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Cervical squamous cancer mRNA profiles reveal the key genes of metastasis and invasion

Yuan Cheng[1], Ding Ma[1], Youyi Zhang[2], Zijian Li[2]*, Li Geng[1]*

[1] Department of Gynecology and Obstetrics, Peking University Third Hospital, Beijing
[2] Institute of Vascular Medicine, Peking University Third Hospital, Key Laboratory of Cardiovascular Molecular Biology and Regulatory Peptides, Ministry of Health, Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education and Beijing Key Laboratory of Cardiovasicular Receptors Research, Beijing (China)

Summary

Purpose of investigation: To investigate mRNA expression profiles associated with cervical squamous carcinoma progression and to identify key genes involved in invasion and metastasis of cervical squamous cancer. *Materials and Methods:* The authors extracted the mRNA expression profile of eight normal cervical tissues by human whole genome microarray. The main functions of differentially expressed genes were identified by gene ontology (GO) analysis. Gene-networks were established based on bioinformatic approaches. Microarray data of the expressions level of key genes verified by qRT-PCR. *Results:* The authors identified 2036 differentially expressed genes between two groups including 1,282 down-regulated genes and 754 up-regulated genes ($p < 0.05$, FDR< 0.05). Gene-network revealed that PDGFRA, CAV1, and GJA-1 were critical for cervical cancer invasion and metastasis. *Conclusions:* PDGFRA, CAV1, and GJA-1 were revealed as key node genes for cervical cancer invasion and metastasis. The results may provide new evidences and ideas for early diagnosis and prognosis assessment of cervical cancer.

Key words: Cervical cancer; Gene expression profile; Microarray; mRNA.

Introduction

Cervical cancer is the third most common malignant tumor in women worldwide, preceded only by breast cancer and colorectal cancer. There is an estimated 500,000 cases of cervical cancer and 260,000 patients that die from the disease each year globally. The highest incidence rate is observed in Eastern, Western and Middle Africa; Central America; South-Central Asia, and Melanesia [1]. Premature mortality caused by cervical cancer in 23 countries is higher than that caused by breast cancer, according to the survey [2]. About 130,000 new cervical cancer cases are diagnosed in China each year, accounting for 1/5 of all cases worldwide. Recent trends suggest increased incidence of cervical cancer among younger women [3,4]. High risk human papillomavirus (HR-HPV) infection is an important risk factor in cervical cancer development. However, only few women infected with HR-HPV eventually develop cervical cancer [5]. It is apparently that oncogene activation, tumor-suppressor gene inactivation, and disruption of gene clusters of cell cycle, apoptosis, adhesion, immunity, and signal transduction occur during the progression from precancerous lesions to infiltrating carcinoma [6]. Clinical data show that the five-year survival rate among patients with cervical intraepithelial neoplasia (CIN) is nearly 100%. The five-year survival rate for patients with Stage I cervical cancer is about 70%-90% and for patients with Stage IV is only around 20% [7,8]. These data indicated that cervical cancer invasion and metastasis to other sites such as lymph node, lung, and bone seriously affects the clinical outcome and prognosis [9]. At present, the carcinogenesis of cervical cancer invasion and metastasis is still unclear, warranting further studies.

Biological processes such as cell division, proliferation, differentiation, and apoptosis are characterized by changes in gene expression. Gene expression is activated or inhibited in response to external environmental or biochemical signaling, producing distinct gene expression profiles, which are termed as "molecule signatures" of specific physiological or pathological conditions. Such signatures elucidate the relationships between gene function and signal transduction pathways and provide insights into various biological activities. With gene microarray technology, expression of tens of thousands of genes is analyzed simultaneously in single experiment, and multiple gene clusters associated with specific diseases and the key players of disease pathogenesis are identified. Rapid automated high-throughput gene analysis is achieved [10]. Screening for differentially expressed genes between normal cervical tissues and cervical cancer tissues with gene expression microarray provides important data underlying pathogenesis and molecular mechanism in cervical cancer development

