Original Article
Discovery of microarray-identified genes associated with the progression of cervical intraepithelial neoplasia

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Abstract: Objective: To identify genes potentially associated with cervical intraepithelial neoplasia (CIN) progression through bioinformatic approaches and clinicopathological verification. Methods: mRNA expression microarray data related to CIN progression were screened from the Gene Expression Omnibus (GEO) database and re-analyzed using bioinformatics approaches. Tissue microarray immunohistochemistry was conducted to assess the significant identified genes in CIN, cervical cancer, and normal tissues. Results: Biological annotation and text mining showed that 14 differentially expressed genes were directly or indirectly related to CIN progression. The expression of 5 up-regulated differentially expressed genes, namely, CCND2, TGFBR2, PRKCB, SH3KBP1 and WNT2B, was examined by tissue microarray immunohistochemistry, with the known CIN progression genes P16 and Ki-67 as the internal reference. Expression of TGFBR2, SH3KBP1, and WNT2B were not detected in CIN and cervical carcinoma, whereas no significant difference in the expression rate of PRKCB was detected (P > 0.05). CCND2, P16, and Ki-67 expression showed a gradual increasing trend in normal, CIN, and cervical cancer. Conclusions: 14 differentially expressed genes were associated with CIN progression, as indicated by the microarray data analysis results. CCND2 may be a new marker for the prediction of CIN progression in addition to P16 and Ki-67.

Keywords: Cervical intraepithelial neoplasia, progression, expression profiling microarray, gene, bioinformatics, immunohistochemistry

Introduction

Cervical cancer is the second most common female malignancy, with poor prognosis and the fourth highest mortality rate in the world [1]. Cervical intraepithelial neoplasia (CIN) is a precursor lesion for cervical cancer, classified histologically as low-grade CIN (CIN1) or high-grade CIN (CIN2 and CIN3). According to laboratory surveys from the College of American Pathologists (CAP), more than 1 million women develop CIN1 each year, and 500,000 are diagnosed with high-grade CIN (CIN2 and CIN3) [2]. Approximately 30% of the patients with high-grade CIN could progress to cervical cancer if left untreated [3, 4], and another 20-40% of those patients will show spontaneous regression [5-7]. The existing pathologic histological

methods are still unable to identify the CIN cases that might progress or spontaneously regress; therefore, the identification of CIN progression markers is necessary for the early prediction and diagnosis of cervical cancer to avoid overtreatment and adverse pregnancy outcomes due to cervical conization for those wishing to retain fertility.

In recent years, data mining has been performed at the molecular level using bioinformatic approaches such as sequence alignment, biochip data extraction, visual mapping, biological data clustering, pathway analyses, statistical analyses, and promoter prediction, and these approaches guide laboratory experiments and promote more rapid genomic annotation [8], providing novel research ideas for

studying the molecular pathogenesis of various diseases. Therefore, bioinformatics approaches are a feasible and potentially valuable approach to data mining and the prediction of gene function on the basis of many large databases and networks [8]. For example, Zou J [9] identified 38 differentially expressed genes and 9 microRNAs associated with ovarian cancer progression through the Gene Expression Omnibus (GEO) [10] database and comprehensive bioinformatic analyses. Liu X [11] identified 6 differentially expressed genes associated with ovarian cancer progression through the GEO database, including ALDH1A2. However, genes associated with CIN progression have never been identified using the GEO database. In this study, using mRNA expression profiles retrieved from the GEO database, we screened for differentially expressed genes that are closely associated with CIN progression and development. The correlation between the differentially expressed genes and CIN progression was examined through comprehensive bioinformatics analyses, including text mining, biological process annotation, biological pathway enrichment, and protein/gene interaction analysis. Based on the results, we identified genes likely to play key roles in the development of high-grade CIN, and analyzed the correlation between these genes, CIN, and cervical cancer based on clinical samples.

Materials and methods

Acquisition and analysis of datasets

Microarray data published prior to January 2016 detailing cervical intraepithelial neoplasia progression-related mRNA expression profiles were retrieved and downloaded from the National Center for Biotechnology Information (NCBI) GEO database (http://www. ncbi.nlm nih.gov/geo). Queries were conducted using "cervical intraepithelial neoplasia" as a keyword. The search was restricted to studies of expression profiling by array and the species to Homo sapiens. We downloaded the mRNA expression microarray datasets GSE63514 [12] and GSE51993 (http://www.ncbi.nlm.nih. gov/geo/query/acc.cgi?acc=GSE51993).

Differentially expressed genes were identified using the GEO2R tool through the GEO database. GEO2R [13] is an R programming language-based dataset analysis tool that was launched in 2012, which is useful to compare two sets of samples under the same experimental conditions to screen differentially expressed genes. In the present study, differentially expressed genes associated with CIN progression were screened using a *p*-value less than 0.05 and a fold change of at least 2 (> 2-fold change) as the threshold.

Bioinformatics analyses

Intersection analysis of multiple genes was performed using Venn Diagram [14] (http:// bioinformatics.psb.ugent.be/webtools/Venn/). Pathway enrichment of the biologically common differentially expressed genes was performed using the DAVID online tool [15-17] (https://david.ncifcrf.gov/home.jsp). Annotation of biologic processes between genes and CIN progression was performed using the Coremine Medical online database (http:// www.coremine.com/medical/) [18]. The protein/gene interaction analysis was performed using the GeneMANIA online tool (http://www. genemania.org/) [19].

