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Genes associated with platinum-resistance in platinum-resistant ovarian cell line

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Summary

Background: In this study the authors aimed to identify genes associated with platinum-resistance in platinum-resistant ovarian cell line. *Materials and Methods:* The transcriptome profile dataset ERA033498 was downloaded. Clean sequencing reads were mapped to human hg19 genome using TopHat. After filtering, reads were then annotated using ANNOVAR tool. Gene expression level was estimated by the value of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) using Cufflinks. R/Limma package was used to identify differentially expressed genes (DEGs) between the platinum-sensitive and -resistant cell lines in each patient, with the threshold of $|\log_2(\text{fold change})| > 2$ and q-value < 0.05. Then, functional annotation and pathway enrichment analysis of DEGs was performed. A protein-protein interaction (PPI) network was also built using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), followed by module analysis, and further functional annotation of the sub-network genes. *Results:* Quality sequencing reads was collected from each sample, with the lowest alignment rate of 95.38%. Totally, 304 DEGs were commonly identified from the two patients, including 247 up-regulated. Four *IFIT* family members were observed to be up-regulated. STAT1, MX1, DDX58, XAF1, and IFIH1 were the top five hubs with the highest connection degrees in the PPI network. *STAT1* may affect platinum-resistance via influenza A, herpes simplex infection, hepatitis C and measles pathways; *IFIH1* may function via RIG-I-like receptor signaling pathway, influenza A, herpes simplex infection, and measles pathways; *IFIT1* via the herpes simplex infection and hepatitis C pathways. *Conclusions: STAT1, IFIH1,* and the four *IFIT* family members may be associated with platinum-resistance in ovarian cancer cells.

Key words: Ovarian cancer; Platinum-resistance; Functional annotation and pathway enrichment analysis; Protein-protein interaction network; Module analysis.

Introduction

Ovarian cancer is the leading cause of death among gynecological cancers in the western world. In most patients, tumors are diagnosed at an advanced stage [1] and platinum-based drugs are often first choice for chemotherapy following surgical cytoreduction. A widely recognized mechanism underlying platinum based chemotherapy is that aquated cisplatin (a major type of platinum-containing drugs) first reacts to guanine nucleotide and forms platinum-DNA monoadducts, which can further form intrastrand and inter-strand crosslinks, subsequently inhibiting the synthesis and replication of DNA [2]. However, relapse occurs in most patients with advanced disease after the initial treatment, due to the development of platinum–resistance [3], which eventually leads to the death of patients.

Many efforts have been made to shed light on the platinum-resistance development in ovarian cancer, and the resistance mechanisms currently presented can be generally divided into two types: reduced cisplatin accumulation and intracellular cisplatin inactivation [4, 5]. The former may be attributed to a decline in drug uptake, an increase in drug efflux, or both in resistant cells. Several genes (e.g. *CTR1*, *ATP7A,* and *ATP7B*) have been proven critical to the cellular cisplatin resistance to, which can impair cisplatin accumulation by affecting its transport of cisplatin [6, 7]. Additionally, genes participating in drug or anticancer drugs transport, such as *MRP* and *MDR1* [8], can also affect cisplatin accumulation. Glutathione (gamma-glutamylcysteinylglycine, GSH) that can detoxify many cellular toxins, including cisplatin and its analogues, is a leading cause of intracellular cisplatin inactivation, by converting cisplatin into cisplatin-thiol conjugates [9-10].

Previous researchers have applied microarrays to identify the gene-expression profiles that are associated with clinical or prognostic outcomes in patients with ovarian cancer [11, 12]. However, chemotherapy resistance may be essentially multi-factorial, thus more molecular markers need to be developed. In the present study, using both plat-

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Table 1. — *Detailed sample information from each patient (downloaded from Sequence Read Archive database, NCBI). For each patient, a platinum resistant sample and a platinum sensitive sample were collected, each in triplicate.*

