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Cytological Immunostaining of HMGA2, LRP1B, and TP63 as Potential Biomarkers for Triaging Human Papillomavirus-Positive Women[1]

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Abstract

Background: Since human papillomavirus (HPV) DNA testing has been promoted as primary screening strategy, the triage method has also evolved from morphological testing to a molecular biomarker detection to improve screening efficiency. In this study, we investigated the performance of three HPV integration hot-spots, HMGA2, LRP1B, and TP63, as potential triage markers in HPV screening tests. *Materials and Methods:* This cross-sectional study was conducted from November 2016 to December 2017 in the First Affiliated Hospital of Sun Yat-sen University. Immunocytochemistry was carried out using residual cervical cell samples from 121 HPV-positive cases (23 normal, 24 cervical intraepithelial neoplasia (CIN) 1, and 74 CIN2+). *Results:* Of the 121 cases, 77 showed completely paired for the three biomarkers. In these 77 cases, receiver operating characteristic (ROC) analysis of HMGA2 showed the best potential for detecting CIN2+ among HPV+ cases (sensitivity 70%; specificity 91.89%; AUC 0.839). TP63 was second most effective biomarker (AUC 0.838; sensitivity 80%; specificity 81.08%). In contrast, LRP1B had the smallest AUC (0.801) among the three biomarkers but had the highest sensitivity (90%) and specificity (56.76%). To test the triage value of combining the three biomarkers, logistic regression was conducted followed by ROC comparison analysis. Promisingly, the combination of the three biomarkers gave the largest AUC of 0.951 with 92.5% sensitivity and 89.1% specificity ($P < .0001$ compared to liquid-based cytology test by Z-test). *Conclusions:* A combination of HMGA2, LRP1B, and TP63 as potential biomarkers may be useful for screening during triage of HPV-positive patients, particularly for detecting CIN2 + .

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Introduction

Cervical cancer is the fourth most common cancer in females worldwide and corresponding screening methods have been evolving for nearly half a century. Currently [1], HPV and cytology co-testing are the main screening methods utilized in most developing countries. However, since 2015, developed countries have gradually implemented the HPV test alone as the primary screening vehicle with flexible cytology triage to provide stronger risk-based stratification effects [2], which attributes to high sensitivity of HPV test. Another benefit of this strategy is that a reproducible HPV test as primary method significantly decreases diagnostic failure caused by the subjective judgment of cytologists, contributing to low-quality screening outcomes [3,4]. However, the high sensitivity also brings up adverse impact of overdiagnosis and overtreatment and further management strategies for HPV-positive patients must be developed, especially to young women. For triage options, cytology test alone was reported reduction in specificity [5] while the sensitivity was still weak [6]. Therefore, it is important to develop additional triage methods to further improve screening strategies.

According to our previous study, HPV integration plays an important role in the progression of cervical lesions and is incorporated at hot spots in the host genome [7]. Integration at hot spots may result in downregulation of tumor suppresser genes such as low-density lipoprotein receptor family (LRP1B) [7] and elevate the expression of oncogenes such as the high mobility group A2 (HMGA2) gene [7] and tumor protein 63 (TP63, isoform ΔNp63 confirmed as oncogene) [8,9]. These genes may serve as potential risk-stratified predictors of cervical cancer.

In this study, we evaluated the levels of three biomarkers in HPV-positive patients by immunocytochemistry to determine if they can be used to inform triage.

Materials and Methods

Study Design

Selected subjects were women attending the outpatient clinic and cervical cancer screening center between November 2016 and December 2017 in the First Affiliated Hospital of Sun Yat-sen University (Figure 1). Patients were co-tested for HPV and liquid-based cytology test (LCT) or they had equivalent HPV test results from another medical institution. Women who were older than 65 years, had been tested repeatedly, or were administered treatment were excluded. Two hundred twenty-seven HPV-positive women were selected for this cross-sectional study and treated according to the 2016 American College of Obstetricians and Gynecologists guidelines. Among the patients who were referred for colposcopy, 121 cases with LCT results were included (23 normal, 24 cervical intraepithelial neoplasia (CIN) I, and 74 CIN2+), and cervical cell samples were collected by the pathology department in BD SurePath LBC preservative fluid (BD Diagnostics, Franklin Lakes, NJ, USA) for immunocytochemistry analysis. Additionally, 10 HPV + LCT- cases without colposcopy referral were used as controls for pretesting. This study was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University (No. [2016]177) and all the participants gave their consent for sampling.

