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RAPID COMMUNICATION

HMGB3 is a potential diagnostic marker for early cervical lesion screening

Despite the spread of effective vaccination strategies, cervical cancer remains the second leading cause of cancer death among women aged 20 to 39.[1] Clinical diagnosis of cervical lesions relies on the P16INK4a (P16) marker, but its sensitivity to low-grade cervical intraepithelial neoplasia (CIN) is limited. Exploring more sensitive and specific molecular markers is still a challenge in cervical lesion screening. In this study, we found that HMGB3 could effectively label pathological cells in different cervical lesions, especially for early CIN. Therefore, HMGB3 has the potential to be used as a novel marker for the early screening of cervical lesions.

We extracted and analyzed the differentially expressed genes (DEGs) in different grades of cervical lesions compared with normal tissues from the GEO datasets. Finally, a total of 34 DEGs co-up-regulated and 14 DEGs co-down-regulated were obtained in all four groups (Fig. S1A, B). GO enrichment indicates that these 48 shared DEGs were significantly enriched in complement activation and alternative pathways (Fig. S2A–C), suggesting that the immune system may be actively involved in the development of cervical lesions. The KEGG pathway enrichment also indicates the immune responses involved in the development of cervical lesions (Fig. S2D). The PPI network was constructed to explore the hub genes (Fig. S3A–D). It was shown that core genes were concentrated in EPCAM, MUC1, MUC16, KRT7, *etc*. Some of these genes have already been reported as potential biomarkers for cervical cancer.[2,3]

We extracted the immunohistochemical (IHC) information for these DEGs from the HPA database and ranked them according to the positive rate of IHC staining (Table S1). Some of them have been reported as markers of cervical cancer, such as EPCAM, KRT7,[2–4] and the IHC results also supported these conclusions (Fig. S4A–C). Notably, the positive rate of HMGB3-labeling was the highest among them, showing nuclear staining but is negative in all normal squamous, basal, and glandular cells (Fig. S5), implying that HMGB3 may be an ideal marker.

Next, we examined the expression of HMGB3 in different grades of cervical lesions with a parallel control study of P16. Hematoxylin-eosin (HE) staining was conducted for pathological re-grading, and immunohistochemical staining was performed on different cervical specimens, including normal epithelium, hyperplasia, CIN1, CIN2, CIN3, and cervical squamous cell carcinoma (CSCC) (Fig. 1A). The results showed that both HMGB3 and P16 were absent in the normal cervical epithelium. However, the positive rate of HMGB3-labeling was 79% in hyperplasia, 97% and 95% in CIN1 and CIN2, respectively, and 100% in CIN3 and CSCC. In contrast, the positive rate of P16-labeling was 100% in CSCC, 97% in CIN3, 70% in CIN2, 33% in CIN1, and only 9% in hyperplasia (Fig. 1B and Table S2). Additionally, we also examined the HMGB3 expression in glandular cells under different pathological grades of squamous epithelium. The results showed that the glandular cells were occasionally positive for HMGB3-staining under normal conditions, weakly positive in hyperplasia, and strongly positive in CIN (Fig. S6). HPA records indicated that HMGB3-staining is negative in normal glandular cells (Fig. S5B); the occasional presence of HMGB3-labeling in normal glandular cells in our study may due to the status of inflammatory irritation, as medical records show these cases are associated with cervicitis.

We observed a 79% positive rate of HMGB3-labeling in squamous epithelial hyperplasia. Although simple hyperplasia is not clinically malignant, clinical follow-up is required. We conducted a retrospective study by collecting tissue samples from patients with a history of two visits with the first diagnosis as hyperplasia. Based on the pathological grading indicated by HE staining, all cases could be divided into three evolutionary types, progression, persistence, and regression. The association of HMGB3-labeling status with disease evolution is shown in Figure 1C. Of the total 46 retrospective cases, 37 (80%) were positive for HMGB3-labeling at the first diagnosis. Of these 37 cases, 18 cases progressed, 11 cases were maintained, and 8 cases regressed at the second diagnosis. Importantly, HMGB3-