Run	BioSample	Clinical information	Individual	Sample name	Clinical history
ERR035533	SAMEA801439	Platinum sensitive	Patient 3	E-MTAB-691:PEO14 ovarian cancer cell line	Presentation
ERR035534	SAMEA801439	Platinum sensitive	Patient 3	E-MTAB-691:PEO14 ovarian cancer cell line	Presentation
ERR035535	SAMEA801439	Platinum sensitive	Patient 3	E-MTAB-691:PEO14 ovarian cancer cell line	Presentation
ERR035536	SAMEA801438	Platinum sensitive	Patient 2	E-MTAB-691:PEO1 ovarian cancer cell line	First relapse
ERR035537	SAMEA801438	Platinum sensitive	Patient 2	E-MTAB-691:PEO1 ovarian cancer cell line	First relapse
ERR035538	SAMEA801438	Platinum sensitive	Patient 2	E-MTAB-691:PEO1 ovarian cancer cell line	First relapse
ERR035539	SAMEA801435	Platinum resistant	Patient 3	E-MTAB-691:PEO23 ovarian cancer cell line	Relapse
ERR035540	SAMEA801435	Platinum resistant	Patient 3	E-MTAB-691:PEO23 ovarian cancer cell line	Relapse
ERR035541	SAMEA801435	Platinum resistant	Patient 3	E-MTAB-691:PEO23 ovarian cancer cell line	Relapse
ERR035542	SAMEA801437	Platinum resistant	Patient 2	E-MTAB-691:PEO4 ovarian cancer cell line	Second relapse
ERR035543	SAMEA801437	Platinum resistant	Patient 2	E-MTAB-691:PEO4 ovarian cancer cell line	Second relapse
ERR035544	SAMEA801437	Platinum resistant	Patient 2	E-MTAB-691:PEO4 ovarian cancer cell line	Second relapse

inum-sensitive and platinum-resistant cell lines from each of two patients, the authors attempted to identify novel genes associated with the development of platinum-resistance in ovarian cancer cells by analyzing a transcriptome sequencing dataset, with the aim to provide potential markers for prediction of response to first-line platinum-based chemotherapy.

Materials and Methods

The authors extracted a transcriptome profile dataset ERA033498 from the public gene expression data repository SRA (Sequence Read Archive, http://www.ncbi.nlm.nih.gov/sra) of National Center for Biotechnology Information (NCBI). In total, this dataset comprised RNA-Seq data from 12 samples from two patients with ovarian cancer. From each patient, both platinum-sensitive cell line and platinum-resistant cell line were collected, each in triplicate. Detailed information about the patient samples is shown in Table 1.

The fast q raw sequencing data were first filtered to obtain quality clean data. Then, the clean sequencing reads were mapped to human hg19 reference genome using TopHat tool [13], and only those with fewer than two mismatches and two gaps were retained. Then, ANNOVAR tool was used to annotate the reads in the order: exon region (UTR5/UTR3), splicing region, intron region, and intergenic region. Finally, the percentage of annotated reads of each type was calculated.

Gene expression level was estimated by the value of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) using the Cufflinks software [14]. The Limma package of R [15] was used to identify differentially expressed genes (DEGs) between the platinum sensitive and platinum resistant cell lines in Patient 2, as well as Patient 3, respectively. Only genes with $|\log_2(\text{fold change})| > 2$ and q-value < 0.05 were considered to be differentially expressed. Clustering analysis of the six samples from each patient using the screened DEGs was performed for validation and the result was shown using heatmaps.

Functional annotation of DEGs was performed using online biological classification software DAVID based on Gene Ontology database [16]. The authors calculated the *p*-value using the hypergeometric distribution. Multiple comparison *p*-value was corrected by controlling false detection rate (FDR) taking q-value < 0.05 as the cut off.

Kyoto Encyclopedia of Genes and Genomes (KEGG) online database(http://www.genome.jp/kegg/pathway.html) was employed to the identify functional and metabolic enrichment pathways of both up- and down-regulated DEGs (q-value < 0.05) [17].

The Search Tool for the Retrieval of Interacting Genes (STRING) database was used to investigate the functional interactions among the encoded proteins of the DEGs. A combined STRING score of > 0.4 was set as the cut-off. The resulting protein-protein interaction (PPI) pairs were then visualized using Cytoscape [18].