Immunohistochemistry

Tissue microarrays CIN481, CIN1201, CR501, and BC10025 were obtained from Alenabio Biotechnology (Xi'an, China) and Fanpu Biotech (Guilin, China) (Table 1). First, the tissue microarray slides were baked at 65 °C for 1 h, and then deparaffinized in xylene and passed through graded alcohol followed by antigen retrieval with

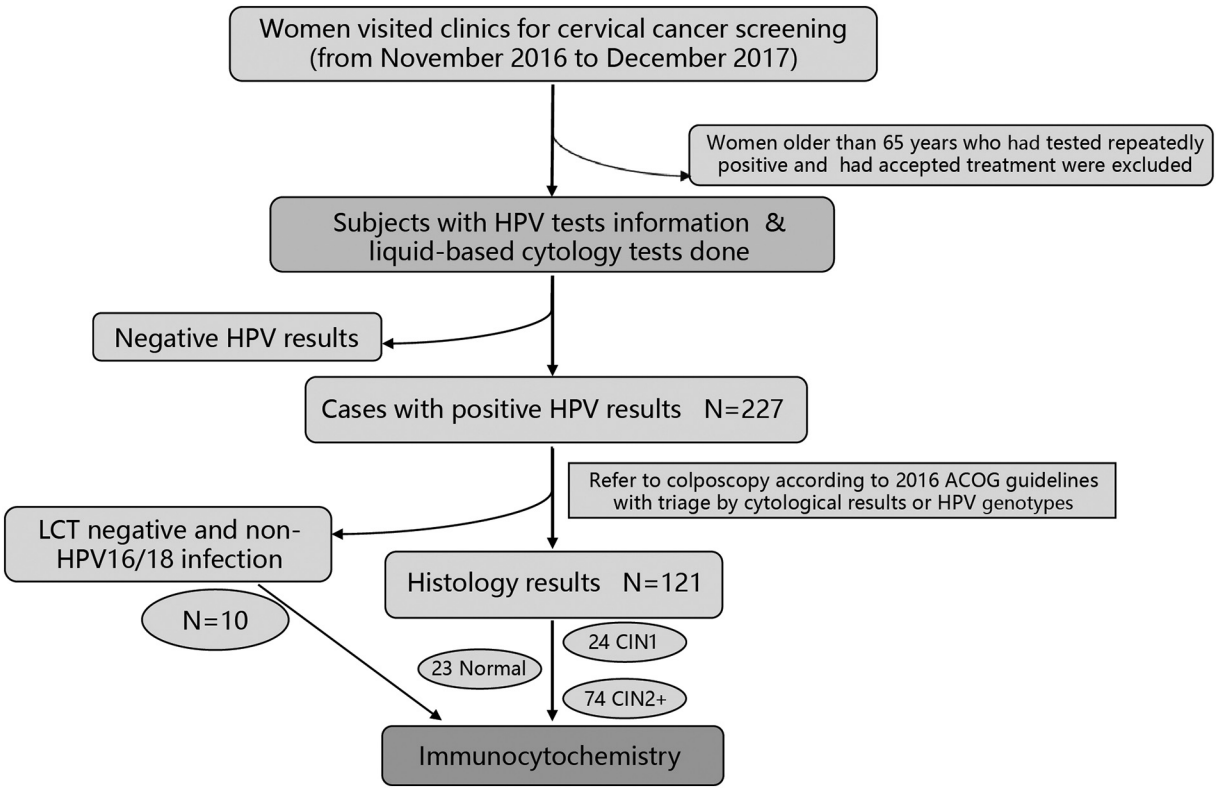

Figure 1. Flowchart of the study. Women with both HPV and LCT test performed at approximately at the same time were chosen. Coloscopy was performed in 121 of the 227 HPV+ patients, with 23 reported as normal, 24 as CIN1, and 74 as CIN2+. Ten cases of HPV+ but negative LCT results were selected as controls. Immunocytochemistry was conducted on 131 samples.

1 mM EDTA, pH 8.0 (Invitrogen, Carlsbad, CA, USA) in a steam pressure cooker at over 120 °C for 2 min. The slides were treated with 3% H_2O_2 to block endogenous peroxidase for 30 min and washed carefully in phosphate-buffered saline with 10% Tween. The slides were first incubated with diluted rabbit anti-LRP1B/TP63 [8] / HMGA2 [10] antibody (LRP1B, ATLAS: HP 1069094, 1:100; TP63, GeneTex, Irvine, CA, USA: GTX102425, 1:350; HMGA2, Protein Tech, Rocky Hill, NJ, USA: 20795-I-AP, 1:250) overnight at 4 °C. After carefully washing the slide with PBS, they were treated with Pika general antibody (Gene Tech, GK500705, Shanghai, China; horseradish peroxidase-conjugated rabbit/mouse antibody) for 30 min, and 3, 3′ diaminobenzidine chromogen was used to detect expression within 3 min. Finally, the slides were counterstained with hematoxylin, dehydrated, and covered.