* *Co-corresponding authors.*

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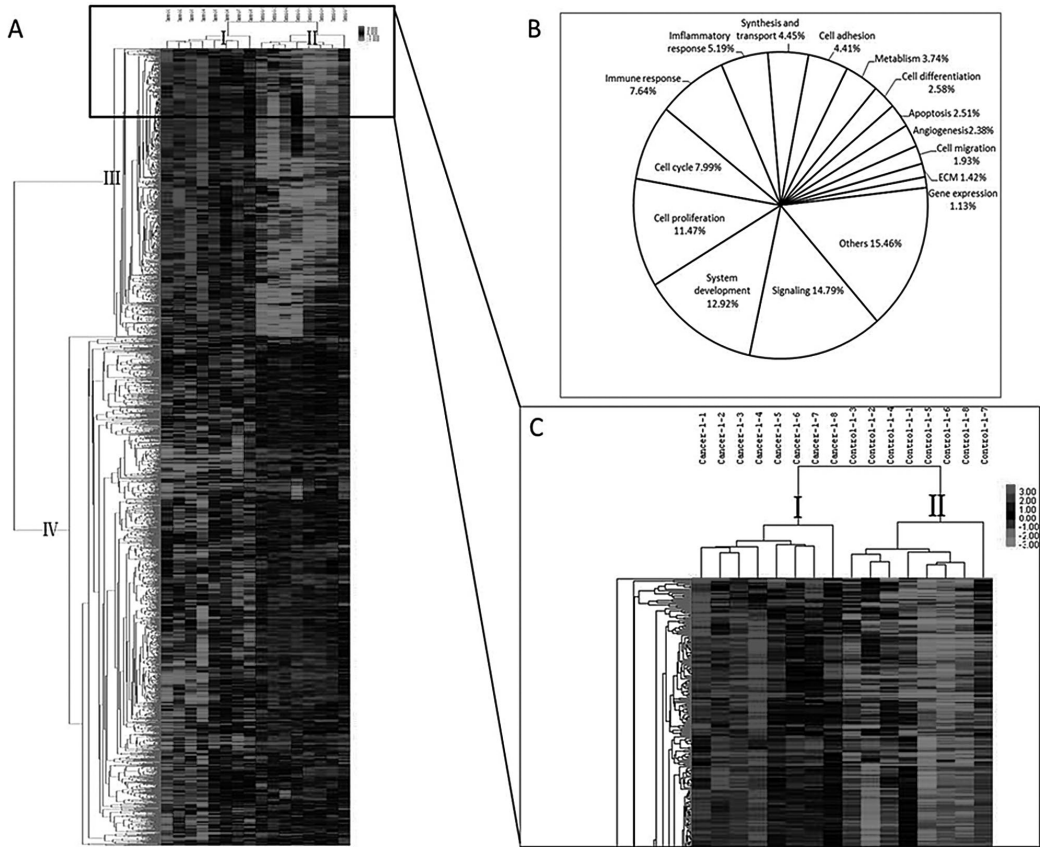

Figure 1. — mRNA expression profile in cervical carcinoma. This map represents the 2,036 differentially expressed mRNA. A) Red icons represent the overexpression of mRNA and green represents decreased expression gene. Each column represents a gene; each row represents a clinical sample. I column for cervical cancer; II columns represent normal tissue. III gene expression in cervical cancer tissues increased, IV on behalf of gene expression in cervical cancer tissues decreased. B) Function classification of the pie for 2,036 genes. C) one part of the amplified A diagram.

and progression. As early as 2004, Sopov *et al.* extracted RNA from 20 CIN3 samples and ten cervical squamous cancer samples, amplified with self-monitoring, analysis and reporting technology (SMART), hybridized with 1,176 tumor-related gene probes in microarray and finally identified 92 differentially expressed genes [11]. In 2006, Chao *et al.* performed gene expression profile analysis of 16 cases of cervical squamous cell carcinoma and nine cases of adenocarcinoma of the cervical cancer by gene microarray and found 653 differential expressed genes [12]. They found a higher expression of CEACAM5, TACSTD1, S100P, and MSLN in adenocarcinoma, while S100A9 and ANXA8, were expressed higher in cervical squamous cell carcinoma. Lyng *et.al* analyzed gene expression profiles in 19 cases of cervical cancer without lymph node metastasis and ten cases of lymph node metastasis [13]. The data showed that eight genes were associated closely with cervical cancer metastasis and clinical prognosis.

Despite the decline in cervical cancer incidence rates due to early screening for HPV and cervical smear test of exfoliated cells, the incidence of cervical cancer is still the highest among malignant tumors of women's reproductive system [1]. Squamous cell carcinoma is the most common pathological type of cervical cancer, accounting for about 75% of all cervical tumors [14]. It mainly occurs in the junction between columnar epithelium of the endocervical canal and the stratified squamous epithelium of the ectocervix (squamocolumnar junction). Therefore, the present study mainly focused on the gene expression profiles of normal cervical tissues and cervical squamous carcinoma tissues to explore the differentially expressed genes in the tumorigenesis. Microarray analysis revealed 2,036 differentially expressed genes, of which 755 genes were upregulated and 1281 genes downregulated. Further exploration of differential mRNAs with gene ontology (GO) analysis and gene regulatory network construction revealed PDGFRA, CAV1, and GJA-1 as the key genes involved in cervical cancer metastasis and invasion, providing evidence and insights for further investigation of cervical cancer pathogenesis and targeted therapy.

