Use of peripheral blood transcriptomic biomarkers to distinguish high-grade cervical squamous intraepithelial lesions from low-grade lesions

CUNHUA ZOU[1*], YALI LYU[2*], JING JIANG[3], YUAN CAO[1], MIN WANG[2], CHANGMEI SANG[1], RUIRUI ZHANG[2], HAIFENG LI[1], CHOONG-CHIN LIEW[4-6], CHANGMING CHENG[2] and SHUPING ZHAO[1]

[1]Gynecology Center, Qingdao Women and Children's Hospital, Qingdao, Shandong 266034; [2]R&D Center, Shanghai Homeostasis Bio-Technology Inc., Shanghai 201203; [3]Gynecology Center, Qingdao Lianchi Maternity and Infant Hospital, Qingdao, Shandong 266034; [4]Golden Health Diagnostics Inc., Yancheng, Jiangsu 224000, P.R. China; [5]Department of Clinical Pathology and Laboratory Medicine, University of Toronto, Toronto, ON M5S 1A8, Canada; [6]Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

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Abstract. It is crucial to classify cervical lesions into high-grade squamous intraepithelial lesions (HSILs) and low-grade SILs (LSILs), as LSILs are conservatively treated by observation, based on an expectation of natural regression, whereas HSILs usually require electrosurgical excision. In the present study, peripheral blood gene expression profiles were analyzed to identify transcriptomic biomarkers distinguishing HSILs from LSILs. A total of 102 blood samples were collected from women with cervical SILs (66 HSIL and 36 LSIL) for microarray hybridization. Candidate gene signatures were identified using AdaBoost algorithms, and a predictive model was constructed using logistic regression to differentiate HSILs from LSILs. To correct for possible bias as a result of the limited sample size and to verify the stability of the predictive model, a two-fold cross validation and null set analysis was conducted over 1,000 iterations. The functions of the transcriptomic biomarkers were then analyzed to elucidate the pathogenesis of cervical SIL. A total of 10 transcriptomic genes (*STMN3*, *TRPC4AP*, *DYRK2*, *AGK*, *KIAA0319L*, *GRPEL1*, *ZFC3H1*, *LYL1*, *ITGB1* and *ARHGAP18*) were identified. The predictive model based on the 10-gene panel exhibited well-discriminated power. A cross validation process using known disease status exhibited almost the same performance as that of the predictive model, whereas null-set analysis with randomly reassigned disease status exhibited much lower predictive performance for distinguishing HSILs from LSILs. These biomarkers were involved in the 'Rho GTPase cycle', 'mitochondrial protein import', 'oncogenic MAPK signaling', 'integrin cell surface interaction' and 'signaling by BRAF and RAF fusions'. In conclusion, peripheral blood gene expression analysis is a promising method for distinguishing HSILs from LSILs. The present study proposes 10 candidate genes that could be used in the future as diagnostic biomarkers and potential therapeutic targets for cervical SILs. A simple, non-invasive blood test would be clinically useful in the diagnosis and classification of patients with cervical SILs.

Correspondence to: Dr Shuping Zhao, Gynecology Center, Qingdao Women and Children's Hospital, 6 Tongfu Road, Qingdao, Shandong 266034, P.R. China
E-mail: zhaosp66@126.com

Dr Changming Cheng, R&D Center, Shanghai Homeostasis Bio-Technology Inc., Building 6, 338 Galilei Road, Shanghai 201203, P.R. China
E-mail: cmcheng2005@163.com

*Contributed equally

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Introduction

Precancerous cervical lesions are defined as localized, identifiable cervical lesions that carry an increased risk of developing into cancer, that are treatable and that can be eradicated to prevent the occurrence of cervical cancer. Large population-based screening programs for precursor lesions have been shown to be highly effective in the prevention of cervical cancer (1,2).

Over the past 100 years, however, the definition of 'precancerous cervical lesions' has been vague and variable, from carcinoma *in situ* (CIS) dysplasia to cervical intraepithelial neoplasia (CIN) (3). Furthermore, according to its potential to develop into cervical cancer, CIN can be divided into grades corresponding to mild, moderate or severe dysplasia and CIS (4,5).

In order to better understand the biology and epidemiology of cervical cancer and to improve the consistency of cervical biopsies, a two-tiered system of nomenclature for squamous intraepithelial lesion (SIL) was developed to replace the three-grade CIN classification (6-10). In this system, low-grade

SILs (LSILs) equate to CIN1, and high-grade SILs (HSILs) equate to CIN3 and to most CIN2 lesions. p16-immunohistochemistry (IHC) was used to further classify CIN2 lesions into LSILs (p16-negative) and HSILs (p16-positive) (11-13). Since most LSILs are expected to regress naturally within ~2 years and are conservatively treated by observation, whereas HSILs usually require excision by electrosurgery, it is important to distinguish LSILs from HSILs (6).

Currently the diagnosis of HSILs and LSILs is based on histopathology. In total, 60% of LSILs will regress naturally without treatment, and the lesions are characterized by features that include hyperplasia of squamous epithelial basement and subbasal cells, mild nuclear disorder and mild atypia (6). LSILs are typically limited to the first one-third of the subepithelial layer and show an absence of p16 staining or positive scattered dots in the epithelium. HSILs, which may develop into infiltrating carcinoma, present with nuclear polarity disorder, an increased proportion of nucleoplasm (higher nuclear-to-cytoplasmic ratio) and increased cellular mitosis (6). Atypical cells extend to the subepithelial two-thirds of the epithelium or even throughout the whole epithelial layer with continuously positive p16 staining (6,14). SIL classification requires tissue biopsy, which is uncomfortable and invasive, and causes patients to be less compliant. Furthermore, it is difficult to distinguish human papilloma virus (HPV) infection from unequivocal LSIL, and HPV infection alone is increasingly being included in the category of LSIL by cytopathologists (15). Although HPV testing and the thinprep cytologic test (TCT) has improved the diagnosis of SIL (16), the final diagnosis still relies mainly on the histopathology. A non-invasive and simplified strategy to discriminate between HSILs and LSILs would greatly facilitate the diagnosis and treatment of these lesions.