Tissue samples and data collection

From the database for the Department of Pathology, Affiliated Tumor Hospital of Guangxi Medical University and Liuzhou People's Hospital, we collected pathology reports of 130 women who underwent both liquid-based cervical cytology and HPV DNA load tests from January 2013 and June 2015. Samples from 68 patients with CIN were obtained after cervical conization. Cancer tissues were taken after radical hysterectomy from 33 early squamous cervical cancer patients without treatment before surgery. Normal cervical tissues were obtained after hysterectomy from 29 uterine leiomyoma patients. The CIN and cervical cancer patients in the group were followed up for an average of 12 ± 3.4 months. All of the hematoxylin and eosin-stained slides from the individual paraffin blocks were reviewed and confirmed by 2 different pathologists. The tissue sample size should be adequate to construct tissue microarray (TMA) blocks. All patients underwent preoperative detection of human papillomavirus (HPV) and cervical cytology. HPV detection was performed using the hybrid capture method, with a relative light unit/positive control critical value (RLU/CO) ≥ 1.0 indicating a positive test. Cytology diagnosis of HPV was

Table 1. Gene expression microarray datasets related to CIN progression

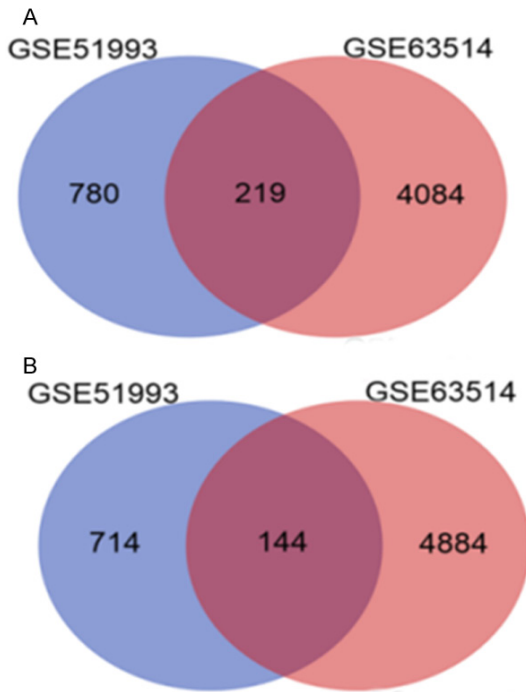| Accession number of the dataset	Organization name	Contributor(s)	Platforms	Samples
GSE 63514	Morgridge Institute for Research	den Boon J	GPL570	24 normal, 14 CIN1, 22 CIN2, 40CIN3, and 28 cancers
GSE 51993	Fudan University	Mo W	GPL10558	7 normal (HPV-), 9 CIN1 (HPV+) and 8 CIN3 (HPV+)

Figure 1. Analysis of the common differentially expressed genes in the two microarray datasets of GSE63514 and GSE51993 by using the Venn diagram tool (A, 219 common down-regulated genes; B, 144 common up-regulated genes).

performed using the 2001 version of the TBS the classification system. The CIN naming system and WHO diagnostic criteria were used for pathologic diagnosis.

TMA construction and immunohistochemistry

The TMA blocks were established using a previously described method [20]. Briefly, hematoxylin and eosin-stained slides from all paraffin-embedded tissue blocks were marked by a pathologist to locate representative areas for TMA samples. Cylinders (2 mm in diameter) were punched from the blocks corresponding to the marked area, and 4-um-thick sections were placed into the tissue microarray paraffin blocks, containing on average 25 to 30 punch biopsies. Immunohistochemistry reactions were performed as follows. Briefly, the TMA

slides were deparaffinized, sequentially dehydrated, pretreated in 3% hydrogen peroxide, boiled with citrate antigen retrieval solution, and incubated in 1% BSA to inhibit nonspecific antibody binding. Then, the TMA slides were incubated in a humidified chamber at 4°C overnight with the primary antibodies. The slides were incubated with a biotinylated antibody, followed by streptavidin-peroxidase for 30 min. Subsequently, the expression staining was visualized by exposing the slides to a diaminobenzidine solution for 1 min. Hematoxylin counterstaining was applied the slides for 10 min, followed by washing with water, dehydration in graded ethanol solutions, clearing with xylene, and mounting.

Immunostaining was assessed by two independent pathologists. CCND2 immunostaining was scored as follows: [21] 0, no staining or staining in < 5% of the cells; 1, staining in $\geq$ 5 to < 25% of the cells; 2, staining in $\geq$ 25 to < 50% of the cells; 3, staining in $\geq$ 50% to < 75% of the cells; and 4, staining in $\geq$ 75% of the cells. Scores of 0 and 1 were considered 'negative', whereas scores $\geq$ 2 were regarded as 'positive'. Positive p16INK4a histology staining was defined as strong and diffuse staining of the basal and para-basal squamous cell compartment in at least the lower third of the epithelial thickness in well-oriented sections [22]. The Ki-67 labeling index was graded into low and high indices at a cut-off value of 20% [23].

Statistical analyses

All statistical analyses were conducted using the SPSS statistical software (version 19.0, SPSS, Chicago, IL, USA). The Chi-square test and Fisher's exact test were applied to compare the expression results in the cervical tissues. Univariate and multivariate analyses of clinical factors affecting the expression of CCND2 were examined using Chi-square tests and logistic regression analyses, respectively. Associations between gene expression and hrHPV were examined using the Chi-square test. Receiver operating characteristic (ROC)

Table 2. The 14 differentially expressed genes that exhibited exactly the same expression pattern trends