Materials and Methods

Source of samples

The study was approved by the Peking University ethics committee (approval No. IRB 00001052-06058). Informed consent was signed by all patients who diagnosed with IB-IIB cervical cancer or benign gynecological diseases before operation for this study. The cervical cancer samples/CCS (n=8) and normal cervical samples/ NCS (n=8) were collected from Peking University Third Hospital. The average age of patients with cervical cancer HPV+ and benign gynecological diseases HPV- was 41.75+3.250 and 45.13 +3.425 respectively. The case information was obtained from medical records and pathology diagnosis.

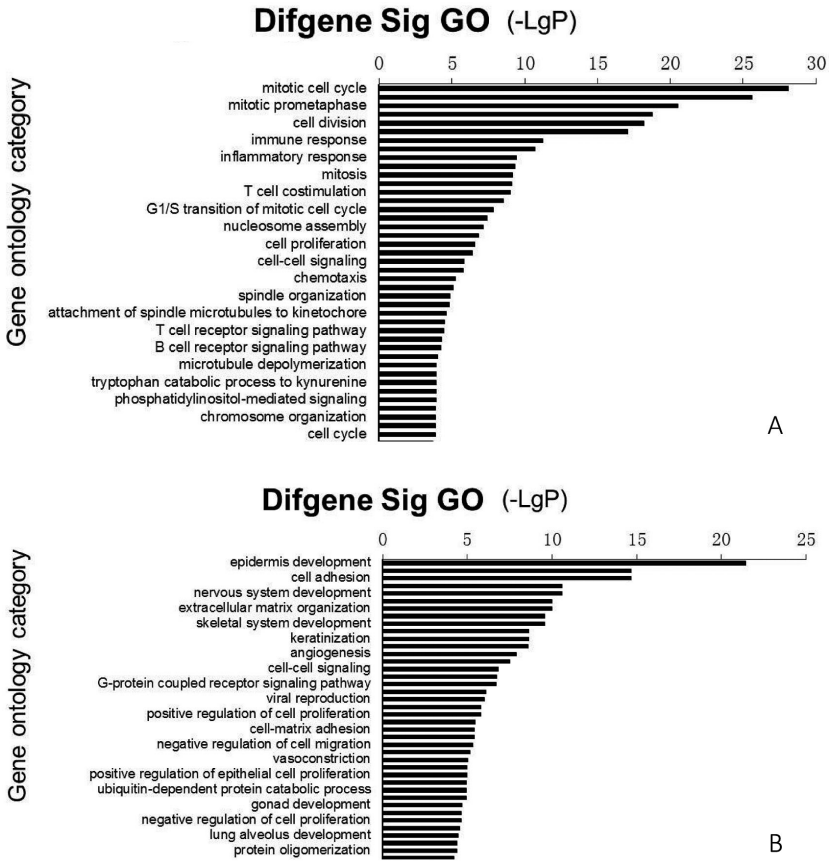

Figure 2. — Histogram of gene function in cervical cancer by GO analysis. The longitudinal coordinates for gene function name, negative logarithmic abscissa for gene function significant levels of *p* value (-LgP). A) upregulation of gene function; B) downregulation of gene function histogram.

Microarray analysis

The authors extracted the microarray expression profile and standardized by Agilent G4450AA feature extraction software 10.0. This study analyzed gene expression profiling on Agilent human whole genome microarray (Agilent G4112F Design ID 014850, 4×44 K format).

GO analysis

GO analysis, the key functional classification of NCBI, was employed to analyze the main function of the genes. Generally, Fisher's exact test and 2 test were used to classify the GO category, and false discovery date (FDR) was calculated to correct the *p* value. The smaller the FDR, the smaller the error in judging the *p* value. FDR was defined as FDR = 1–Nk/T, where Nk refers to cases wherein the *p* value of Fisher's test is less than that of 2 test. Only GOs with $p < 0.05$ and FDR < 0.05 were selected [15, 16].

Pathway analysis

Similarly, Kyoto encyclopedia of genes and genomes (KEGG, Japan), Biocarta (Germany), and Reatome (USA) are open-source for analyzing microarray data on biological pathway [17, 18]. In the present study, these were used to identify significant pathway correlated with cervical cancer.

