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Microarray gene expression profiling for identifying different responses to radiotherapy and chemoradiotherapy in patients with cervical cancer

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Summary

Purpose: Cervical cancer, which is treated by radiotherapy (RT) and chemoradiotherapy (CRT), has high morbidity and mortality in women. This study aimed to identify differences in gene response to CRT and RT. *Materials and Methods:* GSE3578 was downloaded from Gene Expression Omnibus including specimens from 20 RT-treated patients and 19 CRT-treated patients. Differentially expressed genes (DEGs) were identified using siggenes package in R. Protein-protein interaction (PPI) network was visualized by cytoscape. MCODE and cytoscape was used separately to mine and construct modules in the PPI network. Transcription factor (TF)-DEG and miRNA-DEG pairs were predicted and then visualized by cytoscape. *Results:* Total 22 upregulated and 181 downregulated genes were identified in CRT samples. Several functions were enriched for these DEGs. A module involving ZNF449 and ZNF673 was mined from the PPI network of downregulated genes. In the TF-DEG regulatory networks, downregulated GATA3 (which was modulated by SP1) was also a TF, as well as upregulated CDK6 was regulated by several TFs (e.g. GATA3). Hsa-miR-17, hsa-miR-34a, hsa-miR-124, hsa-miR-1185-2-3p, hsa-1185-1-3p, and hsa-let-7f-2-3p were identified as key miRNAs in the miRNA-DEG regulatory network. *Conclusion:* CRT might cure cervical cancer by acting on those molecules that were more sensitive to CRT than CT.

Key words: Cervical cancer; Radiotherapy; Chemoradiotherapy; Protein-protein interaction network; Regulatory network.

Introduction

Cervical cancer is the second most common gynecological malignancy after breast cancer and also the fourth leading cause of death from cancer in women [1, 2]. In 2012, there were approximately 528,000 women with cervical cancer and 266,000 deaths caused by the disease [2]. About 80% of cervical cancer cases were in developing countries [3]. Radiotherapy (RT) and chemoradiotherapy (CRT) are important therapeutic methods for patients with cervical cancer [4, 5].

There has been an increasing interest in the use of RT and CRT to treat cervical cancer over the last decade [6, 7]. RT brings similar survival outcomes for patients with cervical cancer as surgery, but with negative impacts, such as sexual function interruption [8]. Cisplatin-based CRT is the standard modality of care for patients in advanced stages [9]. It is reported that the therapeutic effect of cisplatin-based CRT is better than that of RT [10]. Advantages of CRT over RT in patients with cervical cancer have now been well demonstrated in a series of randomized prospective trials [11]; for instance, absolute improvement in the overall survival and significant reduction in local recurrence [12, 13]. However, few studies have investigated the genetic response to these two therapies. In this study, the authors investigated the microarray expression data of patients with cervical cancer that underwent RT and CRT treatment to analyze the molecular mechanism underlying CRT. Differentially expressed genes (DEGs) between RT and CRT treatment were identified and their underlying functions were predicted. Subsequently, the protein-protein interaction (PPI) network, transcription factor (TF)-DEG network, and miRNA-DEG network were constructed to identify key molecules during CRT treatment.

Materials and Methods

Microarray expression data of biopsy samples from cervical cancer patients (GSE3578) [14] were downloaded from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) which was based on the platform of GPL2895 GE Healthcare/Amersham Biosciences CodeLink Human Whole Genome Bioarray [15, 16]. Squamous cell carcinoma of cervix from 39 patients before and during treatment were collected as biopsy specimens, twice for each patient. Accompanied by ^{192}Ir high dose-rate intracavitary brachytherapy, 20 patients (RT group) received 30.6 Gy of whole pelvic irradiation, and another dose to parametria to reach 50.6 Gy and 19 patients (CRT group) re-

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Table 1. — *The enriched functions for the upregulated genes.*

Category	Term	Count	*p*-value
GOTERM_BP_FAT	GO:0006333~chromatin assembly or disassembly	3	1.35E-2
GOTERM_CC_FAT	GO:0005694~chromosome	4	2.16E-2
GOTERM_CC_FAT	GO:0000785~chromatin	3	2.84E-2
GOTERM_BP_FAT	GO:0022402~cell cycle process	4	4.27E-2

GO: gene ontology; BP: biological process; CC: cellular component.