The histological score (H-score) was used to evaluate the performance of tissue staining. Specifically, immuno-intensity was defined as negative (0), weak (1+), moderate (2+), and strong (3+). The H-score consisted of a sum of the combination of immunoreactivity for intensity multiplied by percentages. The possible range of the H-score was 0–300.

Table 1. Histology information of tissue microarrays CIN481, CIN1201, CR501, and BC10025

Tissue microarray	CIN481	CIN1201	CR501	BC10025
Normal or inflammation	4	10	4	4
CIN1	12	51	-	-
CIN2	11	16	-	-
CIN3	9	20	-	-
Squamous cell carcinoma	10	4	40	30
Adenocarcinoma	2	1	2	-
Adenosquamous carcinoma	-	-	4	10
Cancer adjacent tissue	-	-	-	10
Total	48	102	50	54

Immunocytochemistry

To perform immunocytochemistry analysis of the 121 cervical cell samples, similar procedures and dilution ratios of the three biomarkers were used for immunohistochemistry. Briefly cytological slides of liquid-based cytology were prepared by a ThinPrep 2000 processor (Cytyc Corporation, Boxborough, MA, USA). All slides were used for immunocytochemistry on the same day as they were prepared without baking and deparaffinization. Additionally, 0.5% Triton X-100 (Solarbio, Beijing, China) was added to slides, and the slides were incubated for 30 min before blocking with peroxidase.

The same microscope (Axio Imager Z1, 2010A731, Zeiss, Oberkochen, Germany) with a digital camera was used for all cytological staining evaluation. For each slide, the area with the largest number of cells and highly-stained signal was selected for semi-quantitative analysis under 100× magnification. Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA) was used to calculate the integral optical density (IOD) of selected area. The Log-transformed sum IOD value (Log (IOD[SUM])) of each slide was used for further statistical analysis.

Statistical Analysis

Statistically significant differences ($P < .05$) were evaluated by Kruskal-Wallis H-test and Bonferroni comparisons using GraphPad Prism software version 7.0 (GraphPad, Inc., San Diego, CA, USA) as

well as graphing. Significant degrees were marked as * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .0001$. For each biomarker, a receiver operating characteristic (ROC) was generated with CIN1+ and CIN2+ as the diagnostic results. To examine the combination performance for detecting CIN, stepwise multiple logistic regression was used to construct a model. ROC and logistic regression analyses were performed with MedCalc software (version 15.2.2, Ostend, Belgium).

The optimal cutoff value of each biomarker was determined by the maximal Youden index. The area under the ROC curve (AUC) was used to determine the diagnostic effect with 95% confidence intervals of the proportions calculated. The Z test was applied to compare ROC models ($P < .05$ was considered as significant). For the Pap test, ASCUS+ was used as the cutoff value.

Results

The Three Biomarkers Were Differentially Expressed in Cervical Lesion Tissues

Three slides of CIN tissue microarrays were used for immunohistochemistry analysis to analyze the expression of LRP1B, HMGA2, and TP63. Generally, the expression levels of HMGA2 and TP63 were elevated along with the progression of cervical lesions. Compared to HMGA2, TP63 showed stronger expression which was useful for predicting cervical pathologic grades ($P < .0001$ and 0.0036 respectively) (Figure 2). However, HMGA2 showed a strong ability to distinguish between CIN1 and CIN2+. Specifically, the significance values (*P*-value) of the stratification effect for HMGA2 and TP63 for detecting CIN2+ were 0.0126 and 0.0221, respectively