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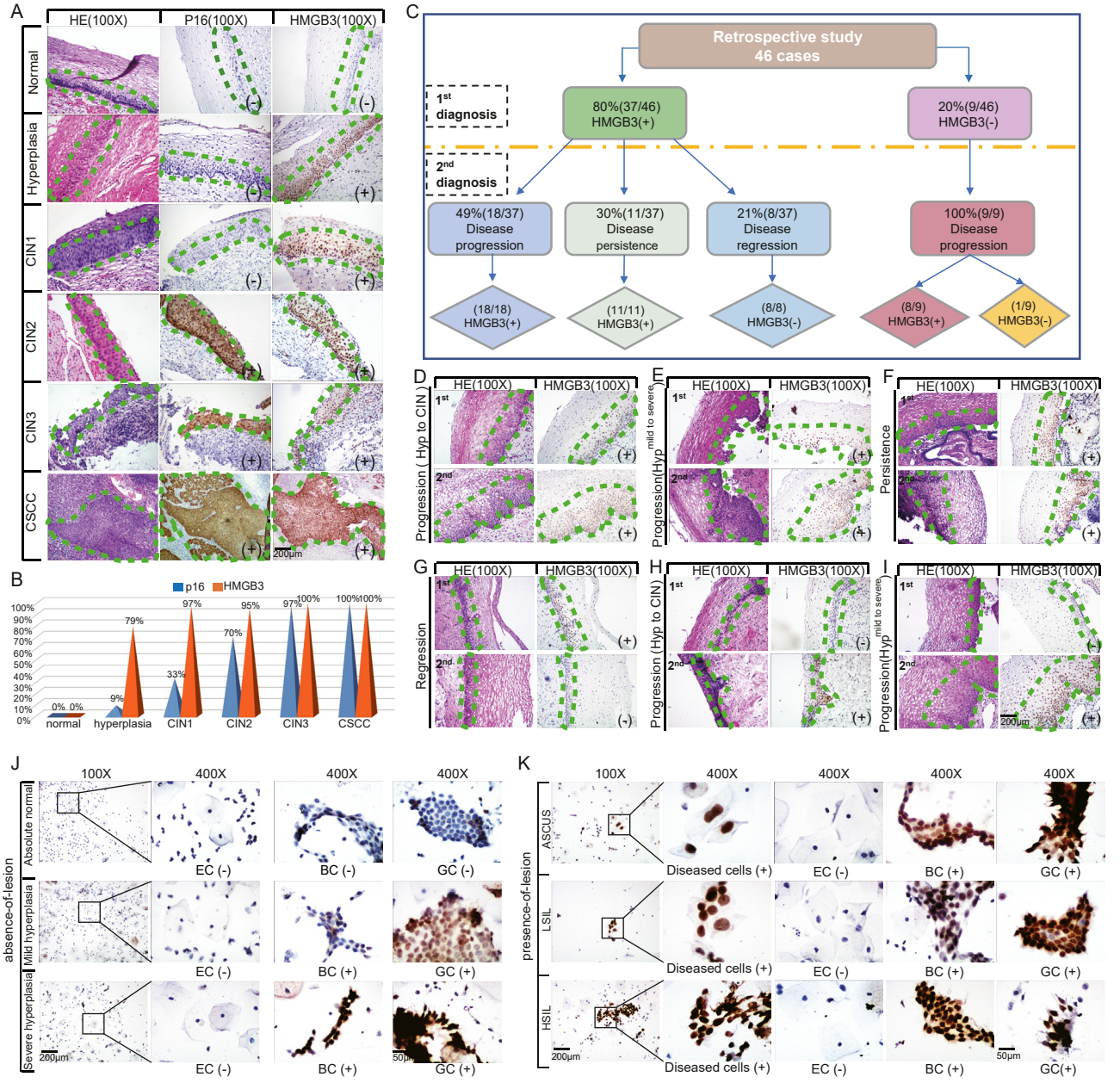