A blood-based method to diagnose cancer by detecting circulating tumor cells or tumor DNA in peripheral blood has been proposed as a simpler, non-invasive strategy for cancer detection (17). This method, however, is not suitable for detecting precancerous lesions or cancer at early stages, as tumor-derived molecules released from a tiny cancer focus would rarely be detectable or would be undetectable. By contrast, we previously reported a novel 'liquid biopsy' (18) for cancer, using peripheral blood transcriptomic biomarkers in the diagnosis of various non-hematological disorders. As a blood-mRNA based rather than tumor dependent diagnostic, this technique is especially useful for detection in the early and pre-cancerous disease stages (19-22).

The present study uses peripheral blood transcriptome profiling to identify candidate genes for distinguishing HSILs from LSILs. The genes identified in this study may be clinically useful as the basis of a new blood test for the diagnosis of SILs, and may further promote our understanding of the pathophysiology of SILs and cervical cancer.

Materials and methods

Ethics. The present study was approved by the Ethics Committee of the Qingdao Women and Children's Hospital (Qingdao, China; approval no. QFELL-KY-2019-46). Sample acquisition for HSILs and LSILs was conducted between July 2019 and October 2019 at the Qingdao Women and Children's Hospital. All 102 participants, including 66 patients with HSILs and 36 with LSILs, were enrolled and provided written informed consent. The inclusion criteria were as follows: i) Ages from 20-65 years; and ii) HPV infection lasting longer than 6 months. The exclusion criteria were: i) Having autoimmune disease or immunodeficiency disease; ii) having cervical cancer or other malignancy; iii) pregnancy; and iv) having taken drugs affecting immune function within the previous 6 months. The age distribution of the patients is listed in Table SI. In order to verify the effectiveness of this method both for identifying transcriptomic biomarkers for LSIL and HSIL and for differentiating SIL from healthy populations, blood samples were also taken from 65 healthy female volunteers without cervical disease collected between July 2019 and October 2019 at the Qingdao Women and Children's Hospital. All healthy volunteers were recruited from patients who underwent a health examination in the hospital and who provided written informed consent.

Study population. A total of 102 blood samples were collected from the women before undergoing cervical tissue biopsy and before they had undergone any form of treatment, including hormone therapy, radio/chemo-therapy or surgery. SIL was categorized based on surgical pathological examination. LSIL was characterized as the proliferation of basal-like cells extending no more than one-third of the epithelial thickness and with normal mitoses. HSIL was characterized as the proliferative cell compartment extending into the middle one-third or the superficial one-third of the epithelium and with abnormal mitoses. All patients underwent HPV testing and TCT before cervical tissue biopsy.

Blood collection, RNA isolation and RNA quality control. Peripheral whole blood (2.5 ml) was collected in PaxGene Blood RNA tubes (PreAnalytix GmbH) and total RNA was isolated using an accessory PaxGene Blood RNA kit (PreAnalytix GmbH) according to the manufacturer's instructions. The isolated RNA quality was accessed using Agilent 2100 Bioanalyzer RNA 6000 Nano Chips (Agilent Technologies, Inc.). All the samples for microarray analysis met the following quality criteria: RNA integrity number ≥7.0 and 28S:18S rRNA ≥1.0. RNA quantity was determined by a NanoDrop 1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.).

HPV test. A single cervical specimen was collected from each participant using a Rovers Cervex-Brush device (Rovers Medical Devices B.V.), and cells were suspended into BD SurePath collection vials containing preservative solution (Becton, Dickinson and Company), according to the manufacturer's instructions. HPV types, including 7 high-risk types 16/44/45/52/53/58/61, were detected with an Aptima HPV assay targeting E6/E7 mRNA (Aptima; Hologic, Inc.) according to the protocol previously described (23,24). The Aptima Auto Detect kit (Aptima; Hologic Inc.) was used to test these specimens with the Panther Fusion system (Hologic, Inc.), according to the manufacturer's instructions.

TCT test. Exfoliated cervical cells were collected from the ectocervix and endocervix with Rovers Cervex-Brush device

(Rovers Medical Devices B.V.) and were analyzed using TCT tests according to the Bethesda classification system 2009 (25).

Microarray hybridization. Whole blood RNA from the 102 samples (66 HSILs and 36 LSILs) was analyzed using Gene Profiling Array cGMP U133 P2 microarray (Affymetrix; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Gene expression signal intensity was processed using Affymetrix Expression Console software (version 1.4.1; Affymetrix; Thermo Fisher Scientific, Inc.) and normalized by MAS5 normalization, in which the global signal intensity value was adjusted to 500 for each microarray to make it possible to compare profiling variations between microarrays.

Microarray data mining. To identify candidate genes that distinguish HSILs from LSILs, the entire 54,675 microarray probe sets were treated according to the following criteria: i) Only the probe sets reliably detected as 'present' in all of the samples were retained; and ii) the probe sets were included within the MicroArray Quality Control (MAQC) list from MAQC Consortium (26). The intensity values of gene expression signals were then log transformed to conform to a Gaussian distribution.

To select gene expression signatures, the ensemble learning strategy AdaBoost method was employed (27). This data mining method does not make restrictive assumptions on the training set, unlike traditional methods. Instead, AdaBoost creates a series of weak classifiers and then combines them into a single strong classifier by assigning each weak classifier its proper weight. In this way, AdaBoost outperforms existing methods in accuracy and training time (27). In the present study, the final 10 genes were selected using the AdaBoost method and then used to construct the predictive model via a logistic regression algorithm. To evaluate the performance of the predictive model for classifying the HSIL and LSIL groups, the model was characterized by the area under the receiver operating characteristic curve (ROC AUC), the sensitivity, the specificity and the accuracy.

As the sample size in this study was small, it was not practical to evaluate the performance of the predictive model by partitioning the entire group of samples into traditional training and test sets. Instead, as in our previously reported study, a 2-fold cross validation process was conducted to avoid data overfitting (28). Half of the LSIL and HSIL samples were randomly selected as a training fold to generate the predictive model; the remainder of the samples were included into a test fold, for prediction over 1,000 iterations.

An additional problem is that the final 10 genes identified may, due to clinical bias and limited sample size, derive from random chance. To avoid this problem, a null set analysis was performed to verify that the results were not due to chance and to reduce the bias owing to the limited number of HSIL and LSIL samples. The cross validation process was tested once again; the model was used to predict the sample cohorts with disease status randomly reassigned (null set). The distributions of diagnostic parameters of sensitivity, specificity, accuracy and ROC AUC were then compared between the two cross validation processes.