Gene-gene network

Gene-gene network was built based on KEGG database. Gene-gene network was evaluated by graph theory method based on their "degree" in the network. The number of genes regulated by mRNA was defined as the degree of the mRNA.

qRT-PCR

Total RNA was extracted from cervical cancer samples and control samples. cDNA was synthesized by Quant reverse transcriptase, dNTP and random primer, and one ug total RNA. The cycling condition of qRT-PCR reaction consisted in an initial two minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 20 seconds at 64 °C, and 30 seconds at 72°C.

Results

Differentially expressed mRNAs in normal and cervical cancer

Tumor formation is a complicated process in which multiple genes and pathways are involved. Gene microarray technology is used for screening of genes involved in tumorigenesis. In this study the authors used Agilent gene expression microarray to analyze the mRNA expression changes between eight normal cervical tissues (HPV-) and eight cervical squamous cancer tissues (HPV+). Microarray data revealed 2,036 differentially expressed genes between two groups (754 genes upregulated and 1,282 genes downregulated) ($p < 0.05$, $FDR < 0.05$) and cluster analysis of differentially expressed genes was performed (Figure 1A). Colors in gene clustering map represent the expression level of mRNAs in tissues. The authors noticed that gene expression profiles in normal cervical tissues and in cervical cancer tissues are quite distinct. The top 25 upregulated

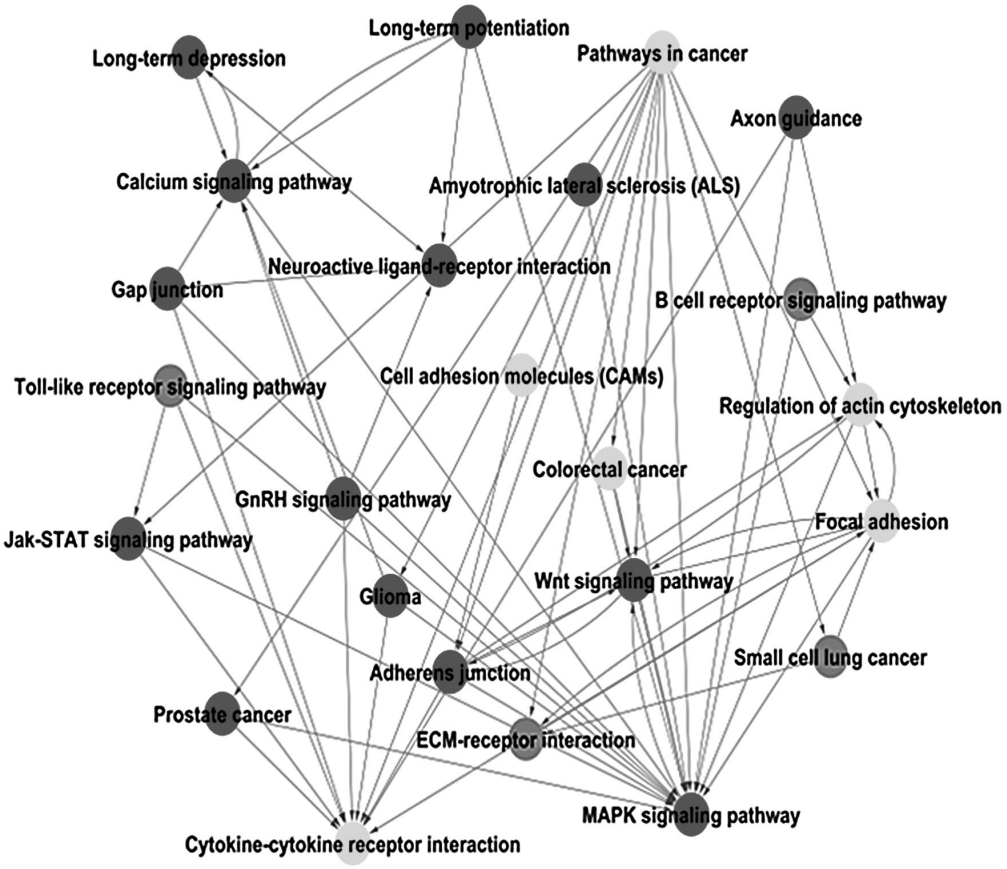

Figure 3. — Pathway network in cervical cancer. The circles represent pathway and the lines are the relationship between pathways. Red represents the expression of the pathway that contains genes that are upregulated; blue represents the expression of the pathway that contains genes that are downregulated; yellow represents the pathway that includes genes that are upregulated and downregulated.