Figure 1 HMGB can effectively label pathological cells in different cervical lesions. **(A)** Representative images of HE staining for cervical tissues at different pathological stages and corresponding IHC assays for P16 and HMGB3. Pathological types for cervical tissue include normal, squamous epithelial hyperplasia, CIN1, CIN2, CIN3, and CSCC. **(B)** The positive rates of HMGB3 and P16-labeling in different pathological stages of cervical tissues were compared. **(C)** Flowchart for the association between HMGB3-labeling and disease evolution. Based on the pathological information obtained by HE staining at the first and second diagnosis, the evolutionary patterns of diseases can be divided into three types, (i): progression, cases that evolve to more severe hyperplasia or CIN; (ii): persistence, cases with a similar hyperplasic degree in both diagnoses; (iii): regression, hyperplasia cases were relieved or regressed on the second diagnosis. **(D—G)** The evolution of Hyperplasia cases with HMGB3-positive staining at the first diagnosis, including (D) progression from hyperplasia to CIN (E) progression to more severe hyperplasia (F) persistence (G) regression. Additionally **(H, I)** the evolution of Hyperplasia cases with HMGB3-negative staining at first diagnosis includes (H) progression from hyperplasia to CIN (I) progression to more severe hyperplasia. Hyp, hyperplasia; CIN, cervical intraepithelial neoplasia. **(J)** The HMGB3-labeling pattern in the absence-of-lesion group for cervical exfoliated cells, included the "Absolute normal subgroup" (all EC, BC, and most of GC were negative but sometimes weakly positive), "Mild hyperplasia subgroup" (negative for EC, weakly positive for BC and GC), and "Severe hyperplasia subgroup" (negative for EC, strongly positive for BC and GC). **(K)** The HMGB3-labeling pattern in the presence-of-lesion group for cervical exfoliated cells, including "ASCUS" (negative for EC, strongly positive for neoplastic cells, BC and GC), "LSIL" (negative for EC, strongly positive for neoplastic cells, BC and GC), "HSIL" (negative for EC, strongly positive for neoplastic cells, BC and GC). EC, epithelial cells; BC, basal cells; GC, glandular cells. The magnification of the images is annotated in the figure.

labeling remained positive in the progressed and persisted cases at the time of the second diagnosis, while it turned negative in all regressed cases (Fig. 1D—G), suggesting that the turnover of HMGB3-labeling from positive to negative indicates the disease regression. In contrast, all 9 patients initially diagnosed HMGB3-negative developed disease progression, with 8 of them turning positive for HMGB3-labeling and only 1 remaining negative (Fig. 1H, I), implying that the turnover of HMGB3-labeling from negative to positive can predict disease progression. Therefore, a follow-up examination of HMGB3 staining can help to determine the prognosis.

As a control, the expression of P16 in these samples was also studied, and the association of P16-labeling status with disease evolution is shown in Figure S7A. At first diagnosis, 7 of the 46 retrospective cases were P16-positive and the remaining 39 cases were P16-negative. Disease progression occurred in 6 of the 7 initial P16-positive cases, and the P16-labeling remained positive in only 4 of them, but it kept positive in 1 regressive case (Fig. S7B a—c). Among the 39 cases initially diagnosed P16-negative, 21 cases progressed at the second follow-up. Among them, only 10 cases turned positive for P16-labeling. Besides, only 2 of the 11 cases with disease persistence turned positive for P16-labeling. In another 7 cases, disease regression occurred and P16-labeling remained negative (Fig. S7B d—f). Therefore, the turnover of P16-labeling was not sufficient in predicting disease prognosis.