Bioinformatics analysis. Gene Ontology (GO) annotations of the candidate biomarkers were queried from the COXPRESdb v7 database (http://coxpresdb.jp) (29). The proteins interacting with the candidate biomarkers were downloaded from the STRING database (https://string-db.org/) with total confidence ≥0.7. Reactome (https://reactome.org/) pathway enrichment analysis using the clusterProfiler R package (version 3.16.0) (30) was performed on signature genes and their correlative proteins. Reactome pathways were identified with a strict cut-off of adjusted $P<0.05$, corrected with the Benjamini-Hochberg method and with a false-discovery rate (FDR) of <0.05. The visualization of the protein-protein interaction network was performed with Cytoscape software (https://cytoscape.org/; version 3.8.0).

Results

Basic and clinicopathological characteristics of patients with HSILs and LSILs. In the present study, a total of 102 samples were collected, including 66 HSIL and 36 LSIL samples. The mean ages of the two groups were not statistically significantly different (two-tailed Student's t-test; $P=0.0642$). Most patients were distributed between the ages of 31 and 40 years (Table SI).

HPV test information for all patients was summarized, as shown in Table SII. In the HSIL group, HPV16 was the most prevalent HPV type and accounted for more than half of all HSIL patients (37/66, 56.1%). HPV16 also predominated in the LSIL group, but its HPV16-positive rate was only one-fifth that found in the LSIL group (8/36, 22.2%). Except for HPV16, other single HPV subtypes comprised <8% of the total.

Peripheral blood gene expression profiling. Gene expression profiling was performed for peripheral blood samples taken from the 66 patients with HSILs and 36 patients with LSILs. Genome-wide expression profiles generated with Affymetrix Gene Profiling Array cGMP U133 P2 microarray were analyzed and correlated as between HSIL and LSIL. Finally, 10 candidate genes were identified as distinguishing HSILs from LSILs: *STMN3*, *TRPC4AP*, *DYRK2*, *AGK*, *KIAA0319L*, *GRPEL1*, *ZFC3H1*, *LYL1*, *ITGB1* and *ARHGAP18*. The corresponding gene symbols and names of the final 10 probe sets are listed in Table I, as well as the fold-change between the two cohorts.

Model construction and performance estimation. Based on the 10-gene panel identified, a predictive model was constructed for discriminating HSILs from LSILs using logistic regression. Fig. 1 shows the performance of the 10 candidate genes separately and the 10-gene panel as a whole for discriminating HSILs from LSILs for the total 102 samples. Use of the 10-gene panel showed better clustering of the HSIL samples.

As the sample size in the present study was too small to be divided into a training set and a test set, the entire data set was used to construct the model. It was found that a 10-gene panel could discriminate HSILs from LSILs (sensitivity, 81.8%; specificity, 83.3%; accuracy, 82.4%). The 10-gene panel exhibited the highest ROC AUC of 0.90 as compared with that of each of 10 candidate genes (0.61-0.75) separately, as shown in Fig. 2A for the total 102 samples. The box-whisker plot (Fig. 2B) also illustrated a well-separated distribution of

Table I. Final 10 candidate genes for distinguishing HSILs from LSILs.

Probe set	Gene symbol	Gene name	Fold-change
222557_at	STMN3	Stathmin-like 3	1.23
212059_s_at	TRPC4AP	Transient receptor potential cation channel, subfamily C, member 4 associated protein	1.19
202968_s_at	DYRK2	Dual specificity tyrosine-(Y)-phosphorylation regulated kinase 2	1.09
222132_s_at	AGK	Acylglycerol kinase	1.09
222468_at	KIAA0319L	KIAA0319-like	1.08
212434_at	GRPEL1	GrpE-like 1, mitochondrial	1.08
213065_at	ZFC3H1	Zinc finger, C3H1-type containing	1.04
210044_s_at	LYL1	Lymphoblastic leukemia-associated hematopoiesis regulator 1	-1.09
1553530_a_at	ITGB1	Integrin β1	-1.23
225173_at	ARHGAP18	Rho GTPase activating protein 18	-1.23

HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade SIL.