Table 2. — *The enriched functions for the downregulated genes.*

Category	Term	Count	*p*-value
GOTERM_BP_FAT	GO:0045449~regulation of transcription	38	2.46E-3
GOTERM_BP_FAT	GO:0007368~determination of left/right symmetry	4	6.50E-3
GOTERM_BP_FAT	GO:0009855~determination of bilateral symmetry	4	6.95E-3
GOTERM_BP_FAT	GO:0009799~determination of symmetry	4	6.95E-3
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	27	8.62E-3
GOTERM_CC_FAT	GO:0043232~intracellular non-membrane-bounded organelle	32	4.16E-2
GOTERM_CC_FAT	GO:0043228~non-membrane-bounded organelle	32	4.16E-2
GOTERM_MF_FAT	GO:0008094~DNA-dependent ATPase activity	4	1.53E-2
GOTERM_MF_FAT	GO:0008134~transcription factor binding	11	1.93E-2
GOTERM_MF_FAT	GO:0004723~calcium-dependent protein serine/threonine phosphatase activity	2	2.72E-2
GOTERM_MF_FAT	GO:0004221~ubiquitin thiolesterase activity	4	3.15E-2

GO: gene ontology; BP: biological process; CC: cellular component; MF: molecular function.

ceived both the same radiotherapy as for the RT group and chemotherapy of cisplatin (40 mg/m^2) for five times in one week. The second biopsy was performed one week later. Both RT and CRT groups received nine Gy to the whole pelvis. In addition, the CRT group also received cisplatin-based chemotherapy. All biopsy specimens were taken from the same site of cervical tumors.

After GSE3578 was downloaded, microarray data was preprocessed. IDs of probes were transformed into gene symbols. For one gene mapped with multiple probes, the average value of these probes were taken as ultimate gene expression value. Then, the authors used preprocessCore package [17] in R to normalize the expression profiles. After that, significance analysis of microarrays (SAM) method in siggenes package [18] in R were used to identify DEGs between RT and CRT samples. The cut-off criterion was set as the false discovery rate (FDR) at < 0.05.

Gene Ontology (GO) analysis is used to perform an functional study for genome or large-scale transcriptomic data [19]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database introduces the working way of molecules or genes [20]. Function and pathway enrichment analysis for DEGs were performed by DAVID software (http://david.abcc.ncifcrf.gov/) [21]. The cut-off criterion was set as *p*-value < 0.05.

The PPI data of DEGs were obtained from the STRING database [22], then the PPI network was visualized by cytoscape [23]. The proteins in PPI network were called nodes and the degree of a node was equal to the number of interactions involving it. Additionally, MCODE [24] and cytoscape separately were used to mine and construct modules for the PPI network.

Combining the information of TF binding sites (TFBS) obtained from UCSC [25], TF-DEG pairs were acquired and TF-DEG regulatory network was constructed using cytoscape.

There are several major miRNA-gene prediction databases including TarBase [26], TargetScan [27], and MiRecord [28]. The authors used these databases to predict the interactions between miRNA and the DEGs. Cytoscape was used to construct the miRNA-DEG regulatory network. MiRNAs which regulated more than two DEGs were selected as key miRNAs in the network.

Results

The authors obtained gene expression matrices of 18002 genes after the data preprocessing procedure. Then, SAM method was used to identify DEGs between CRT and RT samples. Compared with RT samples, total 203 DEGs (including 22 upregulated genes and 181 downregulated genes) were screened in CRT samples. There were more downregulated genes than upregulated genes.

DAVID software was used to carry out enrichment analysis. The significant functions of upregulated genes and downregulated genes are listed in Tables 1 and 2, respectively. The authors found that the upregulated genes were enriched in some GO terms including chromatin assembly or disassembly (p = 1.35E-2), cell cycle process (p = 4.27E-2), chromosome (p = 2.16E-2), and chromatin (p = 2.84E-2). The enriched GO functions for the downregulated genes included regulation of transcription (p = 2.46E-3), determination of symmetry (p = 6.95E-3), and DNA-dependent ATPase activity (p = 1.53E-2). No significant pathways were enriched for these DEGs.