We further performed immunocytochemical (ICC) experiments to assess the efficiency of HMGB3-labeling in cervical exfoliated cells. Based on the immunohistochemical results, HMGB3-positive cells in cervical smear are supposed to include hyperplastic basal and glandular cells, as well as various lesion cells. It is clinically considered that the hyperplastic cells are normal cells due to no significant morphological change,[5] according to the diagnostic criteria that cervical lesion cells are nuclear enlargement with increased nucleo-cytoplasmic ratio. Importantly, HMGB3 just stains the nucleus, which could facilitate rapid differentiation of hyperplastic basal/glandular cells from lesion cells. Thus, based on the ICC staining patterns by HMGB3, cervical smears can be divided into two types: absence-of-lesion and presence-of-lesion. In the absence-of-lesion group (Fig. 1J), HMGB3-labeling showed three staining patterns, including (i) "absolute normal subgroup": negative for all cells, including superficial squamous epithelial cells (EC), basal cells (BC), and most of the glandular cells (GC), but weakly positive for GC (occasionally observed); (ii) "mild hyperplasia subgroup": negative for EC, weakly positive for BC and GC; (iii) "severe hyperplasia subgroup": negative for EC, strongly positive for BC and GC (Fig. 1J). Moreover, in the presence-of-lesion group, the nuclear abnormality was observed in lesion cells, which can be classified into subgroups including "ASCUS", "LSIL", and "HSIL" (Fig. 1K). ICC results showed that HMGB3 can efficiently label different grades of lesion cells, including ASCUS (100%, 15/15), LSIL (98%, 43/44), and HSIL (100%, 16/16). Therefore, HMGB3 staining can assist in the identification of lesion cells in cervical smears.

Our study demonstrated that HMGB3 is more effective than the traditional P16 marker in labeling cervical lesions, especially in the early stages of CIN. Additionally, our retrospective study suggests that follow-up monitoring of hyperplastic cases by HMGB3-labeling can predict disease prognosis. Moreover, HMGB3-labeling can facilitate the rapid screening of lesion cells in cervical smears. Therefore, HMGB3 has the potential to replace P16 as a new biomarker for cervical lesion screening.

Ethics declaration

This study was approved by the institutional ethics review committee of Hefei Institutes of Physical Science, Chinese Academy of Sciences (No. SWYX-Y-2021-47 and SWYX-Y-2022-32).

Author contributions

Conception and design: WY and QC; Development of methodology: WY and QC; Data acquisition: QC, JZ, and WY; Analysis, validation, and interpretation of data: WY, QC, JZ, QD, YL, CF, QW, QS, HW, and HD; Writing, review, and/or revision of the manuscript: WY and QC; Administrative, technical, or material support: JZ, QW, CF, QD, QS, and HW; Study supervision: WY. All authors read and approved the final version of the manuscript. The work reported in the paper was performed by the authors unless specified in the text.

Conflict of interests

Ownership interest/patents: Hefei Institutes of Physical Science, Chinese Academy of Sciences. The authors declare that this research was conducted in the absence of any other commercial or financial relationships that could be interpreted as a potential conflict of interest.

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Data availability

The original contributions presented in the study are included in the article or the Supplementary Material. Further inquiries can be directed to the corresponding authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2023.02.033.

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Qianwen Cui [a,b,c], Jiarong Zhao [a,c], Qingmei Deng [a,c], Yu Liu [a,b,c], Changfang Fu [a,b,c,d], Qiang Wu [e], Qiuyan Sun [c], Haiyan Weng [d], Haiming Dai [a,b,c], Wulin Yang [a,b,c,*]

[a] *Anhui Province Key Laboratory of Medical Physics and Technology, Institute of Health and Medical Technology, Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei, Anhui 230031, China*
[b] *Science Island Branch, Graduate School of University of Science and Technology of China, Hefei, Anhui 230026, China*
[c] *Medical Pathology Center, Hefei Cancer Hospital, Chinese Academy of Sciences, Hefei, Anhui 230031, China*
[d] *The First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui 230001, China*
[e] *Department of Pathology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230601, China*

*Corresponding author. Institute of Health and Medical Technology, Hefei Institutes of Physical Science, CAS, No. 350, Shushanhu Road, Hefei, Anhui 230031, China.
E-mail address: yangw@cmpt.ac.cn (W. Yang)

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