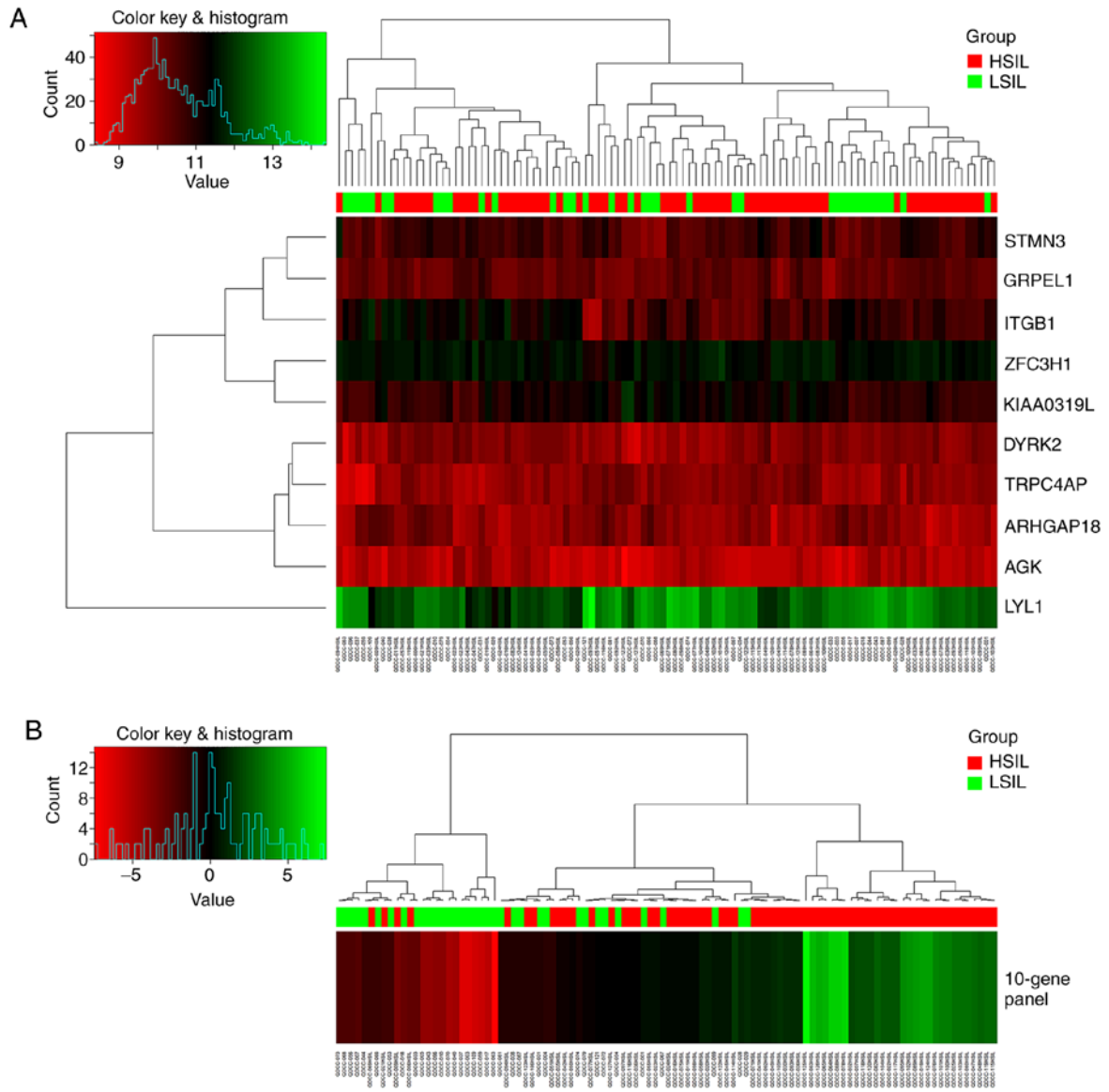

Figure 1. Heat map of gene expression and hierarchical cluster diagram showing the performance of 10 candidate genes and the 10-gene panel for clustering 102 samples in the training set, including 66 HSILs and 36 LSILs. (A) 10 separate candidate genes and (B) the 10-gene panel. The figure was generated using the 'Heatmap' function in R with default settings. HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade SIL.

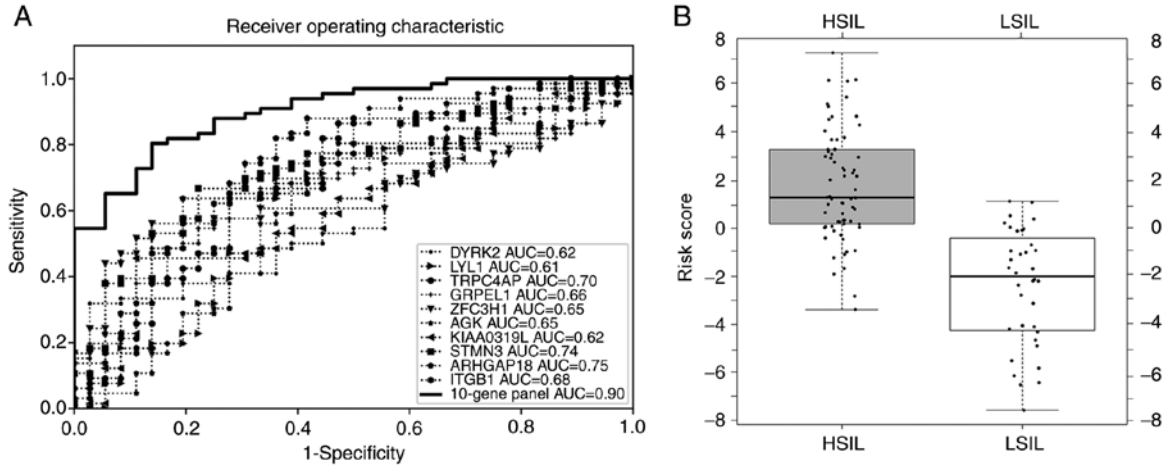

Figure 2. Model evaluation with ROC curve and box-whisker plot. (A) ROC curve for candidate genes of the training set. (B) Box-whisker plot to display the logistic regression scores in HSILs and LSILs in the training set. ROC, receiver operating characteristic; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade SIL; AUC, area under curve.

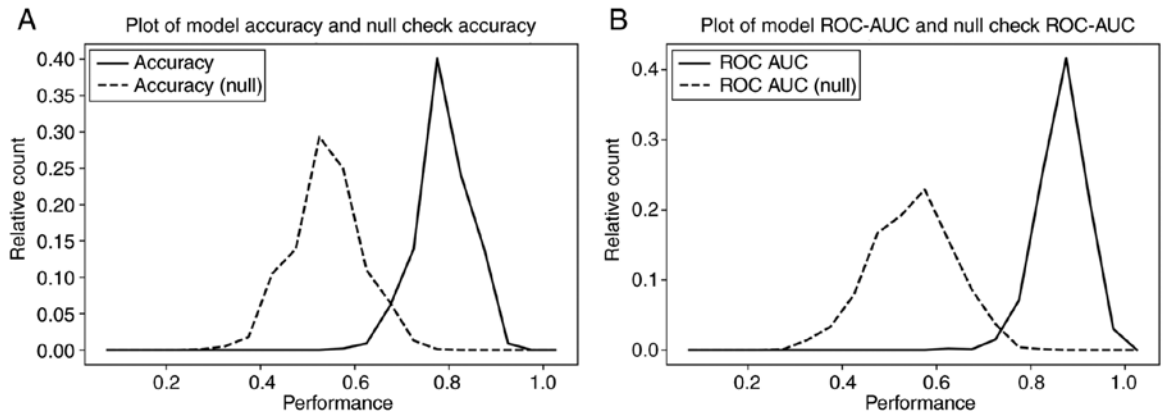

Figure 3. Two-fold cross-validation results over 1,000 iterations based on the known HSIL/LSIL disease status (true set, solid line) and re-assigned disease status (null set, dashed line) of the samples. This chart displays the distributions of (A) accuracy and (B) AUC ROC values over the 1,000 iterations, in which the model accuracy and ROC in the null set analysis were much lower than that in the known HSIL/LSIL status analysis. ROC, receiver operating characteristic; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade SIL; AUC, area under curve.

prediction scores of HSIL and the LSIL, based on the 10-gene panel and logistic regression algorithm.