The number of upregulated genes was so small that no network was constructed for them. The PPI network of downregulated genes is shown in Figure 1. In addition, the authors used MCODE to mine modules in the network, and obtained a significant module (score = 4.2, Figure 2) in-

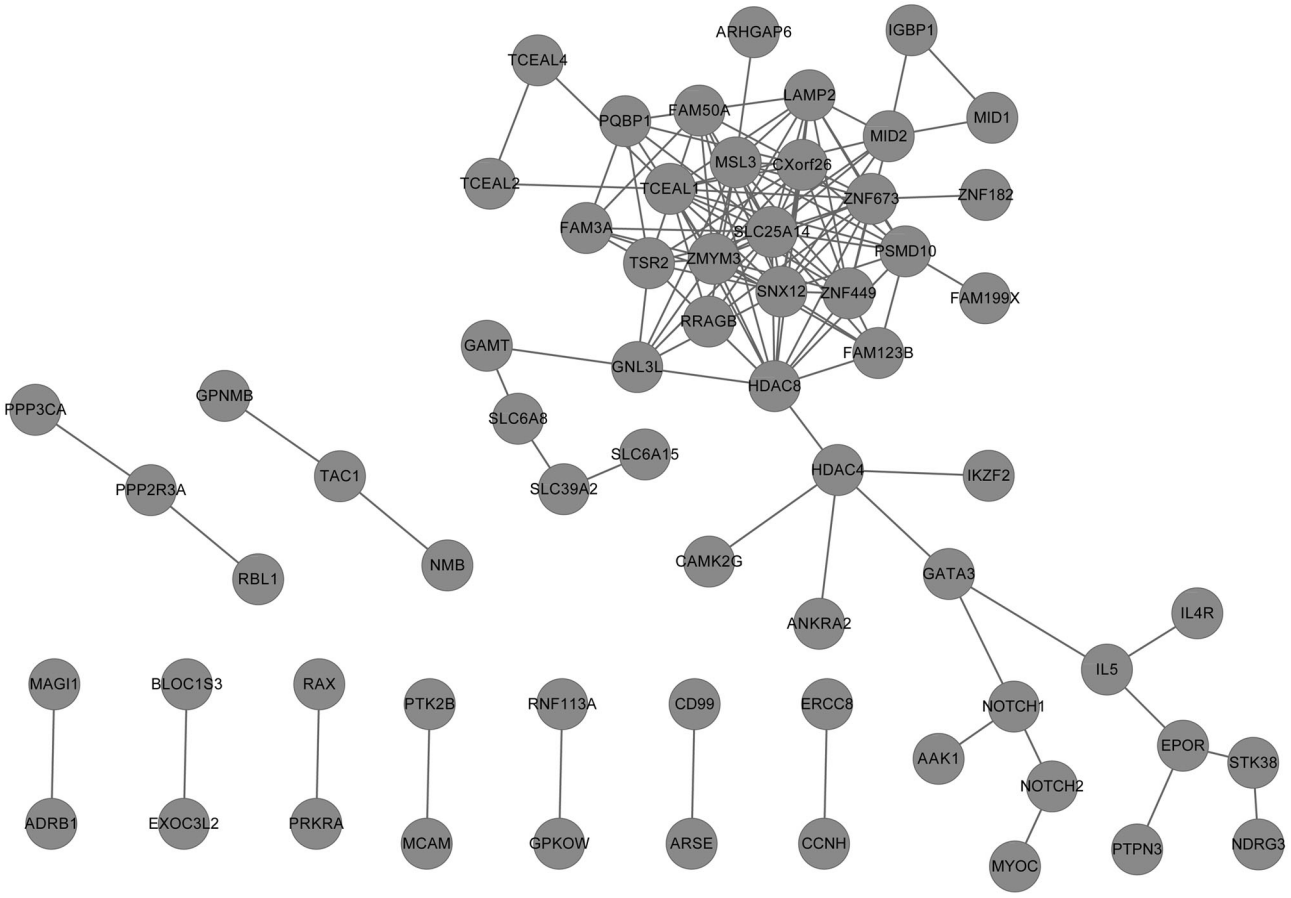

Figure 1. — The PPI network of the downregulated DEGs.

volving ZNF449 and ZNF673. Further enrichment analysis was performed for genes in the module, and the enriched functions included regulation of transcription (p = 1.47E-2) and regulation of RNA metabolic process (p = 3.51E-2) (Table 3).