Genes of the node protein-encoding genes were further subject to functional annotation and pathway enrichment analysis using DAVID based on Gene Ontology (GO) [16] and KEGG [17] databases, respectively.

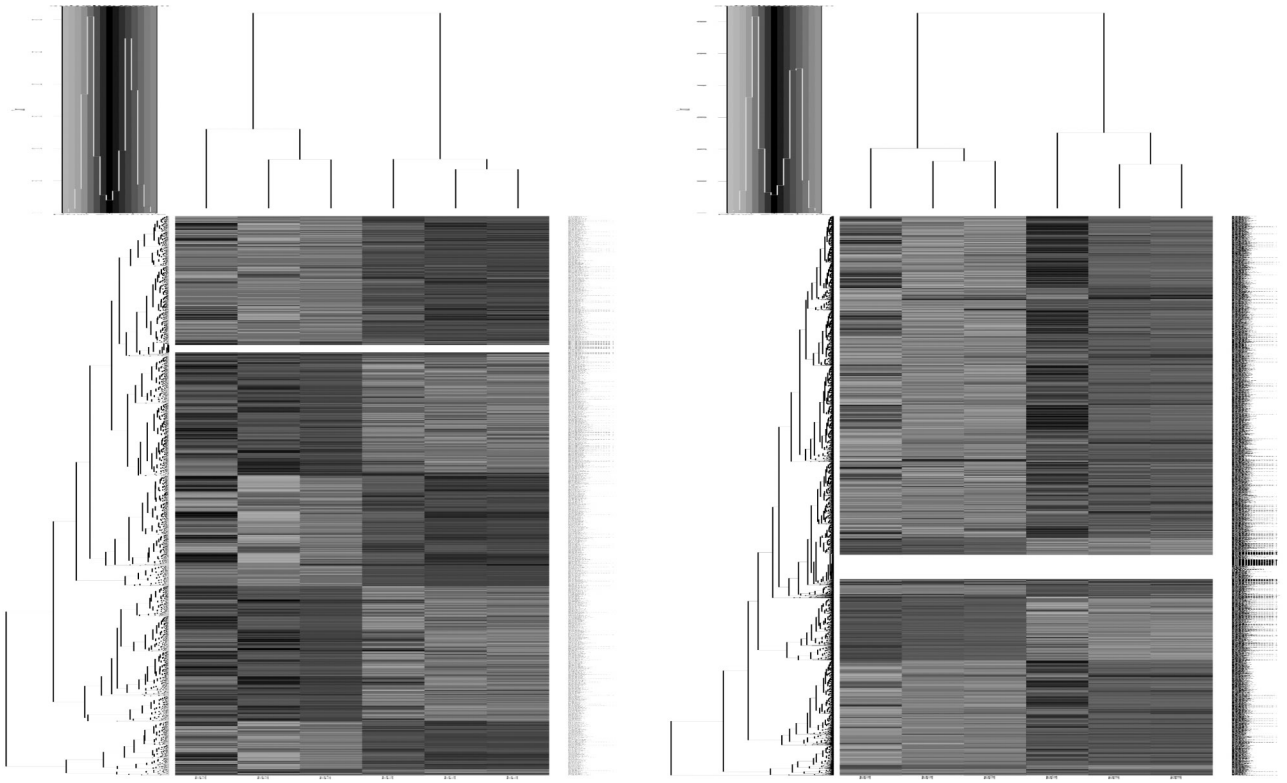
Results

The result of alignment of sequencing reads collected from each sample was assessed, with the lowest alignment rate of 95.38% and in each sample, exons accounted for 63% or higher.

In total, 2,226 and 4,652 DEGs were screened in patients 2 and 3, respectively, including 304 common ones (247 up-regulated and 57 down-regulated). The heat maps using the screened genes revealed that these genes can well distinguish the platinum sensitive samples from the platinum resistant samples (Figures 1A and B). The expressions of four *IFIT* (interferon induced proteins with tetratricopeptide repeats) family numbers, *IFIT1*, *IFIT2*, *IFIT3,* and *IFIT5* were observed to be up-regulated.