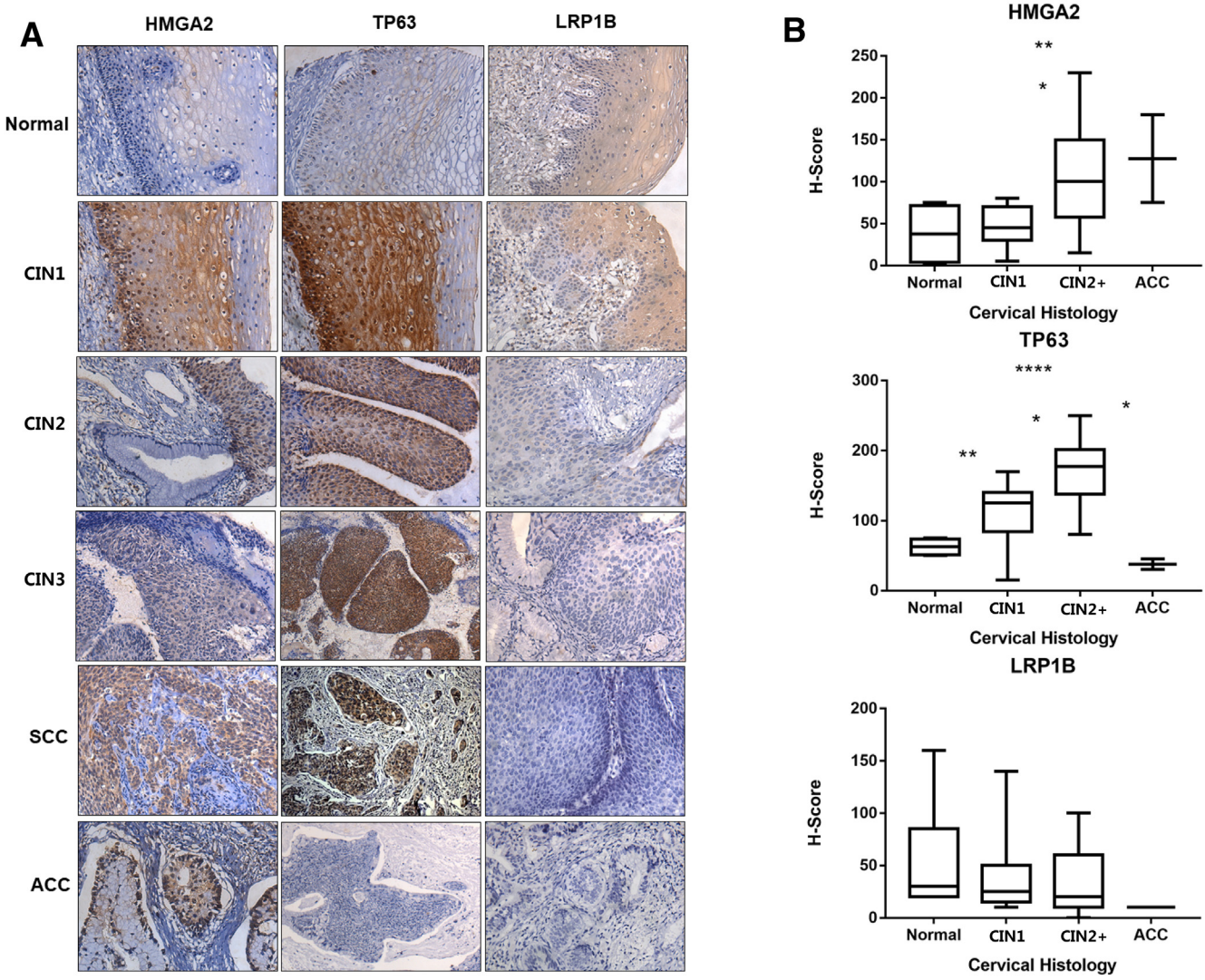

Figure 2. Immunohistochemistry performance of three biomarkers. A, Representative immunohistochemistry results of HMGA2, TP63, and LRP1B in tissue microarrays CIN 481 and CIN 1201. The expression of HMGA2 and TP63 increased along with the lesion degree, while LRP1B exhibited a tumor suppressor pattern. B, H-Score method evaluated the performance of the three biomarkers semi-quantitively on corresponding CIN TMA. Kruskal-Wallis H-test with Bonferroni comparisons was used for analysis Expression of HMGA2 and TP63 differed between CIN1 and CIN2+ (*P*-value .0126 and .0221, respectively), while the general effect of TP63 was stronger than that of HMGA2 (*P*-value <.0001 and .0036, respectively).

Table 2. Performance comparison of three biomarkers and LCT for CIN1+ and CIN2+ detection in two different sample groups

Detection CIN1+ (partially paired samples)					
Strategy	AUC (95% CI)	Maximum Youden Index, %	Sensitivity, %	Specificity, %	n
HMGA2	0.754 (0.656–0.836)	0.5730	61.64	95.65	96
TP63	0.799 (0.703–0.875)	0.6087	69.57	91.30	92
LRP1B	0.786 (0.695–0.861)	0.5397	88.75	65.22	103
LCT	0.719 (0.629–0.799)	0.439	61.29	81.61	121
Detection CIN2+ (partially paired samples)					
Strategy	AUC (95% CI)	Maximum Youden Index, %	Sensitivity, %	Specificity, %	n
HMGA2	0.855 (0.769–0.919)	0.6479	74.55	90.24	96
TP63	0.850 (0.761–0.916)	0.638	78.43	85.37	92
LRP1B	0.782 (0.690–0.857)	0.495	88.14	61.36	103
LCT	0.705 (0.613–0.786)	0.4094	67.61	71.33	121
Detection CIN1+ (completely paired samples n = 77)					
Strategy	AUC (95% CI)	Maximum Youden Index, %	Sensitivity, %	Specificity, %	*Z*-test significance (compared to LCT)
HMGA2	0.745 (0.633–0.837)	0.5306	57.41	95.64	0.9642
TP63	0.780 (0.671–0.867)	0.5982	68.52	91.30	0.6305
LRP1B	0.767 (0.656–0.855)	0.5040	85.19	65.22	0.7203
LCT	0.740 (0.628–0.834)	0.4807	61.11	86.96	-
Logistic Combination	0.882 (0.788–0.944)	0.6651	92.59	73.91	0.0332
Detection CIN2+ (completely paired samples n = 77)					
Strategy	AUC (95% CI)	Maximum Youden Index, %	Sensitivity, %	Specificity, %	*Z*-test significance (compared to LCT)
HMGA2	0.839 (0.738–0.913)	0.6189	70	91.89	0.0196
TP63	0.838 (0.736–0.912)	0.6108	80	81.08	0.0289
LRP1B	0.801 (0.695–0.884)	0.4676	90	56.76	0.0374
LCT	0.664 (0.547–0.768)	0.3277	62.5	72.27	-
Logistic Combination	0.951 (0.877–0.987)	0.8169	92.5	89.1	<0.0001