Based on the TF-gene relationships obtained from UCSC, the authors constructed the TF-upregulated gene regulatory network (Figure 3) and TF-downregulated gene regulatory network, respectively (Figure 4). Importantly, downregulated GATA3 (which was modulated by SP1) was also a TF. Furthermore, upregulated CDK6 was regulated by several TFs (e.g. GATA3) and had a higher degree.

Finally, the authors constructed miRNA-DEG regulatory network for all of the DEGs (Figure 5). They identified six key miRNAs (hsa-miR-17, hsa-miR-34a, hsa-miR-124, hsa-miR-1185-2-3p, hsa-1185-1-3p, and hsa-let-7f-2-3p) which regulated more than two DEGs. Especially, both GATA3 and NLK were targeted by hsa-let-7f-2-3p, hsa-miR-1185-2-3p, and hsa-1185-1-3p.

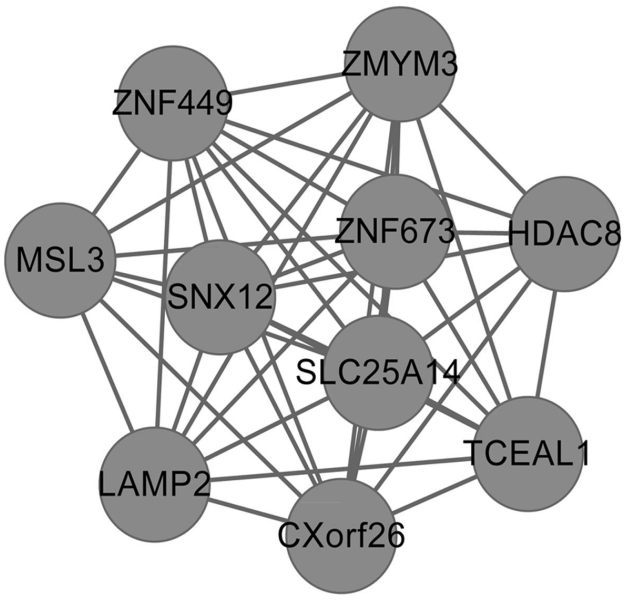

Figure 2. — The significant module in PPI network of the downregulated DEGs.

Table 3. — *The enriched functions for the genes in the significant module.*

Category	Term	Count	*p*-value
GOTERM_BP_FAT	GO:0045449~regulation of transcription	5	1.47E-2
GOTERM_BP_FAT	GO:0006355~regulation of transcription, DNA-dependent	4	3.30E-2
GOTERM_BP_FAT	GO:0051252~regulation of RNA metabolic process	4	3.51E-2
GOTERM_MF_FAT	GO:0003677~DNA binding	4	4.34E-2
GOTERM_MF_FAT	GO:0003700~transcription factor activity	3	4.84E-2

GO: gene ontology; BP: biological process; MF: molecule function.