According to the functional annotation, the up-regulated DEGs were mainly enriched in immune and defense responses, especially in cellular responses to type I interferon (Table 2), while the down-regulated DEGs were observed to be functionally related to the activities of various enzymes, including transferase, NAD+ ADP-ribosyl transferase, ISG15 ligase, and oxidoreductase (Table 2).

According to the pathway enrichment analysis, the up-



A **B**

Figure 1. — Cluster analysis based on the significantly differentially expressed genes screened. Each row, a single gene; each column, a sample. Red indicates a higher expression level, and green indicates a lower expression level; color in-between indicates expression level between the highest and the lowest levels. A) Cluster analysis of differentially expressed genes identified between the platinum-sensitive and -resistant cell lines in patient 2; B) Cluster analysis of differentially expressed genes identified between the platinum-sensitive and -resistant cell lines in patient 3.

regulated DEGs were observed to affect platinum-resistance via ten pathways. Among the DEGS, *IFIH1* may function via RIG-I-like receptor signaling pathway, influenza A and measles pathways; *STAT1* was observed to function via influenza A, pancreatic cancer, and measles pathways (Table 3). Meanwhile, the down-regulated DEGs were observed to function via six pathways.

The constructed PPI network consisted of 162 node proteins forming 584 interaction pairs. *STAT1-*, *MX1-*, *DDX58-*, *XAF1-,* and *IFIH1*-encoded proteins were the top five hubs with the highest connection degrees to other gene-encoding products in the resulting PPI network (Figures 2 and 3). It was discovered that all the nodes in the sub-network were up-regulated, and *STAT1, IFIT1, IFIT2, IFIT3, and IFIT5* were also observed to be among them (Figure 4).

Further functional annotation revealed that protein-encoding genes in the sub-network were also dominantly enriched in defense and immune responses, including responses to type I interferon and cytokine. According to further pathway enrichment analysis, *STAT1* may affect platinum-resistance via four pathways, namely, influenza A, herpes simplex infection, hepatitis C, and measles; *IFIH1* may function via RIG-I-like receptor signaling pathway, influenza A, herpes simplex infection, and measles pathways; an IFIT family member *IFIT1* was observed to function via the herpes simplex infection and hepatitis C pathways (Table 4).

Discussion

According to the differential expression analysis, the up-regulated DEGs identified here were much more than the down-regulated ones, indicating that aberrant up-regulation of genes may have a more significant role in the development of platinum-resistance in ovarian cancer cells. The functional analysis showed that the up-regulated DEGs were significantly related to defense and immune responses, especially to type I interferon, indicating that immune responses, predominantly interferon-mediated

Table 2. — *The top ten GO biological process (BP) terms annotating the differentially up- and down-regulated genes.*