Table 1. — *The interaction network of cervical cancer was significant between the pathway value of degree.*

Path name	Indegree	Outdegree	Degree	Style
MAPK signaling pathway	17	1	18	up
Pathways in cancer	0	11	11	up/down
Cytokine-cytokine receptor interaction	10	0	10	up/down
Focal adhesion	5	5	10	up/down
Adherens junction	4	4	8	up
Calcium signaling pathway	5	3	8	up
Wnt signaling pathway	5	3	8	up
Regulation of actin cytoskeleton	4	3	7	up/down
Gap junction	0	4	4	up
Glioma	1	3	4	up
GnRH signaling pathway	0	4	4	up
Jak-STAT signaling pathway	2	2	4	up
Long-term potentiation	1	3	4	up
Neuroactive ligand-receptor interaction	4	0	4	up
ECM-receptor interaction	3	1	4	down
Colorectal cancer	1	2	3	up/down
Axon guidance	0	3	3	up
Long-term depression	1	2	3	up
Prostate cancer	1	2	3	up
Small cell lung cancer	1	2	3	down
Toll-like receptor signaling pathway	0	3	3	down
B cell receptor signaling pathway	0	2	2	down
Cell adhesion molecules (CAMs)	0	1	1	up/down
Amyotrophic lateral sclerosis (ALS)	0	1	1	up

and downregulated mRNAs suggested that the expression of TCAM1, AIM2, SYCP2, MMP12, CXCL9/10/11, and STAT1 were elevated significantly, while the expression of SPINK5, PPP1R3C, ZNF521, S1PR3, and TGF-β2 declined notably.

According to the NCBI gene functional annotations, the authors performed enrichment analysis of the 2,036 different genes (Figure 1B). Results showed that about 14.79% of the genes contribute to signaling, 12.92% to system development, 11.47% to cell proliferation, 7.64% to immune response, 4.41% to cell adhesion and 1.93% to cell migration.

Significant functional analysis of differential genes GO analysis

GO analysis provides valuable insights into the relationship between gene functions. The authors annotated the functions of 2,036 genes according to GO database (classified with NCBI gene function annotations) to identify significant GO functions, with a $p<0.05$, and $FDR<0.05$. They found 135 upregulated GO functions and 193 downregulated GO functions ($p<0.05$ and $FDR<0.05$). Significant GO functions of downregulated genes are mainly associated with mitotic cell cycle, immune responses, cell proliferation, and inflammations

