. The combination FHIT/C-MYC test had significantly higher specificity

and PPV than was found for any individual test or Pap cytology, which is very appealing for stratified screening triage in HR-HPV positive women.

There are some limitations to our study. The study population was composed of women who asked for cervical lesion screening in outpatient clinics, and the screening results were all HR-HPV-positive. This may have led to biases, such as higher HSIL and CIN3+ proportions in recruited women than in the general population. The cut-off points that we chose in this study might not work well for screening in the general population and should be adjusted if they are applied in clinical practice. Additionally, although C-MYC expression was confirmed to be altered by HPV integration near its gene region and the protein level of C-MYC was previously shown to be higher during the transition from low-grade CIN to high-grade CIN, we did not find a significant difference in diagnostic performance between C-MYC expression and Pap cytology. This could be due to the subjectivity of ICC interpretation and a lack of sufficient recruited subjects. Furthermore, we compared diagnostic performance only between the two evaluated biomarkers and Pap cytology but no other recommended triages, such as p16/Ki67 dual-staining cytology. Further well-designed studies performed in a larger general population will need to compare results between these assays and p16/Ki67 dual-staining cytology to validate the conclusions of our study. Despite these limitations, our study also has some advantages. The ICCs were prepared from residual samples in liquid-based cytology test (LCT) and did not require morphological evaluation by cytologists, thus simplifying procedures and reducing subjectivity. Moreover, the interpretation of ICC for FHIT and C-MYC can be automated by computer. With the development of new technologies, especially artificial intelligence, computer-assisted imaging automatic recognition will continue to make great contributions to cervical cancer screening, and ICC for FHIT and C-MYC could be a potential additional option for stratified screening of HPV-positive women.

In conclusion, our study demonstrates that in histology and cytology samples, FHIT and C-MYC protein expression is correlated with the severity of cervical lesions during the transition from low-grade CIN to high-grade CIN. The utility of FHIT and C-MYC ICC staining in cervical exfoliated cells for detecting CIN2+ in HR-HPV positive women had superior AUC values than were obtained in the LBC test. The sensitivity (but not the specificity) of FHIT was significantly higher than that of the LBC test. Sensitivity and specificity were not significantly different between C-MYC expression and the LBC test. Compared with the LBC test, The combined FHIT and C-MYC ICC staining test achieved superior diagnostic potential (both specificity and PPV) in detecting CIN2+ and may improve the clinical performance of cervical cancer stratified screening.

Acknowledgments

We appreciate the help of Prof. Li Yu and Dr. Tian Tian for pathological diagnoses and guidance.

Conflict of interest

The authors report no conflicts of interest.

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