GO	BPID	Term	*p*-value	Corr. *p*-value	Gene names
Differentially up-regulated genes	GO:0009615	Response to virus	2.53E-09	2.05E-06	BST2;CDK6;IFI16;IFI35;IFIT2;IFIT1; MX1;EIF2AK2;STAT1;TRIM25; ISG15;IFI44;DDX58;HERC5;DDX60; IFIH1;RSAD2;UNC13D
	GO:0060337	Type I interferon-mediated signaling pathway	3.33E-09	2.05E-06	IFI6;IFI27;IFI35;IFIT2;IFIT1;IFIT3; MX1;SP100;STAT1;ISG15;XAF1
	GO:0071357	Cellular response to type I interferon	3.33E-09	2.05E-06	IFI6;IFI27;IFI35;IFIT2;IFIT1;IFIT3; MX1;SP100;STAT1;ISG15;XAF1
	GO:0034340	Response to type I interferon	3.88E-09	2.05E-06	IFI6;IFI27;IFI35;IFIT2;IFIT1;IFIT3; MX1;SP100;STAT1;ISG15;XAF1
	GO:0045087	Innate immune response	8.41E-09	3.55E-06	ASS1;BST2;CAMK2D;MAPK14; IFI6;IFI27;IFI35;IFIT2;IFIT1;IFIT3; MX1;SP100;STAT1UBA7;TRIM25; UBE2L6;ISG15;TANK;IFI30;DDX58; HERC5;XAF1;IFIH1;DHX58;UNC13D
	GO:0032480	Negative regulation of type I interferon production	1.35E-07	4.76E-05	UBA7;TRIM25;UBE2L6;ISG15;DDX58; HERC5;IFIH1
	GO:0032020	ISG15-protein conjugation	5.20E-07	0.000157	UBA7;UBE2L6;ISG15;HERC5
	GO:0006952	Defense response	1.66E-06	0.000439	ASS1;BST2;CAMK2D;MAPK14;IFI6; IFI27;IFI35;IFIT2;IFIT1;IFIT3;ITGB2; MX1;SERPINA1;PLD1;EIF2AK2;SP100; STAT1;UBA7;VDAC1;TRIM25;SPHK1; NMI;UBE2L6;ISG15;TANK;IFI30; DDX58;HERC5;XAF1;IFIH1;DHX58; RSAD2;UNC13D
	GO:0019221	Cytokine-mediated signaling pathway	3.65E-06	0.000849	CAMK2D;IFI6;IFI27;IFI35;IFIT2;IFIT1; IFIT3;MX1;SP100;STAT1;SPHK1; ISG15;IFI30;XAF1
	GO:0032479	Regulation of type I interferon production	4.57E-06	0.000849	UBA7;TRIM25;UBE2L6;ISG15;DDX58; HERC5;IFIH1
	GO:0009653	Anatomical structure morphogenesis	5.34E-08	6.04E-05	BAI2;EPHA4;ETV5;FYN;ID3;IGFBP5; MAP2;MCAM;MSX1;NDN;SLIT3; TBX15;TBX3;AXIN2;BARX2;NRP1; SIX2;CYP26B1;TCF7L1;TWIST2
Differentially down-regulated genes	GO:0051093	Negative regulation of developmental process	2.33E-07	0.000132	BAI2;EPHA4;ID3;TBX3;AXIN2;NRP1; SIX2;TRIB2;TCF7L1;TWIST2
	GO:0045596	Negative regulation of cell differentiation	4.11E-07	0.000155	EPHA4;ID3;TBX3;AXIN2;NRP1;SIX2; TRIB2;TCF7L1;TWIST2
	GO:0009887	Organ morphogenesis	3.29E-06	0.000929	ETV5;ID3;IGFBP5;MSX1;TBX15;TBX3; AXIN2;BARX2;NRP1;SIX2;TWIST2
	GO:0007413	Axonal fasciculation	8.29E-06	0.001875	EPHA4;NDN;NRP1
	GO:0032502	Developmental process	2.26E-05	0.004259	BAI2;EPHA4;ETV5;FYN;HOXC10; ID3;IGFBP5;LOXL2;MAP2;MCAM; MSX1;NDN;CLDN11;SLIT3;TBX15; TBX3;AXIN2;BARX2;NRP1;SIX2; MRAS;TRIB2;CYP26B1;OLFML3; TCF7L1;TWIST2
	GO:0045668	Negative regulation of osteoblast differentiation	5.73E-05	0.008205	ID3;AXIN2;TWIST2
	GO:0048856	Anatomical structure development	5.80E-05	0.008205	BAI2;EPHA4;ETV5;FYN;HOXC10;ID3; IGFBP5;MAP2;MCAM;MSX1;NDN; CLDN11;SLIT3;TBX15;TBX3;AXIN2; BARX2;NRP1;SIX2;MRAS;CYP26B1; TCF7L1;TWIST2
	GO:0048705	Skeletal system morphogenesis	6.70E-05	0.008419	TBX15;AXIN2;BARX2;SIX2;TWIST2
	GO:0030154	cell differentiation	8.17E-05	0.008451	EPHA4;FYN;HOXC10;ID3;IGFBP5; MAP2;MSX1;NDN;SLIT3;TBX3; AXIN2;BARX2;NRP1;SIX2;TRIB2; CYP26B1;TCF7L1;TWIST2

Table 3. — *Result of pathway enrichment analysis of up-regulated genes using KEGG database.*