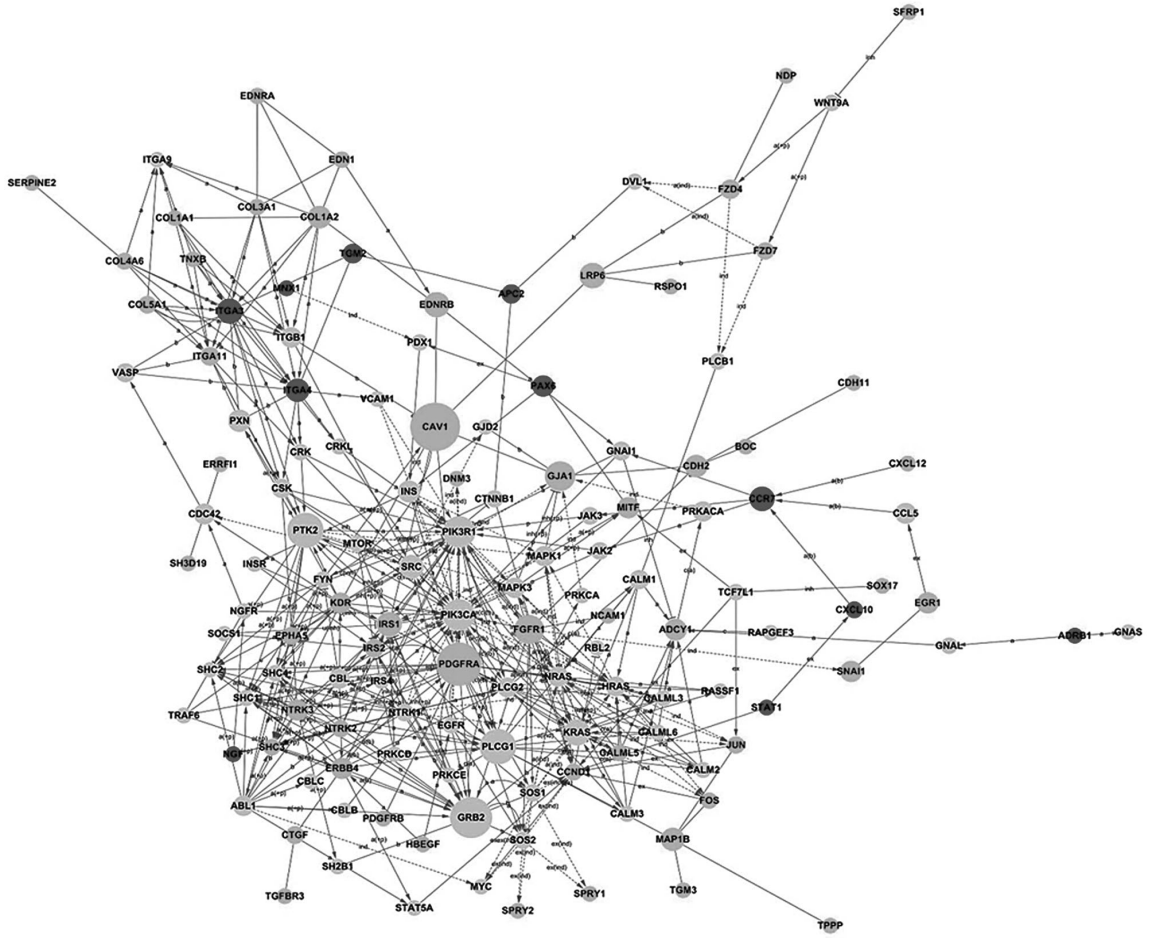

Figure 4. — The gene-network related with invasion and migration in cervical cancer. The circle represents the mRNA. Red label representative gene expression was up-regulated in cervical cancer, the green label representative gene expression was significantly downregulated in cervical cancer, the gray circle represents no changes in expression of genes in the chip.

(Figure 2A), while the significant GO functions of upregulated gene are mainly involved in epidermis development, cell adhesion, extracellular matrix secretion, and keratin differentiation (Figure 2B).

Significant interaction of networks among pathways—Path-Net

The authors further analyzed the 2,036 genes by pathway analysis and constructed Path-Net map depending on the statistical significance (*p value*) of differential gene-associated pathways (Figure 3) (Table 1). They noticed that MAPK signaling, pathways in cancer, cytokine-cytokine receptor interaction, and focal adhesion were critical to the development of cervical cancer. Path-Net map showed that interaction of signaling pathways closely related to cervical cancer migration, invasion, and metastasis including focal adhesion, regulation of actin cytoskeleton, Wnt signaling, adherens junction, and ECM-receptor affected the development and progression of cervical cancer.

Gene-gene networks in cervical cancer metastasis and invasion

Cell adhesion declined significantly in cervical tumorigenesis in the GO and pathway analysis. Such changes favored the invasion and metastasis of cervical cancer. GO-Map and pathway analysis revealed that the interaction of signaling pathways closely related to cervical cancer migration, invasion, and metastasis such as focal adhesion, regulation of actin cytoskeleton, Wnt signaling, adherens junction, and ECM receptor affected the development and progression of cervical cancer. The authors therefore, listed the different genes involved in cell adhesion, focal adhesion formation and cytoskeleton, which are related to cervical cancer invasion and metastasis. In combination with signal transduction pathways and gene-protein relationships in KEGG database, they constructed gene regulatory signal transduction network (Figure 4) (Table 2). Depending on the value of centrality (a larger score representing higher influence of a specific gene in signal trans-

Table 2. — *Cervical carcinoma metastasis and invasion of regulatory networks of mRNA related degree value.*

Gene_symbol	Description	Centrality	Degree	Indegree	Outdegree
CAV1	Caveolin 1, caveolae protein, 22kDa	0.03516	9	8	8
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	0.02962	27	10	22
GJA1	Gap junction protein, alpha 1, 43kDa	0.01494	8	8	3
FGFR1	Fibroblast growth factor receptor 1	0.01448	13	4	12
LRP6	Low density lipoprotein receptor-related protein 6	0.01053	4	4	4
EDNRB	Endothelin receptor type B	0.01031	4	3	3
ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	0.00975	14	10	8
IRS1	Insulin receptor substrate 1	0.00952	15	12	5
CCR7	Chemokine (C-C motif) receptor 7	0.00940	6	3	3
ITGA4	Integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	0.00814	14	9	8
MAP1B	Microtubule-associated protein 1B	0.00811	5	5	5
COL1A2	Collagen, type I, alpha 2	0.00754	9	4	9
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	0.00728	4	4	4
ADCY1	Adenylate cyclase 1 (brain)	0.00662	10	9	2