In order to validate the stability of the model, a 2-fold cross-validation method was performed, iterated 1,000 times. For each iteration, the samples were randomly and equally divided into training and test folds. The predictive model was constructed based on the samples in the training fold and then its performance was evaluated on the sample in the test fold. The distributions of accuracy and AUC ROC values over the 1,000 iterations exhibited good prediction performance, with a model mean accuracy of 82% (Fig. 3A) and ROC AUC of 0.9 (Fig. 3B) (solid line). To rule out the possibility that the final 10-gene panel was merely derived from chance and clinical bias, a null set analysis process was performed for 1,000 iterations, similar to the aforementioned 2-fold cross-validation process, but with HSIL/LSIL status randomly re-assigned. Comparing the prediction results with the known HSIL/LSIL status of the samples, it was found that the null set analysis results were significantly lower, with a model accuracy of 52% (Fig. 3A) and ROC AUC of 0.58 (Fig. 3B) (dashed line). The distribution of each analysis resulted in 2 well-separated curves with <5% overlap, from which it was concluded that the performance of the 10-gene panel for distinguishing HSILs from LSILs was unlikely to be merely the result of random chance.

To further validate efficiency, this data mining method was performed in an analysis of blood-based gene expression profiles of women with cervical SILs and healthy volunteers. Transcriptomic biomarkers were identified and were found to exhibit good performance for differentiating cervical SIL patients from healthy volunteers. Detailed information is presented in Fig. S1 and Table SIII.

Protein-protein interaction and functional enrichment categorization. Proteins interacting among the 10 candidate genes were downloaded from the STRING database individually, with a total confidence ≥0.7, followed by the protein-protein interactions of the total 149 proteins thus identified. The protein-protein interaction network is shown in Fig. 4.

The genes corresponding to the selected 149 proteins were functionally categorized based on Reactome pathways. Reactome pathways were identified with a strict cut-off of

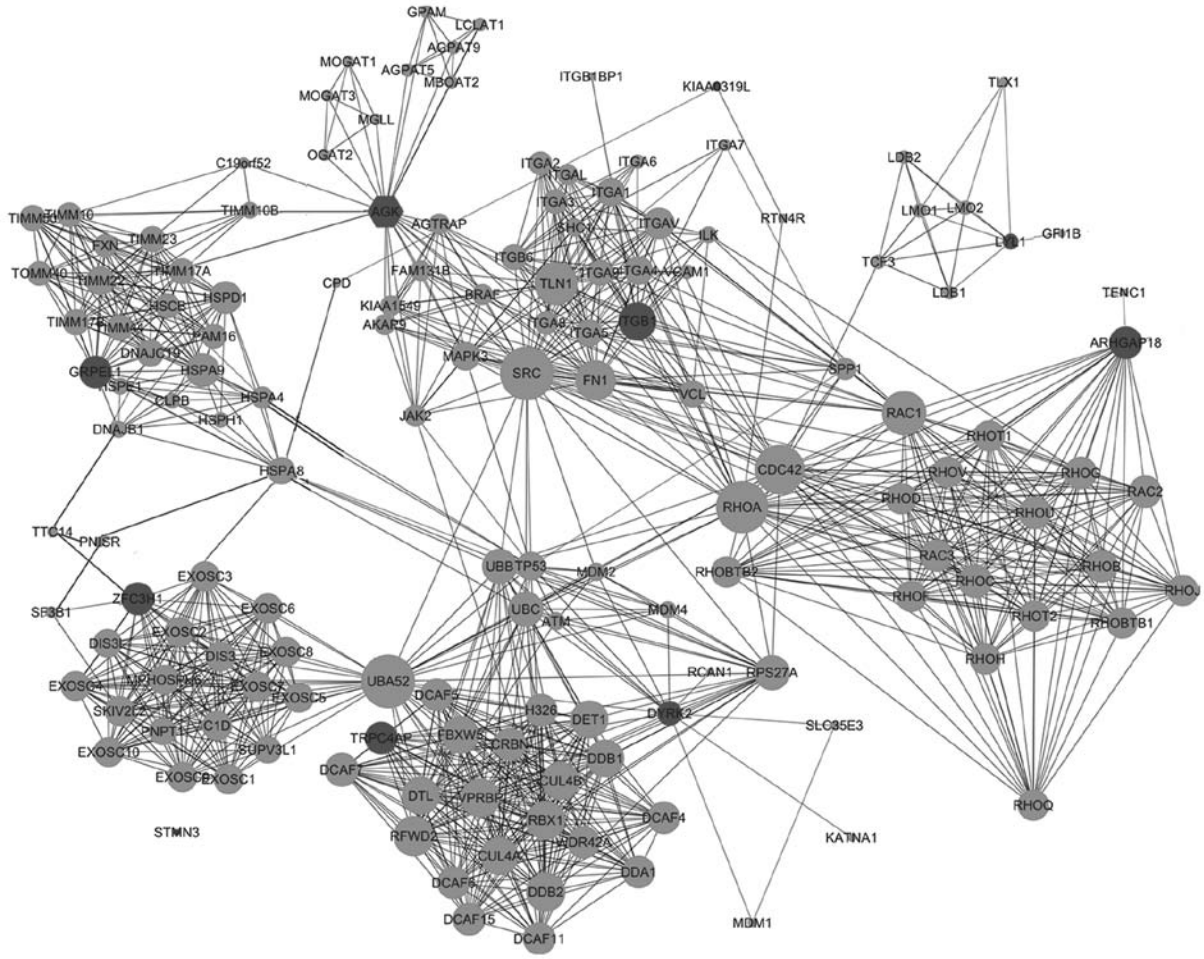

Figure 4. Protein-protein interaction network of the 10 candidate genes for high-grade squamous intraepithelial lesions. Interaction map of 10 transcriptomic biomarkers (dark circles) and their interacting proteins (light circles), with total confidence ≥0.7.