Gene name	Signal pathway	LogFC*		Expression pattern	Literature related to CIN progression
		GSE63514	GSE51993		
CCND2	Wnt	0.0446	0.504157	Upregulated	-
NFATC1	Wnt	0.879	0.168807	Upregulated	-
PRKCB	Wnt	0.847	0.462753	Upregulated	-
PPP2R5D	Wnt	0.488	0.266376	Upregulated	-
WNT2B	Wnt	0.847	0.511759	Upregulated	-
SH3KBP1	Endocytosis	0.578	0.429254	Upregulated	Highly expressed in cervical cancer [24]
HSPA6	Endocytosis	1.18	0.435959	Upregulated	-
PIKFYVE	Endocytosis	0.266	0.126757	Upregulated	-
RABEP1	Endocytosis	0.483	0.224578	Upregulated	-
TGFBR2	Endocytosis	0.0341	0.153118	Upregulated	-
KDELR1	Vibrio cholerae infection	-0.212	-0.23526	Downregulated	-
MUC2	Vibrio cholerae infection	-0.0647	-0.2518	Downregulated	-
KCNQ1	Vibrio cholerae infection	-0.156	-0.21546	Downregulated	-
TJP2	Vibrio cholerae infection	-0.26	-0.49813	Downregulated	-

*A positive logFC value shows that the gene is upregulated, and a negative logFC value indicates that the gene is downregulated. - No reports have been reported or retrieved.
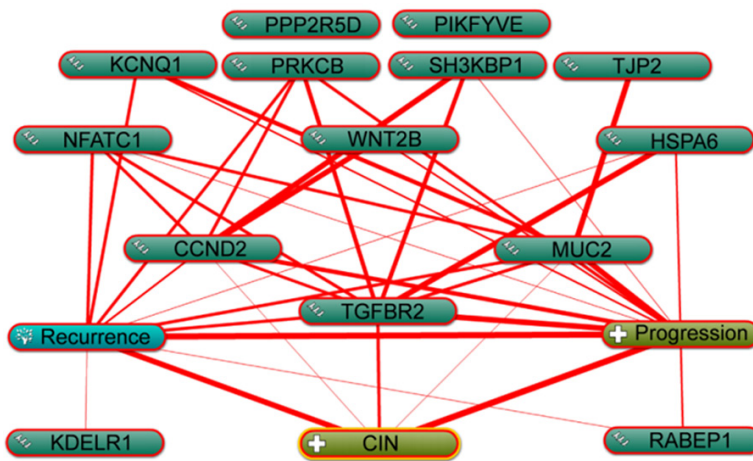

Figure 2. Function prediction and analysis of the 14 identified genes related to progression and recurrence using the Coremine tool. The thickest red line indicates the strongest association.

base were GSE63514 [12] and GSE51993. The data of GSE63514 included 14 CIN1 lesions, 22 CIN2 lesions, 40 CIN3 lesions, and 28 cancer specimens, whereas the GSE-51993 data comprised 7 normal (HPV-), 9 CIN1 lesions (HPV+) and 8 CIN3 lesions (HPV+) (**Table 1**). The CIN and normal samples served as the experimental group and control group, respectively. By using adjusted *P* value < 0.05 and a fold change > 2 as the threshold, the GSE63514 and GSE51993 datasets were screened using the GEO2R tool to reveal the differentially expressed genes between the experimental and control groups. A total of 999 up-regulated differentially expressed genes and 858 down-regulated differentially expressed genes were identified from the GSE51993 dataset, and 5,028 up-regulated differentially expressed genes and 4,303 down-regulated differentially expressed genes from the GSE63514 dataset. The up-regulated and down-regulated differential expression genes in the two datasets were intersected using the Venn Diagram tool, and 144 commonly up-regulated genes and 219 commonly

curve and logistic regression analyses were introduced to evaluate the diagnostic capabilities of the identified genes. All tests were performed using a *P* value of 0.05 for significance.

Results

Screening of differentially expressed genes and pathway enrichment

In the present study, the mRNA expression profiling datasets screened from the GEO data-
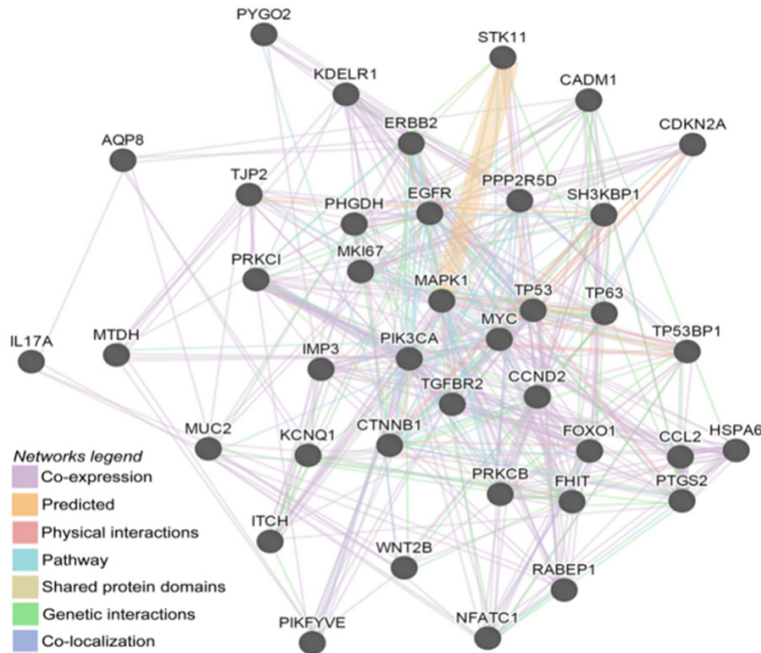
Figure 3. Protein/gene interaction network between the 24 known genes and the 14 screened genes generated using the GeneMANIA tool. The types of interactions between proteins/genes are indicated in the network legend of the figure.