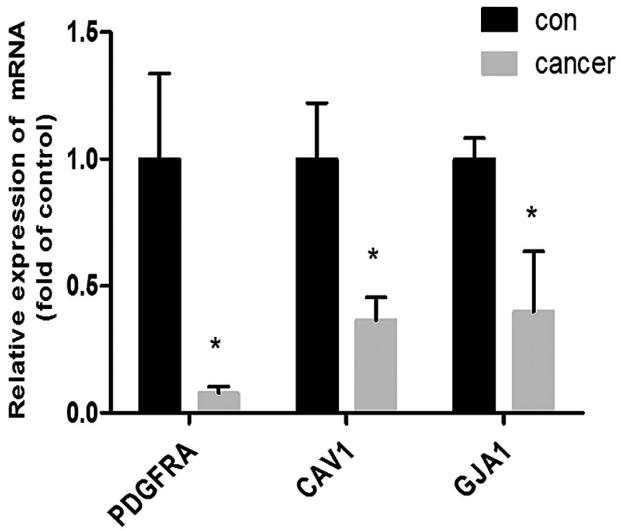

Figure 5. — Expression of PDGFRA, CAV1, and GJA-1 in cervical tissues verified by qRT-PCR. Comparisons of the expression of PDGFRA, CAV1 and GJA-1 in control and cervical cancer tissues by real-time RT-PCR. The β-actin is the reference. *$P < 0.05$, compared with controls.

duction) and degree (representing the number of genes interacting with a specific gene in a network) of genes in networks, they noticed that platelet-derived growth factor receptor alpha (PDGFRA), caveolin-1 (CAV1), gap junction protein, alpha 1 (GJA1), and fibroblast growth factor receptor 1 (FGFR1) play a central role. They also noticed that members of integrin family including integrin alpha-3/4 (ITGA3/4) and collagen, type V, alpha 1/2 (COL5A1/COL5A2) are important in the network.

Microarray data verification

To evaluate the microarray data, the authors used qRT-PCR to analyze gene expression profiles in normal cervical tissues and cervical cancer tissues. They checked the expressions level of PDGFRA, CAV1 and GJA1, the critical genes involved in cervical cancer metastasis and invasion, and confirmed the reliability of microarray data (Figure 5).

Discussion

Cervical cancer is one of the most common cancers of the female reproductive system. Despite the decreasing incidence due to improved screening approaches such as cervical smear test, the prognosis of cervical infiltrating and metastatic carcinoma remains poor with a high recurrence rate. The five-year survival rate after surgery is still very low [19, 20]. Currently, the development of effective tools to predict invasive cervical cancer and metastasis is still ongoing, requiring additional studies. Genetic screening contributes to the understanding of cervical cancer pathogenesis by identifying novel biomarkers and candidate genes essential for tumorigenesis. The present study has established gene expression profiles of eight normal cervical tissues and eight cervical squamous cancer tissues. The authors discovered several well-known tumor-associated genes such as MMP-12, STAT1, and CXCL9, which were upregulated significantly in tumor samples and promoted tumor proliferation, metastasis, and invasion [21-23]. Multiple known cervical cancer-related genes such as CKS2, TBX3, and LAM2 were also identified. Lyng *et al.* performed microarray analysis of cervical cancer with or without lymph node metastasis. The results suggested that CKS2 and TBX3 were closely related with survival rate of metastatic carcinoma [13]. Imura *et al.* identified Lam-5 as an efficient biomarker in cervical infiltrating carcinoma diagnosis [24]. In addition, multiple genes such as TCAM1, AIM2, SYCP2, SPINK5, and PPP1R3C changed significantly in this study, which were not identified in previous studies. Testicular cell adhesion molecule 1, known as TCAM1, is highly conserved in the evolution of mammals.

Higher expression of TCAM1 in cell membrane has been shown to be related with female infertility [25], but no reports have shown the role of TCAM1 in cervical cancer. These novel genes provide new ideas and challenges in elucidating the pathogenesis of cervical cancer.

Tumor invasion and metastasis is a complicated process involving multiple genes. Abnormal changes include cytoskeletal remodeling, diminished cell adhesion, and extracellular matrix degradation [26, 27]. Normal epithelial cells stick together by cell adhesion molecules to form tight connections. Local infiltration of carcinoma in situ is associated with decreased adhesion forces between tumor cells mediated by cell adhesion molecules [28]. Cell migration and transformation during invasion and metastasis is promoted by elevated cellular deformability. Cellular cytoskeleton is mainly composed of microtubules, microfilaments, and intermediate filaments. Microtubule depolymerization is the major driving force of cell movement. Previous studies showed that family members of the Rho Family GTPase (Rac1, Cdc42, RhoA, and RhoC) participate in cellular signal transduction and regulate the remodeling of cytoskeleton [29-31]. The Wnt-β-catenin signal pathway has been shown to be closely related with tumor invasion and metastasis. In tumor cells, E-cadherin overexpression blocked the transcriptional regulation by β- catenin and switched off the expression of target genes to inhibit the proliferation and migration of tumor cells [32]. In this study, GO analysis and Path-Net analysis of differential genes in cervical cancer indicated that cell adhesion, cell movement, and cell cytoskeleton regulation are important in the development of cervical cancer. A network of genes associated with cervical cancer metastasis and invasion was constructed with genes involved in focal adhesion, Wnt signaling pathway, regulation of actin cytoskeleton and Rho family. Network analysis revealed the central role of PDGFRA, CAV1, FGFR1, and GJA1 in the whole network. The authors also checked the expression level of PDGFRA, CAV1, and GJA1 in cervical tissues with qRT-PCR and identified that the expression of these three key genes in cervical cancer was consistent with microarray data.