P<0.05 and FDR of <0.05. When the top 10 significantly enriched pathways were analyzed, it was found that the 10 candidate genes and their interactive proteins were mainly engaged in the 'Rho GTPase cycle', 'mitochondrial protein import', 'oncogenic MAPK signaling', 'integrin cell surface interaction' and 'signaling by BRAF and RAF fusions', as shown in Fig. 5A. Since the Rho GTPase proteins act as binary switches by cycling between active GTP-bound and inactive GDP-bound conformations (Rho GTPase cycle) (31), the 'signaling by Rho GTPase' pathway mainly reflects the 'Rho GTPase cycle' and thus could be aligning to the pathway of 'Rho GTPase cycle' in the present study. The interactions of the engaged processes and the related candidate genes of each process are also indicated in Fig. 5B.

Discussion

The present study was undertaken to explore a blood transcriptomic profiling method for distinguishing cervical HSILs from LSILs. Peripheral blood samples of patients with cervical HSILs and LSILs were collected, and blood transcriptomic features were identified using the AdaBoost algorithm. A panel of 10 candidate genes that successfully discriminated cervical HSILs from LSILs, with an accuracy of 82.4%, was identified. Functional enrichment analysis indicated that the candidate genes were mainly involved in the 'Rho GTPase cycle', 'mitochondrial protein import', 'oncogenic MAPK signaling', 'integrin cell surface interaction' and 'signaling by BRAF and RAF fusions'. These preliminary results are promising; however, further research is needed to validate the findings in larger cohorts and in a multi-site validation clinical study.

Most types of cervical cancer are caused by HPV infections, and HPV is detected in ~99.7% of cervical cancer cases (32). Not all HPV infections, however, lead to cervical cancer. Most of these infections are temporary, resulting only in LSILs; it is only persistent HPV infection that may result in an HSIL (33). The comparative risks of progression to cervical cancer differ sharply between HSILs and LSILs (34). From a clinical perspective, the management of the two types of lesion is also different, with HSILs regarded as true cervical cancer precursor lesions, requiring colposcopy and treatment (35). Currently, the diagnosis of HSIL is mainly dependent on colposcopy and biopsy, but neither test is completely satisfactory. A non-invasive test would be welcome to distinguish HSILs from LSILs during the premalignant stage and to prevent cervical cancer.

In current clinical practice, the tools used for cervical cancer screening and diagnosis include HPV testing, cytology testing, colposcopy and cervical biopsy. Although combinations of

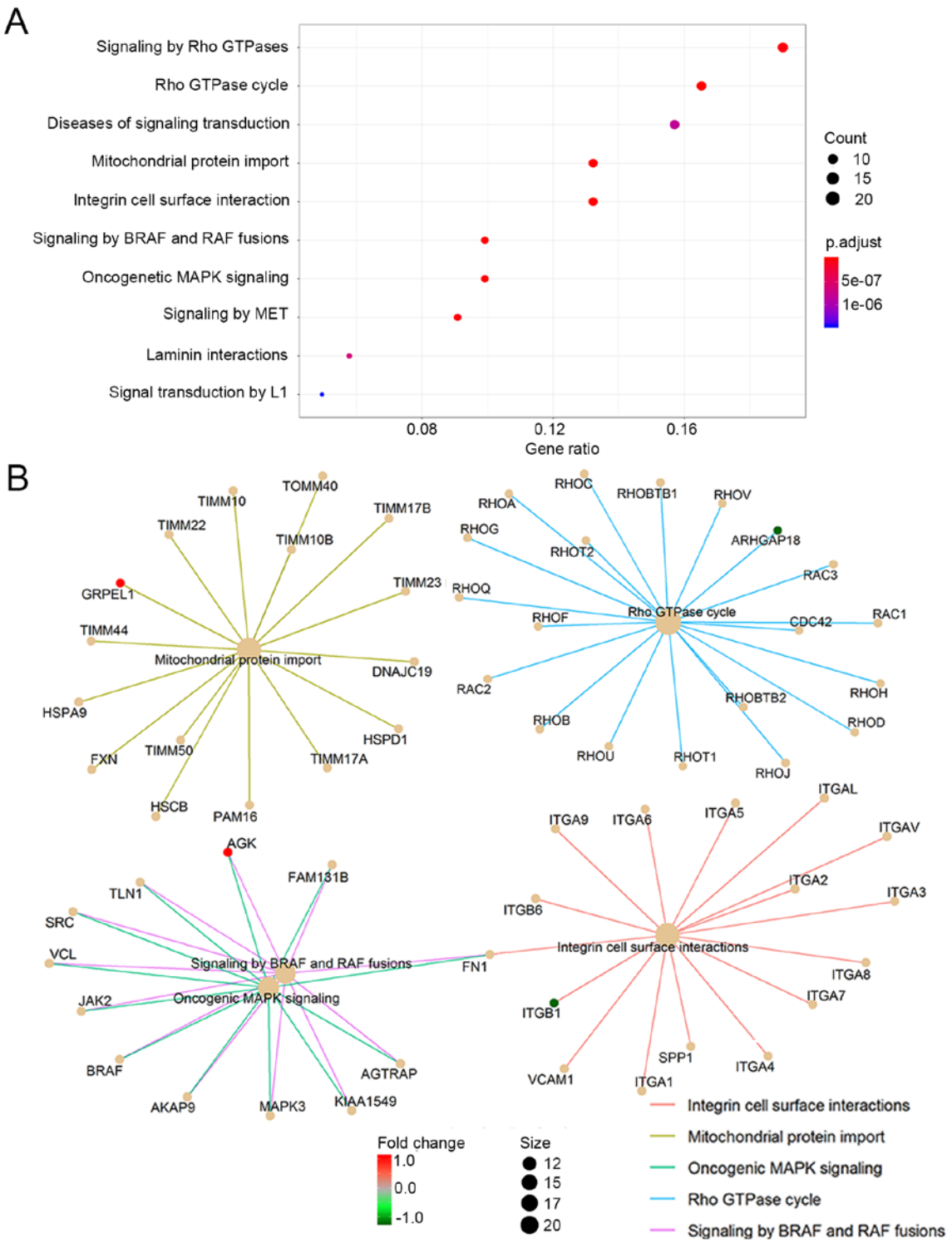

Figure 5. Reactome pathway analysis of the 10 candidate genes. (A) The top 10 significantly enriched Reactome pathways. (B) The top 5 significantly enriched Reactome pathways and their associated genes (red, upregulated candidate genes; green, downregulated candidate genes; light orange, genes corresponding to the proteins interacting with the candidate genes).

different techniques have improved the screening and diagnosis of cervical cancer, the sensitivity, specificity, reliability and repeatability are still unsatisfactory due to sampling errors and/or screening errors, and the final diagnosis is still dependent on cervical biopsy (36). Thus, the development of a sensitive, non-invasive approach for the screening and diagnosis of HSILs and LSILs is essential to complement existing techniques.