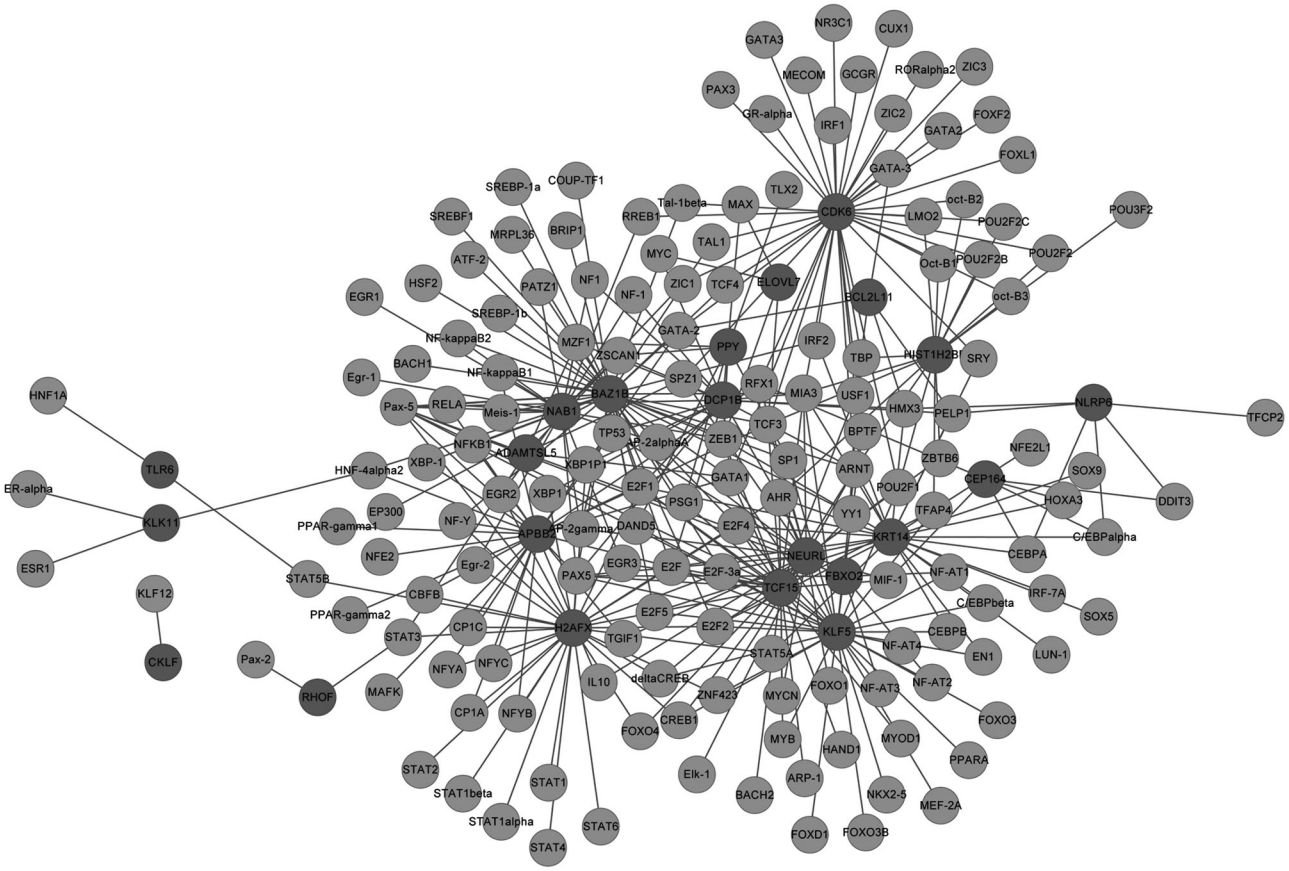

Figure 3. — The TF-upregulated gene network. Blue nodes indicate TFs, and red nodes represent upregulated genes.

Discussion

High-throughput gene expression profiling analyzed by bioinformatics methods has provided new understandings of pathogenesis, diagnosis, and treatment of many human diseases including cervical cancer. In this study, based on microarray data, some key molecules contributing to the therapeutic effect of CRT were detected. Total 203 DEGs (including 22 upregulated genes and 181 downregulated genes) were identified in CRT samples compared with RT samples. The majority of upregulated genes were related to chromatin assembly or disassembly, chromosome, chromatin, and cell cycle process. Meanwhile, downregulated genes were mainly enriched in regulation of transcription, determination of symmetry, transcription factor binding, and non-membrane-bounded organelle.

A significant module was obtained from the PPI network of downregulated genes. Functional enrichment analysis indicated that proteins in the module were enriched in some important biological processes, such as regulation of transcription and regulation of RNA metabolic process. ZNF449 and ZNF673 in the module were members of zinc finger protein family. Zinc finger protein family plays important roles in the recognition and binding to DNA, protein, and RNA. Previous researches have shown that some