genes using the COREMINE tool. A co-occurrence analysis of the literature was conducted using the gene names and "Cervical Intraepithelial Neoplasia", "Progression", and "Recurrence" as keywords. The results revealed that all of the commonly differentially expressed genes were present in the text-mining networks, except PPP2R5D and PIKFYVE. Among the 12 present genes, 3 genes (CCND2, TGFBR2 and MUC2) were directly related to CIN progression. In addition to significant correlations with "Cervical Intraepithelial Neoplasia", "Progression", and "Recurrence", the genes were also significantly associated with each other, with the relationship between TGFBR2 and WNT2B presented as an example in **Figure 2**.

down-regulated genes were found to be significantly differentially expressed in the two datasets (**Figure 1**). Biological signaling pathway enrichment was then performed on the above 144 up-regulated and 219 down-regulated genes using DAVID to identify the biological significance of the common differentially expressed genes in the regulation of CIN progression at the unitary level. The pathway enrichment identified 3 significant signaling pathways, the Wnt, endocytosis, and *Vibrio cholerae* infection pathways, according to their *P* values. Fourteen differentially expressed genes were found among the 3 pathways that exhibited an identical expression pattern trend between the 2 datasets (**Table 2**). Among them, only SH-3KBP1 has been shown to be highly expressed in cervical cancer[24]; none of the other genes have been previously reported.

Bioinformatics analysis of the correlation between the differentially expressed genes and CIN progression

To reveal the biologic significance of the 14 commonly differentially expressed genes in the regulation of CIN progression at the unitary level, biological process annotation and text mining were performed on the above-described

Protein/gene interaction analysis

An advanced search using the keywords 'Cervical Intraepithelial Neoplasia' and 'Progression' was performed in the PubMed database, and 24 protein-coding genes were found to be significantly correlated with CIN progression, including CDKN2A [25], AQP8 [26], CADM1 [27], CCL2 [28], CTNNB1 [23], EGFR [29], ERBB2 [29], FHIT [30], IL17A [31], IMP3 [32], MAPK1 [33], MKI67 [34], MTDH [35], MYC [30], PHGDH [36], PIK3CA [33], PRKCI [37], PTGS2 [29], STK11 [33], TP53 [33], TP53BP1 [38], TP63 [39], FOXO1 [40] and ITCH [41]. The relationships between the 14 potentially differentially expressed genes and 24 previously reported protein genes were analyzed using GeneMANIA (http://www.genemania.org/), a web-based tool for the rapid and accurate prediction of gene function based on multiple networks derived from different genomic/proteomic data sources [19]. The protein/gene interactions in the network include pathway, co-localization, co-expression, physical interaction, genetic interaction, and shared protein domains. As shown in **Figure 3**, all of the 14 screened genes interacted with the 24 reported genes either directly or indirectly, indicating that these genes may be functionally relevant. Accordingly, the

Table 3. Antibodies used for immunohistochemical staining

Antibodies	Type	Dilution	Source
CCND2	Rabbit Monoclonal Antibody	1:50	Santa Cruz Biotechnology (sc-754)
PRKCB	Rabbit Monoclonal Antibody	1:200	Abcam (ab18512)
WNT2B	Rabbit Polyclonal Antibody	1:200	Santa Cruz Biotechnology (sc-134152)
SH3KBP1	Rabbit Monoclonal Antibody	1:200	Abcam (ab151574)
TGFBR2	Mouse Monoclonal Antibody	1:200	Abcam (ab78419)
P16	Mouse Monoclonal Antibody	1:100	Cell Signaling technology (#2407)
Ki-67	Rabbit Monoclonal Antibody	1:400	Cell Signaling technology (#9027)

Table 4. Expression of different genes in normal, CIN, and cervical cancer tissues

Gene	Normal (n = 29)	CIN (n = 68)	Cervical cancer (n = 33)	X^2	P
CCND2				18.649	0.000
Negative	25 (86%)	48 (71%)	12 (36%)		
Positive	4 (14%)	20 (29%)	21 (64%)		
PRKCB				4.577	0.101
Negative	24 (83%)	46 (68%)	19 (58%)		
Positive	5 (17%)	22 (32%)	14 (42%)		
P16				68.63	0.000
Negative	24 (83%)	21 (31%)	3 (9%)		
Positive	5 (17%)	47 (69%)	30 (91%)		
Ki-67				19.54	0.000
Negative	23 (79%)	29 (43%)	7 (21%)		
Positive	6 (21%)	39 (57%)	26 (79%)		

sis, was examined by tissue microarray immunohistochemistry, with the known CIN progression genes P16 and Ki-67 as the internal reference. Each primary antibody is summarized in **Table 3**. The positive expression rates of CCND2 in normal, CIN, and cervical cancer tissues were significantly different at 14%, 29% and 64%, respectively, and their expression increased gradually with the increase in cervical lesion grade (**Table 4** and **Figure 4**). The positive expression rates of P16 and Ki-67 in normal, CIN and cervical cancer tissues were also significantly different (P < 0.05) at 17% and 69%, 91% and 21%, and 57% and 79%, respectively. The increase in cervical lesions also indicated a gradual increase in the trend (**Table 4** and **Figure 4**). There was no significant difference in the expression rate of PRKCB (P > 0.05), whereas WNT2B, SH3KBP1 and TGFBR2 were not detected in the CIN and cervical cancer tissues. The results did not change significantly after CIN was subdivided into the CIN1, CIN 2, and CIN 3 subgroups and compared with the normal and cervical cancer groups (**Table 5**).