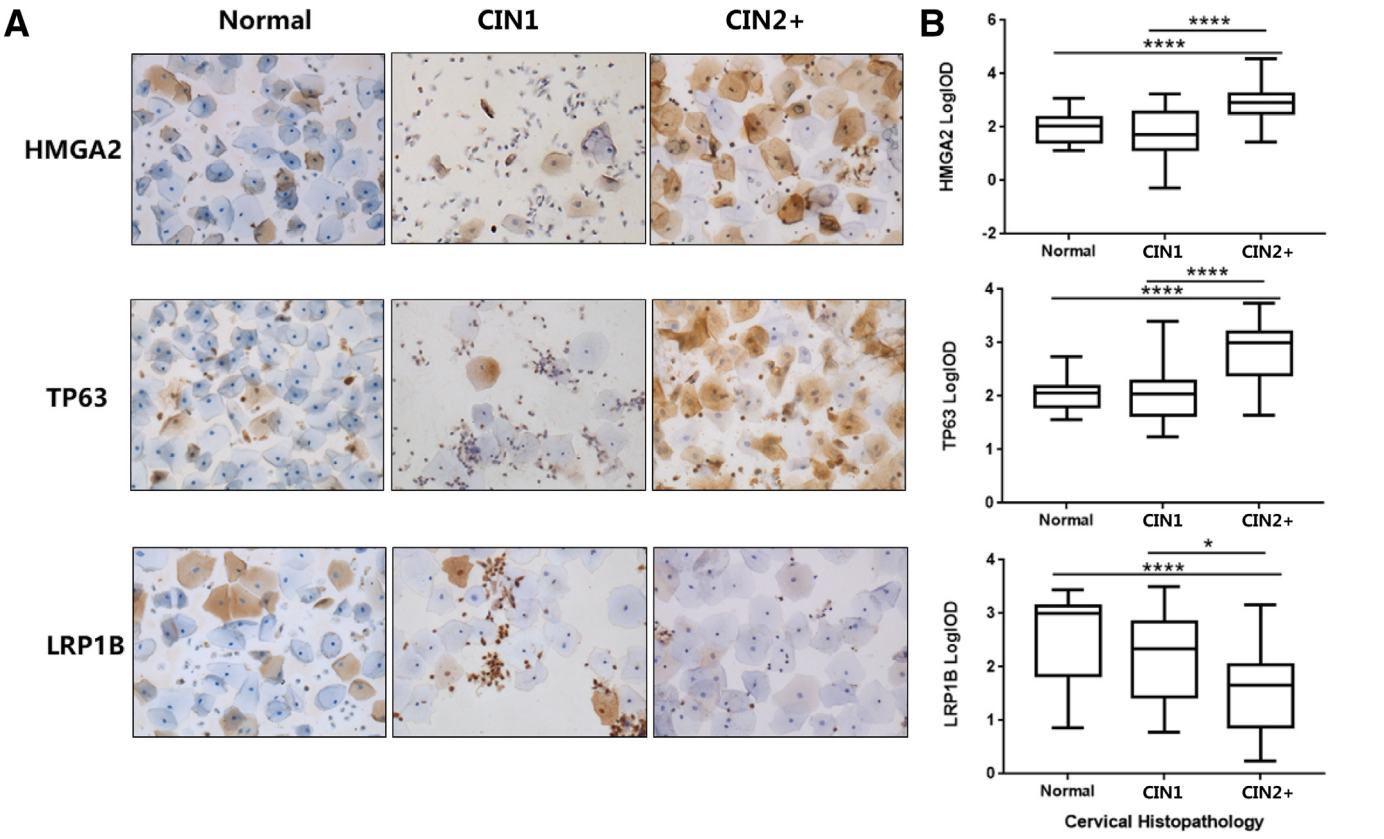

Figure 3. Immunocytochemistry performance of three biomarkers. A, Representative immunocytochemistry of three biomarkers on residual cervical cell samples from normal to CIN2+. Consistent with the immunohistochemistry results, HMGA2 and TP63 acted as oncogenic biomarkers and LRP1B showed a decreased expression pattern along with progression of cervical lesions. B, Image-Pro Plus (IPP) semi-quantitative results of three biomarkers. Log-transformed Sum IOD value (Log (IOD[SUM])) of each slide was used for Kruskal-Wallis H-test with Bonferroni comparisons. Expression of HMGA2 and TP63 significantly differed between CIN1 and CIN2+, as well as normal and CIN2+ ($P < .0001$). LRP1B showed a lower ability to distinguish between CIN1 and CIN2+ (*P*-value .0105).

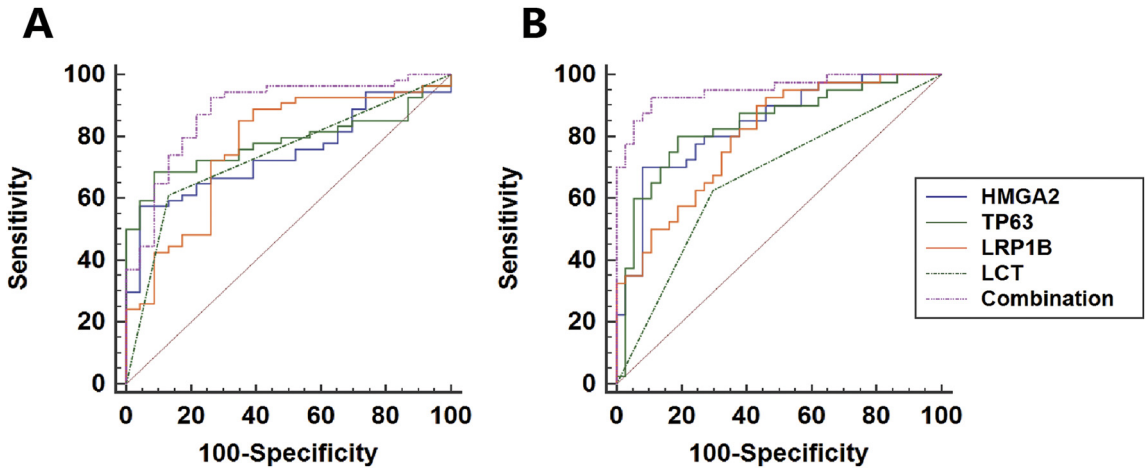

Figure 4. Receiver operating characteristic curve of three biomarkers and LCT for detection of CIN1+ **(A)** and CIN2+ **(B)**.

(Figure 2). Notably, HMGA2 is also expressed in cervical adenocarcinoma (Figure 2). To support this result, we also immunohistochemically evaluated HMGA2 and TP63 in the tissue microarrays BC10025 and CR501, respectively. The intensity score revealed that HMGA2 showed stronger staining in cervical adenocarcinoma and squamous carcinoma compared to in tissue adjacent to cervical carcinoma and normal cervical tissues (S1 Fig). Additionally, TP63 was significantly overexpressed mainly in cervical squamous carcinoma. However, both HMGA2 and TP63 showed weak non-specific expression in the normal cervix, which may contribute to the low sensitivity of this test (Figure 2 and Table 2).

In contrast, LRP1B expression was observed, although staining signal was not strong in the normal cervix, it showed relatively lower levels in CIN. However, the staining of LRP1B showed no significant association with the progression to cervical lesions (Figure 2). Based on this result, we predicted that downregulation of LRP1B occurs in the early stages of cervical morphological changes during the progression from normal cells to CIN. Additionally, LRP1B may serve as a sensitive biomarker for cervical lesion detection. However, its downregulation may be difficult to detect by immunohistochemistry methods.

The immunohistochemistry results of the tissue microarrays indicated that HMGA2 and TP63 can serve as histological diagnostic biomarkers for cervical lesions.