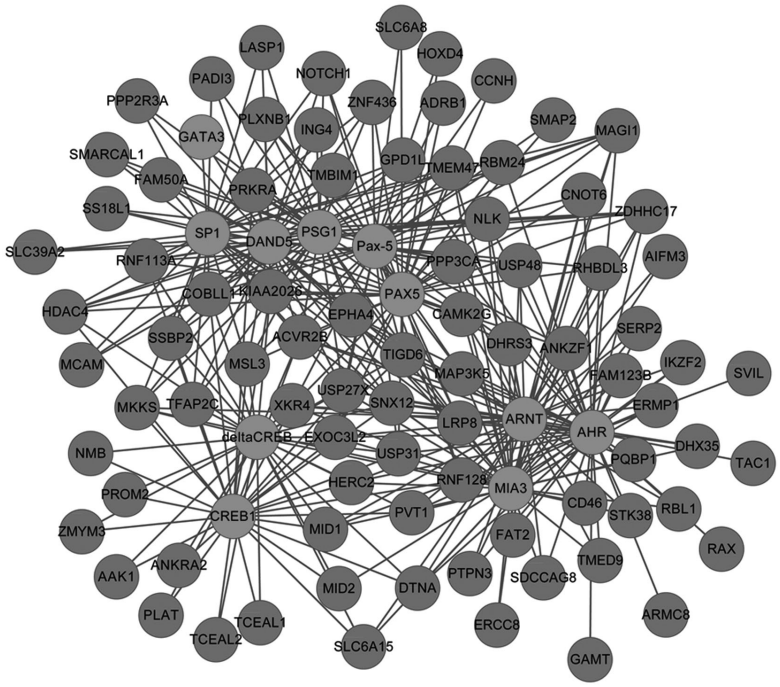

Figure 4. — The TF-downregulated gene network. Blue nodes indicate TFs, and green nodes represent downregulated DEGs.

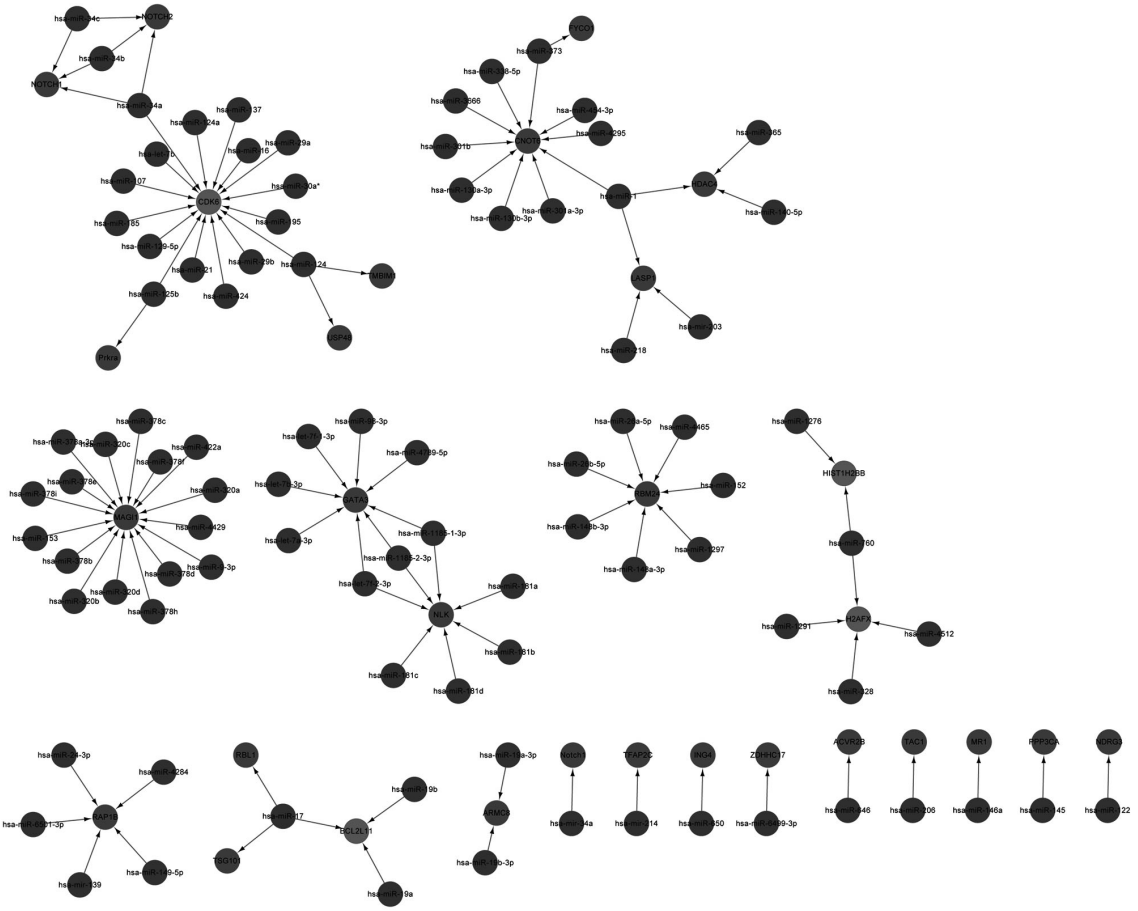

Figure 5. — The miRNA-DEG regulatory network. Blue nodes indicated miRNAs, red nodes represent upregulated DEGs, and green nodes stand for downregulated DEGs.