The present study was based on our previous blood transcriptome profiling study (37) and on our previous reports in cancer research (19,21,22,37-39). In the present study, a 10-gene panel (Table I) was identified that can successfully distinguish HSILs from LSILs. A predictive model performed well in the training set (Figs. 1 and 2), with a sensitivity of 81.8%, specificity of 83.3% and accuracy of 82.4% (Table II). The 10 candidate genes (10-gene panel) used to construct

Table II. Predictive performance of the training set.

Performance	HSILs, n	LSILs, n	Sensitivity, %	Specificity, %	Accuracy, %	ROC AUC
Positive prediction	54	6				
Negative prediction	12	30				
Total	66	36	81.8	83.3	82.4	0.9

HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade SIL; ROC, receiver operating characteristic; AUC, area under the curve.

the predictive model were: *STMN3*, *TRPC4AP*, *DYRK2*, *AGK*, *KIAA0319L*, *GRPEL1*, *ZFC3H1*, *LYL1*, *ITGB1* and *ARHGAP18*. The last 3 genes *(LYL1*, *ITGB1* and *ARHGAP18)* were downregulated in HSILs as compared with LSILs, while the other 7 genes were upregulated.

To verify that this method is also efficient for identifying blood-based transcriptomic biomarkers suitable for discriminating HSILs/LSILs from healthy populations, the blood gene expression profiles of samples from healthy women and samples from women with cervical SIL were analyzed. It was found that the biomarkers identified by the present data mining method were able to differentiate SILs from healthy samples with relatively high accuracy, similar to the HSIL and LSIL classification results. Detailed information is provided in Fig. S1 and Table SIII. This result also confirmed the feasibility of applying blood-based transcriptomic biomarkers for SIL and cervical cancer screening.

To further explore the function of the 10-gene panel, the protein-protein interaction network was mapped and a total of 149 proteins were identified interacting with the candidate genes. The genes corresponding to the 149 interactive proteins were then functionally categorized into several biological processes, including the 'Rho GTPase cycle', 'mitochondrial protein import', 'oncogenic MAPK signaling', 'integrin cell surface interaction' and 'signaling by BRAF and RAF fusions', as shown in Fig. 5A. Of these, GRPEL1 is involved in 'mitochondrial protein import', ARHGAP18 in 'Rho GTPase cycling', ITGB1 in 'integrin cell surface interaction', and AGK in 'oncogenic MAPK signaling' and 'BRAF and RAF fusions', as indicated in Fig. 5B. These findings suggest that these processes may be associated with the progression of LSILs to HSILs.

Mitochondrial protein import is essential for maintaining cellular homeostasis. During this process, the mitochondria and nucleus co-ordinately communicate with each other, and the adaptive responses to stress depend on modulation of mitochondrial import (40). Dysfunction of this process plays a role in human disorders such as mitochondrial neuropathies, myopathies and cancer (41-43). It has been reported that cervical cancer HeLa cells can revert from an apoptotic state even after the induction of widespread mitochondrial-membrane permeabilization, and that mitochondrial protein import plays a role in the anastatic process of HeLa cells (44). Since most mitochondrial proteins are encoded in the nucleus and are imported into the matrix compartment where they are properly folded, the process is facilitated by mitochondrial heat shock protein 70 (mtHsp70) (45) GrpE-like 1 (GrpEL1), as a putative nucleotide exchange factors ortholog, acts as a hetero-oligomeric subcomplex to associate with mtHsp70 and regulate mtHsp70 function (46). Therefore, the GrpEL1-related mitochondrial protein import process may be a potential therapeutic target for cervical cancer and precancerous lesions.

The best characterized members of the Rho GTPase family, RhoA, Rac1 and Cdc42 (47), are associated with abnormalities in Rho GTPase function that have major consequences for tumorigenesis (48), cancer progression (49) and cancer immune suppression (50). The Rho GTPase member, Rac1, is expressed in the nucleus of epithelial cells in SIL and cervical cancer cell lines, and inhibition of Rac1 can decrease cellular proliferation, suggesting that Rho-GTPases may have a role in cervical cancer progression (51). One study demonstrated that stimulation of G12 proteins is capable of promoting cervical cancer invasion through RhoA/ROCK-JNK activation (52). RhoA has also shown a positive correlation with the progression and metastatic potential of cervical cancer, both *in vivo* and *in vitro* (53). *ARHGAP18* is one of the crucial factors for the regulation of RhoA, whose knockdown and overexpression in HeLa cells in a previous study resulted in the inhibition and promotion of cell migration, respectively. Furthermore, it was also required for the polarization of cells for migration (54). Since *ARHGAP18* was the most downregulated candidate gene in the present study, we speculate that this gene may be a biomarker associated with a good prognosis in cervical cancer, as has been reported in breast cancer (55).

Integrins belong to the family of heterodimeric cell surface receptors and trigger various cellular responses by forming physical crosstalk between the inside and outside of cells, and these receptors can bi-directionally control the signals for cell adhesion, migration, proliferation, survival and differentiation (56). Due to their crucial role in physiological functions, integrins also play a pivotal role in tumorigenesis (57). Integrins are heterodimers that include 8β and 18α subunits. ITGB1 protein, namely the integrin β1, which is a member of the β sub-family, forms dimers with different α subunits (α1-7 and αV) 55(t.). ITGB1 protein together with another integrin, ITGA6 protein, was found to be downregulated in Pap-cell smears of high-risk human papillomavirus-positive squamous cervical carcinoma in a previous study (58). The present results were consistent with this, as the gene expression of *ITGB1* was also decreased in HSILs compared with that in LSILs. There was also a contradictory study, which suggested that *ITGB1* was negatively mediated by miR-183-5p in cervical cancer cells; since miR-183-5p serves as a latent anti-oncogene, the investigators regarded *ITGB1* as a metastasis-promoter gene (59). The precise role of *ITGB1* in SILs and cervical cancer thus requires further research clarification.