Combining Immunocytochemistry of LRP1B, HMGA2, and TP63 Showed a Stronger Triage Effect than LCT for CIN2+ Detection

To explore the dilution ratio of the three biomarkers, we first tested the immunocytochemistry method using Siha, HeLa, 10 HPV+ LCT- cervical cell samples and HEK293T cell lines. Consistent with the histological results, LRP1B, HMGA2, and TP63 showed the same expression patterns. We then conducted immunochemistry analysis of cervical cell samples using the same dilution ratio. For the 121 HPV+ cases with histology results, we analyzed the slides semi-quantitatively. Consistent with the immunohistochemistry results, HMGA2 and TP63 were significantly overexpressed in CIN2+ compared to in CIN1 or normal cell samples, indicating their potential triage value. Unexpectedly, LRP1B performed better in HPV+ cytology than in cervical lesion tissues, with $P < .0001$ and .0105 for distinguishing normal tissue from CIN2+ and CIN1 from CIN2 respectively (Figure 3).

Importantly, not all of the 121 cases with LCT results were suitable for evaluating the three markers mainly because of the poor condition of the slides. Therefore, we analyzed the ROC in two groups: i) partially paired samples (n = 121) and ii) completely paired samples for three biomarkers (n = 77). For the second group, logistic regression analysis was performed to examine the performance when combining the three biomarkers; the results were compared to those of the *Z*-test (Table 2). The ROC curves were plotted for LRP1B, HMGA2, TP63, combination of the three biomarkers, and LCT results for detecting CIN1+ and CIN2+ (Figure 4).

Notably, the triage effect of LCT in the first group (n = 121) was consistent with that reported previously [5], achieving 61.29% sensitivity and 81.61% specificity for detecting CIN1+ and 67.71% and 71.33%, respectively, for CIN2+. Additionally, the AUC of LCT was larger for detecting CIN1+ than for CIN2+. In contrast, either single or combined biomarker patterns performed better for detecting CIN2+ than for CIN1+. For detecting CIN1+, although the three biomarkers alone and logistic regression of TP63 and LRP1B resulted in a larger AUC than that obtained for LCT, the results of the *Z*-test were not significant. For CIN2+, all three markers and logistic regression of the three biomarkers showed a stronger triage effect than LCT and the logistic regression combination was significant ($P < .0001$) (Table 2).

Specifically, in the group of 77 cases used to detect CIN2+ among the HPV+ cases, the AUC of LCT was 0.664 with a 62.5% sensitivity and 72.27% specificity. For a single biomarker, ROC analysis of HMGA2 showed the best potential (sensitivity 70%; specificity 91.89%; AUC 0.839). Moreover, TP63 was the second-best biomarker with an AUC of 0.838, sensitivity of 80%, and specificity of 81.08%. LRP1B showed the smallest AUC of 0.801 among the three biomarkers but had the highest sensitivity of 90% and specificity of 56.76%. Therefore, HMGA2 and TP63 shared high specificity and relatively low sensitivity, while LRP1B showed high sensitivity and low specificity. Together, the logistic regression of three biomarkers had a comprehensive effect compared to any of three biomarkers for detecting CIN2+ among HPV+ cases (Table 2).

The logistic combination model for detecting CIN1+ involved only TP63 and LRP1B evaluated by stepwise multivariate regression with the variate of $P > .1$ excluded (Logit(p) = −1.40418 LRP1B+ 2.42022 TP63–1.4989, *P*-value of likelihood ratio test <.0001). The AUC, sensitivity, and specificity of this model were 0.882, 92.59%, and 73.91%, respectively, while the logistic model for detecting CIN2+ that included all three biomarkers with AUC, sensitivity, and specificity increased to 0.951, 92.5%, and 89.1%, respectively (Logit(p) = 1.76981 × HMGA2–2.07416 × LRP1B+ 2.11305 × TP63–4.9213, *P*-value of likelihood ratio test <.0001).

In conclusion, a combination of HMGA2, TP63, and LRP1B may be useful as HPV+ triage biomarkers for CIN2+ (Figure 4).

Discussion

Cervical cells collected for screening are a convenient and reliable source of material. Several clinical researches have been conducted to improve cervical cancer screening strategies. Because HPV is necessary but not the only determining factor in the progression to cervical cancer, an increasing number of screening methods have begun to focus on molecular alterations during tumorigenesis [11–15]. For instance, p16/Ki67 and microRNAs have been reported as promising for improving the sensitivity and specificity of HPV-positive triage effects [14,16]. Relative changes in these molecular biomarkers may occur before morphological transformation, which can serve as a better indicator than the Pap test for the triage of patients who are HPV-positive. In this study, we selected our previously identified hot spot integration genes, HMGA2, TP63, and LRP1B, to assess their triage effects by immunocytochemistry and semi-quantitative evaluation. Generally, ROC analysis demonstrated that the semi-quantitative immunostaining values of the three biomarkers gave greater AUC values than the LCT test for distinguishing between both CIN1+ and CIN2+ among HPV+ cases.