zinc finger proteins were associated with cervical cancer. For instance, Wang *et al.* suggested that the zinc finger protein ZNF268 is overexpressed in human cervical cancer and may be a novel therapeutic target or a diagnostic marker for cervical cancer [29]. The study of Huang *et al.* shows that ZNF582 is frequently methylated in CIN3 and worse lesions, and can be used as a potential biomarker for the molecular screening of cervical cancer [30]. Therefore, the present authors inferred that ZNF449 and ZNF673 might also be associated with cervical cancer, since proteins in the same family might have similar functions.

In the TF-downregulated gene regulatory network, downregulated GATA3 was also a TF. GATA3 belongs to the GATA zinc finger transcription factor family, which functions as a tumor suppressor gene [31]. Previous researches show that GATA3 is associated with cervical cancer [32-34]. Thus, the downregulation of GATA3 in this study suggested an effective response to CRT treatment. Furthermore, GATA3 was regulated by SP1 in the TF-downregulated gene regulatory network. SP1, as the first TF found in mammalian cells, belongs to C2-H2 zinc-finger family. It plays an important role in cervical cancer [35, 36], and participates in resistance to cisplatin [37]. Downregulation of SP1 increases Noxa expression in response to cisplatin, which will then promote the apoptosis of cancer cells [38]. Therefore, SP1 might be a specific gene response to CRT treatment in cervical cancer. In the TF-upregulated gene regulatory network, CDK6 was regulated by several TFs (e.g. GATA3) and had a higher degree. Kohrt *et al.* suggested that CDK6 bound and promoted the degradation of the EYA2 protein, which has been found to be overexpressed in cervical cancer [39], indicating that CDK6 might be involved in CRT treatment of cervical cancer.

By analyzing the miRNA-DEG regulatory network, six miRNAs (hsa-miR-17, hsa-miR-34a, hsa-miR-124, hsa-miR-1185-2-3p, hsa-1185-1-3p, and hsa-let-7f-2-3p) were obtained to be sensitive to CRT treatment. Wilting *et al.* suggest that hsa-miR-124 is methylated in cervical cancer cell lines and may be used as a valuable marker for improved detection of cervical cancer [40]. Reduced levels of hsa-miR-34a have been detected in cervical cancer [41], and also the expression level of hsa-miR-1185 [42]. Accordingly, these miRNAs might be the therapy response factors during CRT treatment. A study of Tsuchida *et al.* demonstrates that the miR-17–92 miRNA cluster yields six mature miRNAs, including hsa-miR-17 and hsa-miR-20a [43]. Kang *et al.* concludes that hsa-miR-20a can promote migration and invasion of cervical cancer cells [44]. Therefore, the present authors deduced that hsa-miR-17 might be a candidate target for CRT treatment of cervical cancer. There is no literature regarding hsa-let-7f-2-3p in cervical cancer, but it targeted the same two DEGs (GATA3, NLK) as hsa-miR-1185-2-3p and hsa-1185-1-3p. Thus, the present authors speculated that hsa-let-7f-2-3p might also be sensitive to CRT treatment of cervical cancer.

In conclusion, a comprehensive bioinformatics analysis was conducted to identify genes which might be sensitive to CRT treatment of cervical cancer. The authors screened 203 DEGs in CRT samples compared with RT samples. Several molecules (ZNF449, ZNF673, CDK6, GATA3, SP1, hsa-miR-17, hsa-miR-34a, hsa-miR-124, hsa-miR-1185-2-3p, hsa-1185-1-3p, and hsa-let-7f-2-3p) which were more sensitive to CRT treatment than RT treatment of cervical cancer, were identified. Therefore, the authors inferred that CRT treatment might be able to cure cervical cancer by acting on cancer related molecules. However, future studies are still needed to validate the outcomes of this study.

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