Term	Id	Sample number	*p*-value	Corrected *p*-value	Genes
RIG-I-like receptor signaling pathway	hsa04622	7	0.000478	0.070568	TRIM25;DHX58;MAPK14;IFIH1;DDX58;TANK;ISG15
Influenza A	hsa05164	11	0.000806	0.070568	TRIM25;MAPK14;TMPRSS4;EIF2AK2;PIK3R1;IFIH1;DDX58; VDAC1;STAT1;RSAD2;MX1
Pancreatic cancer	hsa05212	6	0.002496	0.145575	CDK6;SMAD2;PIK3R1;STAT1;PLD1;E2F3
Bacterial invasion of epithelial cells	hsa05100	5	0.017654	0.546463	DNM3;CD2AP;PIK3R1;CTNNA1;PTK2
Measles	hsa05162	7	0.017891	0.546463	CDK6;EIF2AK2;PIK3R1;STAT1;DDX58;IFIH1;MX1
Histidine metabolism	hsa00340	3	0.018736	0.546463	ABP1;ALDH3B2;ALDH3B1
Arginine and proline metabolism	hsa00330	4	0.025448	0.636206	ABP1;P4HA2;ASS1;CKB
Cell cycle	hsa04110	6	0.035536	0.75192	TTK;CDK6;SMAD2;SKP1;E2F3;CDKN2C
Glioma	hsa05214	4	0.03867	0.75192	CAMK2D;CDK6;PIK3R1;E2F3
Phenylalanine metabolism	hsa00360	2	0.047691	0.804393	ALDH3B2;ALDH3B1

Table 4. — *Result of pathway enrichment analysis of genes encoding proteins in the sub-PPI network based on KEGG database.*

Term	Id	Sample number	*p*-value	Corrected *p*-value	Genes
RIG-I-like receptor signaling pathway	hsa04622	7	5.92E-07	5.15E-05	TRIM25;DHX58;MAPK14;IFIH1;DDX58;TANK;ISG15
Influenza A	hsa05164	8	3.36E-05	0.001463	TRIM25;MAPK14;EIF2AK2;STAT1;DDX58;IFIH1;RSAD2;MX1
Herpes simplex infection	hsa05168	6	0.001914	0.044238	EIF2AK2;IFIT1;STAT1;DDX58;IFIH1;SP100
Hepatitis C	hsa05160	5	0.002542	0.044238	DDX58;EIF2AK2;IFIT1;MAPK14;STAT1
Measles	hsa05162	5	0.002542	0.044238	DDX58;EIF2AK2;MX1;STAT1;IFIH1

responses, are involved in the development of platinum-resistance. The overexpression of four *IFIT* members *IFIT1*, *IFIT2*, *IFIT3* and *IFIT5* was observed in the platinum-resistant cell line in each patient. Generally, the IFIT proteins are produced during viral infection, interferon treatment, and pathogen recognition by immune cells, which are supposed to contribute to immunity acquisition during viral infection [19], either by specifically binding to viral nucleic acids or directly binding to eukaryotic initiation factor 3 (eIF3), and subsequently preventing eIF3 from initiating the translational process. Additionally, *IFIT1* was speculated to contribute to platinum-resistance development via the herpes simplex infection and hepatitis C pathways. However, none of the *IFIT* members have been reported to have a role in platinum-resistance development before.

IFIH1 (interferon induced with helicase C domain) encodes a putative RNA helicase, which contains a conserved motif Asp-Glu-Ala-Asp (DEAD). *IFIH1* may contribute to platinum-resistance development via RIG-I-like receptor signaling pathway, influenza A, herpes simplex infection, and measles pathways. So far, there is no report on the role of *IFIH1* in the development of platinum-resistance either.