A clinical analysis of CCND2 expression was performed based on the immunohistochemical protein expression and clinical data. The univariate analysis showed that high expression of CCND2 was positively correlated with high-grade CIN (P < 0.05) but not significantly associated with age, age at first sexual intercourse, number of sexual partners, contraceptive methods, number of pregnancies, number of births, P16 level and Ki-67 level (P > 0.05). Logistic regression analysis that included CCND2 as a

screened genes may also be associated with CIN progression. Particularly, CCND2 was found to interact directly with 16 CIN progression relevant genes including CDKN2A, CADM1, CCL2, CTNNB1, ERBB2, IMP3, MYC, PHGDH, PIK3CA, PRKCI, PTGS2, TP53, TP53BP1, TP63, FOXO1 and ITCH. Moreover, the 14 screened genes also interacted with each other through various means. For example, CCND2 directly interacted with PRKCB, SH3KBP1, TGFBR2 and KDELR1; TGFBR2 directly interacted with CCND2 and 7 other genes; PRKCB directly interacted with CCND2 and 6 other genes; and SH3KBP1 directly interacted with CCND2 and 5 other genes.

Expression of different genes in cervical lesions and the analysis of clinical factors

The expression of 5 up-regulated differentially expressed genes, including CCND2, TGFBR2, PRKCB, SH3KBP1 and WNT2B, which were significantly associated in the above text-mining networks and protein/gene interaction analy-
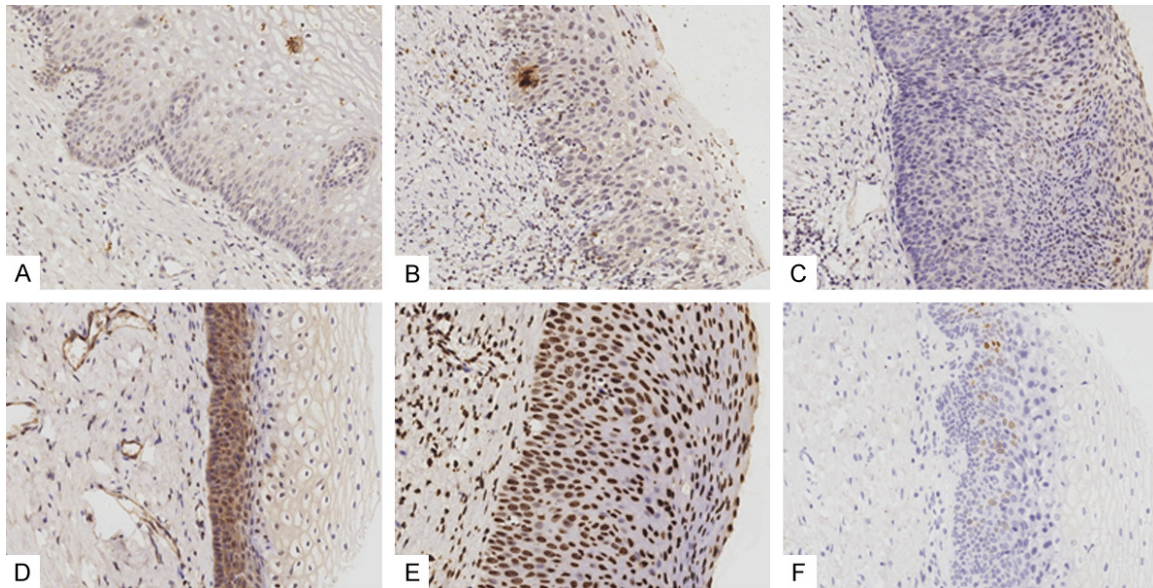
Figure 4. Representative positive examples of immunohistochemistry of identified genes on CIN tissues. A. CCND2 (CIN1); B. CCND2 (CIN2); C. CCND2 (CIN3); D. PRKCB (CIN1); E. P16 (CIN3); F. Ki-67 (CIN2). Immunohistochemistry, DAB, brown stain, magnification × 20.

Table 5. The expression of each gene in normal, CIN1, CIN2, CIN3 and cervical cancer tissues

Gene	Normal (n = 29)	CIN 1 (n = 14)	CIN 2 (n = 23)	CIN 3 (n = 31)	Cervical cancer (n = 33)	P
CCND2						0.000
Negative	25 (86%)	12 (86%)	19 (83%)	17 (55%)	12 (36%)	
Positive	4 (14%)	2 (14%)	4 (17%)	14 (45%)	21 (64%)	
PRKCB						0.30
Negative	24 (83%)	10 (71%)	16 (70%)	20 (65%)	19 (58%)	
Positive	5 (17%)	4 (29%)	7 (30%)	11 (35%)	14 (42%)	
P16						0.00
Negative	24 (83%)	6 (43%)	7 (30%)	8 (26%)	3 (9%)	
Positive	5 (17%)	8 (57%)	16 (70%)	23 (74%)	30 (91%)	
Ki-67						0.00
Negative	23 (79%)	6 (57%)	8 (35%)	15 (48%)	7 (21%)	
Positive	6 (21%)	8 (43%)	15 (65%)	16 (52%)	26 (79%)	

dependent variable indicated a significant difference in the CIN grade, with an odds ratio (OR) of 6.847 ($P < 0.05$) (**Table 6**).

The correlation between CCND2 expression and hrHPV infection was analyzed. The results indicated that CCND2 was significantly associated with hrHPV infection ($P < 0.05$). The logistic regression analysis that included CCND2 as the dependent variable showed that hrHPV was statistically significant, with an OR of 3.898 ($P < 0.05$) (**Table 6**).

The relation between hrHPV and the expression of genes in CIN and cervical cancer tissues was analyzed. The expression of CCND2, P16 and

Ki-67 in hrHPV-positive CIN tissues was significantly higher than that in hrHPV-negative CIN tissues ($P < 0.05$), but not in cervical cancer tissues ($P > 0.05$) (**Table 7**). These results indicated that the expression of the three genes in CIN increased significantly with hrHPV infection, suggesting that during the progression of CIN to cervical cancer, the expression of CCND2, P16 and Ki-67 were closely associated with hrHPV infection.