HPV integration is a functional step of related carcinogenesis and HPV DNA may randomly scatter in the human genome during the early phases. However, the integration hot spots provide advantages for long-term virus survival and selection, making them predominant in invasive cervical cancers. The carcinogenetic mechanism of these viral-benefit integration hot spots results from downregulation of tumor suppressors or upregulation of oncogenes. Therefore, genes such as HMGA2, TP63, and LRP1B can be used to predict HPV transforming infection, and our results support the application of immunostaining cytology as triage method. Additional molecular tests for identifying HPV integration hot spots should be conducted.

HMGA2 plays an important role in regulating DNA activities in embryogenesis but is not detectable in adult cells [17]. Based on our previous study, HMGA2 was found at 7.8% frequency as a new HPV integration hot spot accompanied by significant upregulation in cervical cancer tissues [7]. A recent study reported dramatically increased expression of HMGA2 in CIN and cervical cancer cells and that it had a stronger diagnostic effect than the HPV 16 copy number. The underlying mechanism is linked to inhibition of cell apoptosis [18]. Additionally, high HMGA2 expression was found to contribute to the metastasis and poor prognosis of various cancers, including gastrointestinal, breast, oral cavity, lung, and ovarian cancers [19–23]. We found that HMGA2 expression slightly different between CIN1 and normal cells in both tissue and cytology analyses (Figures 2 and 3), corresponding to its decreased triage sensitivity (57.41% in the second group in Table 2). However, its significant difference between CIN2+ and CIN1 indicates its potential as a diagnostic tool for detecting CIN2+ among HPV+ cases with 70% sensitivity and 91.89% specificity.

LRP1B is a tumor suppresser gene located in the region 2q22.1–22.2 with 91 exons and a length of more than 500 kb [24]. As previously reported, HPV integrates into the LRP1B intron with a frequency of 5.8%, leading to down-regulation of its expression in cervical cancer [7]. A study suggested that LRP1B inhibits tumor cell growth by increasing Stat3 phosphorylation and $p21^{C1P1}$ levels as well as by inactivating the phosphorylation of *SRC* [25]. Inactivation of LRP1B contributed to tumorigenesis of lung, gastrointestinal, thyroid oral, urothelial, ovarian, and colon cancer [26–30]. In our study, LRP1B did not show a strongly decreased expression pattern from normal to CIN cervical tissues (Figure 2). However, it performed much better in HPV+ cytology by immunostaining as a triage biomarker, indicating a close relationship between HPV infection and LRP1B downregulation. As observed in the larger sample group, the triage effect of LRP1B for CIN1+ and CIN2+ was the same. Additionally, the AUC of LRP1B was not stronger than that of TP63 for detecting CIN1+ and ranked last for detecting CIN2+. Regardless, as a tumor suppresser, its high sensitivity and low specificity for detecting cervical precancers among HPV+ cases may compensate for the limitations of the other two biomarkers, which shared low sensitivity and high specificity.

TP63 is closely associated with cancer progression but its two isoforms, ΔNp63 and Tap63, have different effects. The former is considered as an oncogene that inhibits the tumor suppressive activities of the latter [31]. Given the similarities between TP63 and p53 gene, a previous study showed that Tap63 functions as a tumor suppressor gene by activating major apoptosis pathways such as p53 [32]. In contrast, upregulation of ΔNp63 can induce tumorigenesis by disrupting Tap63 and p53 transactivation function and is related to various types of squamous cell cancer [33,34]. Moreover, TP63 and its position at 3q28 were observed as frequent HPV integration spots in both cervical cancers and oropharyngeal squamous cell carcinomas [9,35,36]. Over-expression of ΔNp63 was reported in cervical cancer and is useful for differentiating cervical lesion severity [15,37]. Based on these findings, we chose ΔNp63 as a potential biomarker. We found that this protein was the primary biomarker for detecting CIN1+ and achieved nearly the same AUC as HMGA2 for CIN2+ prediction, indicating its powerful triage effect.

In this study, we observed expression changes in three biomarkers, HMG2, LRP1B, TP63, immunocytochemically in different degrees of HPV + cervical lesions to evaluate their triage potential. The three biomarkers were all HPV integration hot spots with related expression changes observed in previous studies. Our results demonstrated that combining the three biomarkers for evaluation by logistic regression improved the sensitivity to 92.5% and specificity to 89.1%, which is suitable for triage HPV screening.

However, there were some limitations to our study. Immunocytochemistry analysis lacks objectivity compared to other quantitative molecular tests of DNA, mRNA, protein, and methylation. Additionally, our sample size was small, which could impact our results.

Importantly, the combination of immunocytochemistry and morphological methods may improve the current triage methods for HPV screening. Our study provides an alternative method to traditional cytological tests by taking advantage of the expression of HPV integration hot spots.

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Not applicable.

Declarations of Interest

None.

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