STAT1 encodes STAT1 protein, a member of the STAT family. Once activated by IFNs and other cell signals via phosphorylation, STAT1 is translocated to the cell nucleus where it initiates the transcription of a wide array of IFN-stimulated genes, which is thus thought to be important for cell viability in response to different cell stimuli and pathogens. Stronach *et al.* observed STAT1 overexpression in clinically resistant cells, and concluded that STAT1 can be activated by HDAC4 following cisplatin treatment in cells with acquired clinical platinum resistance [20]. Previously, Kramer *et al.* reported the acetylation of STAT1 at lysine residues 410 and 413 in melanoma cells and showed increased acetylation following HDAC inhibition or IFN-α treatment [2]. In the present study, *STAT1* in the platinum-resistant cell line was up-regulated compared to the platinum-sensitive cell line collected from each patient, implying more STAT1 was deacetylated in platinum-resistant

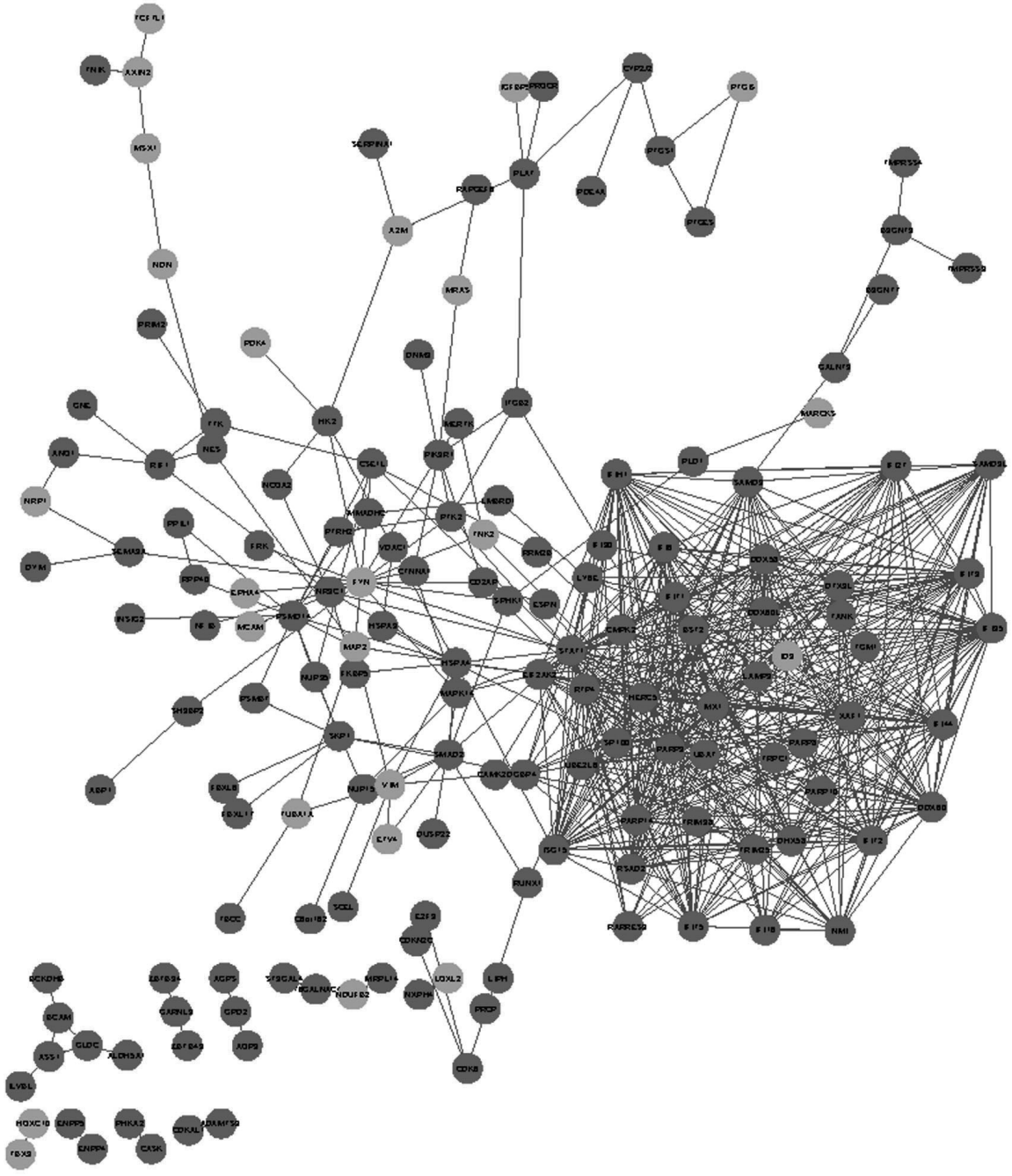


Figure 2. — The protein-protein interaction network. A red circle represents an up-regulated differentially expressed gene (DEG); a blue one represents a down-regulated DEG.