Oncogenic MAPK signalling, as well as BRAF and RAF fusions with AGK, were the other biological processes identified in the present study. AGK protein, namely acylglycerol kinase, is a novel lipid kinase, which produces lysophosphatidic acid from monoacylglycerol (60). *AGK* is a cancer-related gene that is overexpressed in various human cancer types (61-64). Sun *et al* found that AGK protein and mRNA expression was significantly upregulated in cervical cancer cell lines and cancer tissues and demonstrated that the AGK protein expression level was an independent prognostic factor for the survival of patients with early-stage cervical squamous cell cancer (65). In the present study, it was also found that *AGK* was upregulated in HSILs, which suggested that *AGK* plays a role in the progression of LSILs to HSILs, and can be used as a prognostic marker for SIL and cervical cancer evolution.

On the journey of cancer as it progresses from precursor lesions towards full-scale malignancy, gene expression undergoes changes. Those genetic events in turn establish an environment permissive for neoplastic progression (66). We propose that the downregulation of *ITGB1* and *ARHGAP18* in women with HSILs leads to the dysfunction of cell adhesion and the promotion of cell migration. Dysfunction of cell adhesion then allows cells to migrate and to be more invasive, which occurs during the transformation of LSILs into HSILs. We also suggest that the upregulation of *AGK* and *GRPEL1* in women with HSILs may disrupt the process of protein localization and transport (67,68), processes that establish and maintain proteins during oncogenesis. The present findings thus indicate that protein transport and cell migration may play important roles in the progression of LSILs to HSILs.

Conceptual progress for the hallmarks of cancer has been made over the past decade. In the present study, the downregulated genes, *ITGB1* and *ARHGAP18*, were found to be involved in the integrin cell surface interaction and the Rho GTPase cycle pathways. Integrin, as one of the family of heterodimeric cell surface receptors, can mediate cell adhesion to the extracellular matrix, which is a network of macro-molecules. Integrins can bi-directionally control the signals for cell adhesion, migration, proliferation, survival and differentiation (56). The activation of Rho GTPases can trigger changes in the organization of the cytoskeleton, thereby regulating cell polarity and cell-cell junctions (47). RhoA has also shown a positive correlation with the progression and metastatic potential of cervical cancer (53). In brief, these two enriched pathways identified in the present study are associated with cell invasion and metastasis, two important hallmarks of cancer (69).

The upregulated genes, *AGK* and *GRPEL1*, are involved in the oncogenic MAPK signaling and mitochondrial protein import pathways. Accumulating evidence shows that the RAS/RAF/MAPK cascade is a critical pathway for cancer cell proliferation, differentiation and survival (70). Mitochondrial protein import is essential for maintaining cellular homeostasis (71). *GRPEL1* acts as a hetero-oligomeric subcomplex to interact with mtHsp70, facilitating the process in which mitochondrial proteins are encoded and imported into the matrix (45), and regulating mtHsp70 function (46). In brief, these two enriched pathways identified in the present study are associated with selective growth and proliferative advantage, which are hallmarks of cancer (69,72).

A question arises as to any possible correlations that may be identified between blood gene expression and specific HPV types. In the present study, HPV16 was the most prevalent HPV type and accounted for more than half of all HSIL patients (37/66, 56.1%). A limitation of this study, however, is that only 20 samples in the HSIL cohort presented with other types of HPV, a number inadequate for statistically significant correlation analysis between blood gene expression and specific HPV type. This issue will be studied in future in larger cohorts.

Another notable future project would be to study variations in blood-based gene expression profiling between women with HSIL and women with cervical cancer. Such a study would be of clinical benefit for diagnosing cervical cancer.

In conclusion, the present study identified a panel of 10 blood transcriptomic biomarkers that discriminate cervical HSILs from LSILs, and which could form the basis of a novel blood test for SIL classification. Gene function investigation has indicated that these genes are mainly engaged in the 'Rho GTPase cycle', 'mitochondrial protein import', 'oncogenic MAPK signaling', 'integrin cell surface interaction' and 'signaling by BRAF and RAF fusions'. We propose that these biological processes play important roles in the progression of LSILs to HSILs. We also suggest that these processes may be associated with cell invasion and metastasis, selective growth and proliferative advantage, which are important hallmarks of cancer. Moreover, the 10 candidate genes identified in this study (*STMN3*, *TRPC4AP*, *DYRK2*, *AGK*, *KIAA0319L*, *GRPEL1*, *ZFC3H1*, *LYL1*, *ITGB1* and *ARHGAP18*) may participate in events leading to precancerous cervical lesions and require additional investigation. The genes identified here may also be used as diagnostic biomarkers and targeted therapy for cervical SILs.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CZ was responsible for conception, study design and acquisition of data. YL was responsible for conceptualization, study design and writing the original draft of the manuscript. YC, CS, HL and JJ were involved in acquisition and analysis of data. MW was responsible for analysis and interpretation of data. RZ was responsible for analysis and interpretation of data. CC was involved in conception and design of the study, analysis

and interpretation of data, drafting of the initial manuscript, revising the manuscript for important intellectual content and providing approval for the final version of the manuscript to be published. CCL was responsible for conceptualization and designing of the study, resources and revising the manuscript for important intellectual content. SZ was responsible for conception and design of the study, acquisition and analysis of data and revising the manuscript for important intellectual content. All authors have read and approved the manuscript.

Ethics approval

This study was approved by the Ethics Committee of the Qingdao Women and Children's Hospital (institutional review board no. QFELL-KY-2019-46). Sample acquisition for HSILs and LSILs was conducted between July 2019 and October 2019 at the Qingdao Women and Children's Hospital. All 102 participants, including 66 HSIL and 36 LSIL patients, were enrolled and provided written informed consent. All 65 healthy volunteers also provided informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors wish to declare the following competing interests: CC, YL, ME and RZ are employees of Shanghai Homeostasis Bio-Technology Inc., who sponsored this research. CCL was the scientific consultant at Shanghai Homeostasis Bio-Technology Inc., and the chair and founder of Golden Health Diagnostics. The authors declare that they have no competing interests.

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