Combined detection of genes in CIN diagnosis

The area under the curve (AUC) of CCND2 for the diagnosis of CIN was 0.58 ($P > 0.05$), and for P16 and Ki-67 it was 0.76 and 0.68, respec-

Table 6. Logistic regression analysis of factors affecting CCND2 expression

	CCND2 (+) (n = 20)	CCND2 (-) (n = 48)	OR	P
Age			0.797	0.760
≥ 40	13	34		
< 40	7	14		
Primary Sex Age			0.514	0.332
< 19	11	20		
≥ 20	9	28		
Sexual Partners			0.005	0.945
1	14	34		
≥ 2	6	14		
Contraceptive			0.137	0.712
Yes	17	39		
No	3	9		
Pregnancy Times			0.021	0.884
≥ 3	4	7		
< 3	16	31		
Birth Times			1.511	0.219
≥ 3	7	10		
<3	13	38		
hrHPV			3.898	0.048
Negative	3	19		
Positive	17	29		
P16			1.103	0.294
Negative	8	13		
Positive	12	35		
Ki-67			0.677	0.410
Negative	7	22		
Positive	13	26		
CIN Grade			6.847	0.033
I	2	12		
II	4	19		
III	14	17		

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

Table 7. Relationship between the expression of each gene and hrHPV infection

	CIN		P value	Cervical cancer		P value
	hrHPV (-)	hrHPV (+)		hrHPV (-)	hrHPV (+)	
CCND2			0.048			0.610
Negative	19	29		2	10	
Positive	3	17		2	19	
P16			0.018			0.330
Negative	11	10		1	3	
Positive	11	36		3	26	
Ki-67			0.016			0.190
Negative	14	15		2	5	
Positive	8	31		2	24	

CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus.

tively, suggesting that CCND2 was less valuable for the diagnosis of CIN than P16 and Ki-67. The sensitivities of CCND2, P16 and Ki-67 in the diagnosis of CIN were 29.4%, 69.1% and 57.4%, respectively, and the specificities were 86.2%, 82.8% and 79.3%, respectively (**Table 8**).

Using normal and CIN as the dependent variables, CCND2, Ki-67, P16, age, hrHPV status, age at first sexual intercourse, number of sexual partners, contraceptive methods, number of pregnancies and number of births were selected by the logistic diagnostic model. The results showed that only Ki-67, P16 and hrHPV status were statistically significant (P < 0.05), and the OR values were 10.34, 15.49 and 13.70, respectively, indicating that the diagnostic value of Ki-67, P16 and hrHPV-positive status were 10.34, 15.49 and 13.70 times greater than those of hrHPV-negative status, respectively (**Table 9**).

Results of the follow-up

Sixty-eight CIN patients in the group were followed up for an average of 12 ± 3.4 months. The results indicated that 2 patients had recurrence, and their pathology before surgery was CIN3. The immunohistochemical analysis showed that these two patients were P16 and Ki-67 positive, with one patient being CCND2 positive. Moreover, recurrence was not detected in 33 of the cervical cancer patients during the follow-up.

Discussion

CIN is a precursor lesion of cervical cancer. The occurrence and progression of CIN

Table 8. Sensitivity, specificity, and accuracy of p16, Ki-67, and CCND2 in CIN diagnosis

Gene	Sensitivity (%)	Specificity (%)	Positive predictive values	Negative predictive values	AUC (95% CI)	P
CCND2	29.4	86.2	83.3	34.2	0.58 (0.47-0.68)	0.0684
P16	69.1	82.8	90.4	53.3	0.76 (0.66-0.84)	< 0.0001
Ki-67	57.4	79.3	86.7	44.2	0.68 (0.58-0.77)	0.0002

Table 9. Result of CIN diagnostic logistic regression model

Factor	B	S.E.	Wals	P	OR	95% CI
ki67	2.34	0.76	9.51	0.002	10.33595	2.34-45.60
P16	2.74	0.75	13.52	0.000	15.49167	3.60-66.75
hrHPV	2.62	0.74	12.37	0.000	13.70814	3.19-58.97
Constant	-8.748					

is a complex process with multi-step and multi-factor participation. At present, many of the mechanisms of CIN have not been illuminated at the molecular level. Accurate predictions of the progression rates of CIN lesions are limited due to a lack of sensitive markers for neoplastic progression. Therefore, it is very important to further explore the regulatory mechanism of CIN progression at the molecular level. To our knowledge, this study is the first to use GEO profile data mining to identify the signaling pathways and key genes related to the regulation of CIN progression. In this study, using integrated bioinformatics and clinical sample data analyses, three signaling pathways, including the Wnt, endocytosis, and *Vibrio cholerae* infection pathways, were identified to be significantly associated with CIN progression, and CCND2, which is part of the Wnt pathway, may be a potential key gene associated with CIN progression. This study has a certain role at the molecular level in further studies of CIN progression. CCND2 is expected to be a new molecular marker for CIN progression, in addition to P16 and Ki-67, to guide clinicians to stratify CIN therapy.

The reanalysis and reuse of public data has a potentially great effect in scientific research [42]. The GEO database is a publically available international repository containing high-throughput microarray and second-generation sequencing functional genomic datasets that are submitted by the research community. By 2012, the GEO database comprised more than 32,000 public series submitted directly by 13,000 laboratories, hosting 800,000 samples derived from > 1,600 organisms [13]. The data-

sets presented to the GEO database often comply with journal or grant directives that require data to be made publicly available in a MIAME-supportive [43] database. As a result, the GEO database has supporting data and links to nearly 20,000 published manuscripts [13]. In this study, through the rigorous screening of qualified GEO database microarray data, two sets of CIN-related mRNA expression profiles (**Table 1**) were chosen to analyze the pathways and genes involved in CIN progression, further revealing the molecular mechanism of CIN progression.