cell line. Whether *STAT1* overexpression is an outcome of acetylation needs further experimental validation. Interestingly, *HDAC11* and *HDAC9* were observed to be up-regulated in patients 2 and 3, respectively, although their aberrant expression was not commonly observed in both patients. Similarily, *HDAC4*, *HDAC11,* and *HDAC9* may have similar roles in regulating *STAT1* expression as histone deacetylase. This also needs further experimental proofs. Additionally, *STAT1* was supposed to affect platinum-resistance via four pathways, influenza A, herpes simplex infection, hepatitis C, and measles

As *STAT1, IFIH1,* and *IFIT1* were all observed to function via the herpes simplex infection pathway, it thus can be inferred that this pathway may have a particularly critical

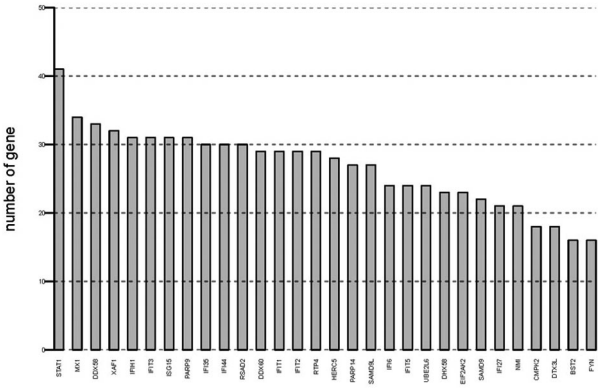


Figure 3. — Ranking of node proteins in the protein-protein interaction network according to the connection degree.

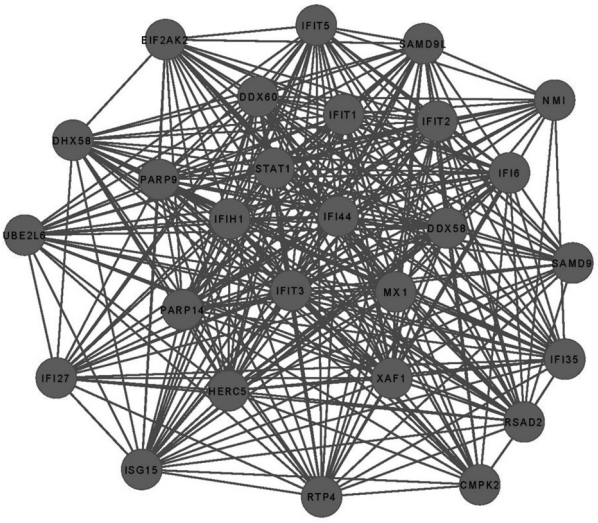


Figure 4. — A sub-module of the protein-protein interaction network. A red circle represents an up-regulated differentially expressed gene (DEG); a blue one represents a down-regulated DEG.

role in the development of platinum-resistance. However, up to now, the role of this pathway has never been reported in the platinum-resistance development in ovarian cancer.

Conclusion

Taken together, the aberrant up-regulation of genes seems to be more critical to the development of platinum-resistance in ovarian cancer cells. The overexpression of *STAT1, IFIH1* and four *IFIT* members (*IFIT1*, *IFIT2*, *IFIT3,* and *IFIT5*) is associated with the platinum-resistance development in ovarian cancer cells. Furthermore, *STAT1, IFIH1,* and *IFIT1* may affect the development of platinum-resistance via altering more than one pathways, especially via the herpes simplex infection pathway, thus these three genes can be considered as potential markers for prediction of response to first-line platinum chemotherapy. However, most genes identified in the present study have never been reported to relate to platinum-resistance in the ovarian cancer previously. Thus, solid experimental proof is required.

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