PDGFRA belongs to receptor tyrosine kinase family. Ligand binding to PDGFRA is related to cell growth and division [33, 34]. Studies have shown that PDGF-PDGFRA signaling is associated with vascular smooth muscle cell migration and remodeling though Akt, ERK1/2, and EGFR [35, 36]. Taja-Chayeb *et al.* analyzed the expression level of PDGFRA in 36 cervical cancer samples (32SCC, 4ASC) with immunohistochemistry and found downregulation of PDGFRA expression in 58.4% of all cervical cancer samples [37]. With microarray and qRT-PCR analysis, the present authors found that PDGFRA expression in cervical cancer samples decreased significantly compared with normal samples. Regulatory network analysis of tumor invasion and metastasis-associated genes revealed that the indegree value of PDGFRA-related signal transduction pathways [10] and the outdegree value [22], suggest the indispensable role of PDGFRA in the entire regulatory network. GO and pathway analysis showed that PDGFRA is mainly involved in extracellular matrix organization and positive regulation of cell migration, which is essential for tumor metastasis and invasion. The authors also found that PDGFRA participated in cervical cancer invasion and metastasis by regulating the downstream genes such as SRC, PIK3CA, KRAS, and GRB2.

In recent years, loss of tumor suppression gene CAV1 was associated with tumor proliferation, invasion, metastasis, disrupted signal transduction. and multiple drug resistance [38, 39]. Miotti *et al.* found that CAV-1 deletion leads to E-cadherin redistribution inside cells, reduced cellular interaction. and elevated ovarian carcinoma cell metastasis [40]. Song *et al.* showed that reduced CAV1 expression in lung cancer cell line NCI-H460 upregulated β-catenin expression and downregulated E-cadherin expression significantly, indicating the inhibitory role of CAV-1 in tumor invasion and metastasis in NCI-H460 cell line [41]. In this present study, the authors found that in comparison with the normal tissue groups, CAV1 declined significantly in eight cervical cancer tissues. GO and pathway analysis identified the significant role of CAV1 in canonical Wnt receptor signaling pathway regulation. In Wnt signaling pathway, β-catenin activated the transcription of downstream targets such as c-myc, cyclin D1, MMP-7, CD44, Claudin-1, and participated in the regulation of cell adhesion, migration, angiogenesis and so forth [42,43]. Studies by Williams *et al.* revealed a negative correlation between CAV1 and MMP expression [44]. The present authors found that CAV-1 deletion may have direct or indirect interaction with β-catenin or TGF-β to promote the invasion and metastasis of cervical cancer cells.

Connexin 43 protein, encoded by GJA1 gene, is involved in cell-cell signaling and related signal transduction pathways. Previous studies revealed that reduced or deficient connexin 43 expression resulted in abnormal cell-cell communication, diminished surveillance, and regulation of cellular function, excessive cell proliferation and elevated cell migration [45-47]. Crespin *et al.* reported that in neuroblastoma, connexin 43 affected cell morphology through regulation of actin cytoskeleton [48]. The present results showed that GJA1 expression declined significantly in cervical cancer tissues compared with normal cervical tissues. Network analysis of tumor invasion and metastasis-associated genes indicated that GJA1 participated in the process of cervical cancer adhesion, migration and metastasis to promote malignant transformation of cervical cancer.

Conclusion

In conclusion, gene microarray and bioinformatics analysis showed that PDGFRA, CAV1, and GJA1 are critical for cervical cancer metastasis and invasion. The results elucidate the key role of genes involved in cervical cancer de-

velopment and progression, providing insights into the underlying mechanism, and offer evidence for further identification of novel biomarkers and key genes in cervical cancer.

Acknowledgments

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Address reprint requests to:
LI GENG, M.D.
Department of Gynecology and Obstetrics
Peking University Third Hospital
Huayuan-Bei Road No. 49, Haidan District
100191 Beijing (China)
e-mail: gengli1957@bjmu.edu.cn