Based on the above data mining and bioinformatics analyses, this study identified three potential CIN progression-associated signaling pathways, including the Wnt, endocytosis and *Vibrio cholerae* infection pathways, which is, to some extent, consistent with previous research results. For example, endocytosis is involved in the signal transduction of a variety of cellular physiological activities, including cell growth and differentiation, cell chemotaxis, and immune responses [44]. Dynamin 2, the core gene in the endocytosis pathway, was proved to be a helpful biomarker in grading CIN lesions and is a candidate biomarker for the detection of low-grade CIN with high sensitivity [45]. Combined with the endocytosis pathway gene SH3KBP1, which is highly expressed in cervical cancer [24], the results suggest that the endocytosis signaling pathway may also play an important role in CIN progression. Recent research has shown that the Wnt signaling pathway is significantly associated with the promotion of cervical cancer cell proliferation and cell migration/invasion [46, 47], as well as the progression of cervical cancer by promoting the epithelial-mesenchymal transition [48], which has also been suggested in other tumors [49-51]. Given the close relationship between CIN and cervical cancer, it has been proposed that alterations in the Wnt pathway are involved in
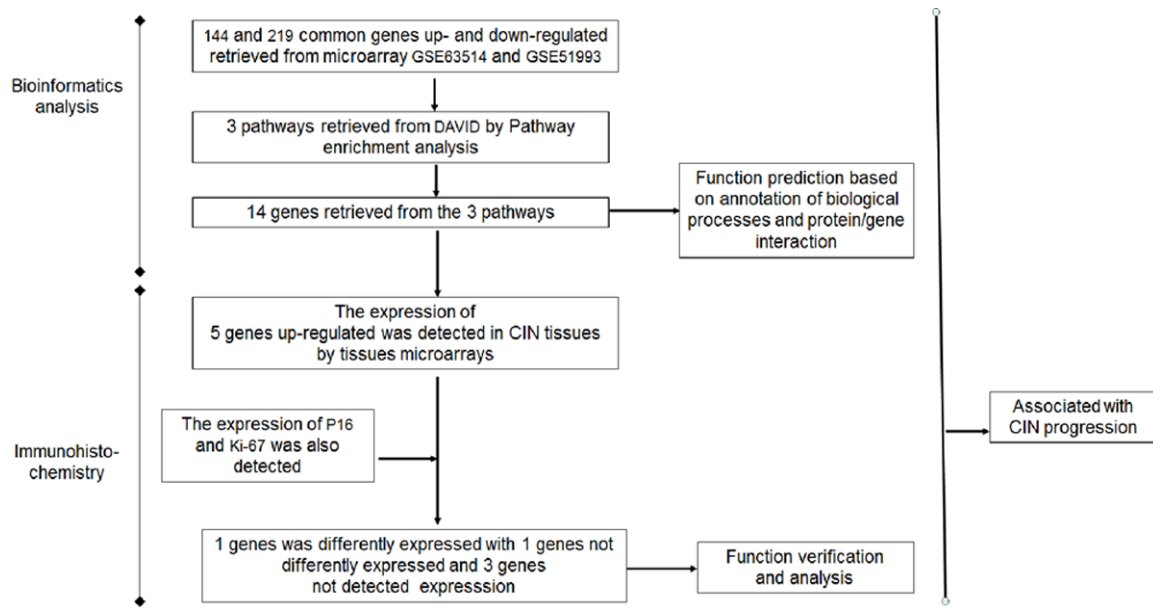
Figure 5. Procedure for identification of biological processes and genes associated with CIN progression.

CIN progression, and studies of CIN progression based on this signaling pathway may provide a new solution for blocking CIN progression. Although there are no reports on the relationship between the *Vibrio cholera* infection pathway and CIN, cholera toxin (CT) as a carrier/adjuvant may enhance human T-cell responses to HPV oncoprotein *in vitro* using dendritic cells (DCs) as antigen-presenting cells [52]. Based on the notion that hrHPV may be responsible for both cervical carcinoma and CIN, the *Vibrio cholera* infection pathway may also be involved with cervical lesions.

In terms of the 14 differentially expressed genes in the three signaling pathways, SH3-KBP1 was found to be highly expressed in cervical cancer tissues [24], but the other 13 genes were not reported in CIN and cervical cancer. Moreover, through biological annotation, it was found that some of the differentially expressed genes were directly associated with CIN progression. Although not all of the genes were directly related, most of them were associated with tumor progression and recurrence. In addition, these genes weresignificantly related to each other, suggesting that the 14 identified genes may play an important role in the progression of CIN. Three genes, CCND2, TGFBR2 and MUC2, which were directly associated with CIN progression, may be directly involved in the regulation of CIN progression. Based on Gene MANIA, it was found that all of

the selected genes not only interacted with the 24 known CIN progression genes but also interacted with each other in a variety of ways, further supporting that these screened genes may be functionally associated with and participate in the regulation of CIN progression at the unitary level.

Of the 14 differentially expressed genes, CCND2 and TGFBR2 may have the most significant associations with CIN progression. First, only three genes were identified as having a direct association with CIN progression by biological annotation, and these included CCND2 and TGFBR2. Second, in the complete protein/gene interaction network, comprising 24 known genes, CCND2 and TGFBR2 exhibited the greatest numbers of interactions, interacting with 16 and 12 of the 24 known genes, respectively. These known genes have been reported to be associated with the progression of CIN, suggesting that CCND2 and TGFBR2 may be more closely related to CIN progression. Moreover, CCND2 directly interacted with 4 of the screened differentially expressed genes, and TGFBR2 also had a direct interaction with 8 of the screened differentially expressed genes in the protein/gene network. For the above reasons, CCND2 and TGFBR2 may be important candidate genes for CIN progression regulation. However, the results need to be confirmed by further studies.

To verify the relationship between the differentially expressed genes and CIN progression, this study conducted immunohistochemical detection using a tissue microarray. The selected genes for immunohistochemical analysis included the significant genes from the bioinformatics analysis and the reported known genes P16 and Ki-67. Results indicated that the up-regulation of CCND2 was positively correlated with CIN grade, and that hrHPV-positive infection status also increased with CIN grade. For this reason, it was speculated that CCND2 may play a role in CIN progression to cervical cancer. CCND2 (cyclin D2) is one of the cyclin family members that regulates the cell cycle. CCND2 activates cell cycle-dependent kinase 4 (CDK4) and CDK6 in the cell cycle, forms a complex with CDK4 and CDK6 as a regulatory subunit, and plays a key role in regulating cell cycle transition from the G1 phase to S phase [53]. Previous studies have confirmed that CCND2 is highly expressed in tumors, including ovarian cancer and testicular cancer, and CCND2 knockout inhibits cancer cell proliferation [54]. A recent report indicated that mutations in the vicinity of the CCND2 gene were closely related to the occurrence of colorectal cancer [55]. Du et al. [56] found that CCND2 down-regulated the cell cycle G1/S transition, which inhibited the proliferation of HeLa cells. However, to date, no studies have investigated the relation between CCND2 and CIN. Our study showed that the expression of CCND2 was significantly associated with CIN grade and hrHPV infection status, indicating that CCND2 may be a new marker for the early diagnosis of CIN progression.

Four significantly up-regulated genes in the bioinformatics analysis, PRKCB, WNT2B, SH3KBP1 and TGFBR2, were not expressed or differentially expressed in cervical cancer tissues. The reason may be due to the low specificity of the high-throughput database and the differences between the screening and validation methods. Therefore, other methods, such as RT-PCR and western blotting, should be employed for functional verification.

The tumor suppressor protein p16 may be one of the most studied markers in CIN progression. P16 is the only biomarker currently used clinically for CIN diagnosis and has been recommended by the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology (ASCCP) [57]. However, P16 has not been applied clinically for the diagnosis of CIN progression and prognosis. Several studies have found that the expression of P16 is associated with the progression of cervical lesions [25, 58, 59]. Our study also indicated that P16 was significantly associated with CIN progression, and P16 may be a marker of CIN progression.

Recently, the expression of the proliferating antigen gene Ki-67 was found to increase gradually in CIN1, CIN2, CIN3, and squamous cell carcinoma by immunohistochemistry [60], which is consistent with the results of our study. Based on our analysis of the differences in Ki-67 expression in normal, CIN, and cervical cancer tissues, Ki-67 may predict both low-grade and high-grade CIN progression. However, some scholars have found that Ki-67 was irrelevant for evaluating CIN progression [61], indicating that more studies to prove its usefulness as a progression marker are needed.

CCND2, Ki-67, and P16 were positively correlated with the hrHPV infection status in CIN and cervical cancer tissues, suggesting that these three genes are not only associated with CIN progression but are also synergistic in their carcinogenic effects with hrHPV. However, correlation analysis showed that CCND2 was not associated with Ki-67 and P16, suggesting that the mechanism of action of CCND2 and HPV is not related to that of Ki-67, P16, and HPV. Therefore, the specific mechanism of the involvement of these three genes in CIN progression needs to be further studied.

Most cases of CIN can be accurately diagnosed through cervical biopsy specimens using hematoxylin and eosin (HE) staining. However, when the lesions are also combined with immature metaplasia, inflammatory epithelial hyperplasia and epithelial atrophy, the diagnosis of CIN is difficult using only HE staining. Therefore, as a means of screening cervical lesions, P16, Ki-67 and other immunohistochemical markers have been gradually incorporated to compensate for the inadequacy of simple HE staining. However, there are still a small number of high-grade CIN cases that do not express the above genes [62], which was confirmed in our study. The ROC curve analysis showed that CCND2 alone was not valuable for the diagnosis of CIN,

but when combined, the sensitivity for CIN diagnosis increased. This indicates the need for multiple immunohistochemical assays to avoid clinically over-treating false positive cases and inadequately treating false negative cases.

Several limitations of this study should be considered. First, this study was only cross-sectional design, not a prospective study that can follow up patients with CIN for progression analysis; therefore, there might have been confounders that were not recognized or controlled. The small sample size may have affected the results. Finally, hrHPV-negativity of the CIN samples might have led to a bias in the effect.

Conclusions

In this study, differentially expressed genes were screened using bioinformatics approaches and the GEO database, and using pathway enrichment, biological annotation and protein/gene interaction analyses, three pathways with 14 differentially expressed genes related to CIN progression were identified. The Wnt signaling pathway may be an important pathway in the development of CIN, and CCND2 and TGFBR2 may be important candidate genes associated with the regulation of CIN progression. The study of CIN progression based on signaling pathways and genes may provide new solutions to block the progression of CIN. The molecular marker CCND2, which was verified by subsequent tissue microarray immunohistochemistry, may provide an important basis for further studies and clinical applications (**Figure 5**). The next steps will be to increase the number of cases, extend the follow-up time, and utilize other experimental methods to study the molecular mechanism of CCND2 up-regulation associated with CIN progression.

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Disclosure of conflict of interest

None.

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