Prediction of key genes in ovarian cancer treated with decitabine based on network strategy

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Abstract. The objective of the present study was to predict key genes in ovarian cancer before and after treatment with decitabine utilizing a network approach and to reveal the molecular mechanism. Pathogenic networks of ovarian cancer before and after treatment were identified based on known pathogenic genes (seed genes) and differentially expressed genes (DEGs) detected by Significance Analysis of Microarrays (SAM) method. A weight was assigned to each gene in the pathogenic network and then candidate genes were evaluated. Topological properties (degree, betweenness, closeness and stress) of candidate genes were analyzed to investigate more confident pathogenic genes. Pathway enrichment analysis for candidate and seed genes were conducted. Validation of candidate gene expression in ovarian cancer was performed by reverse transcriptase-polymerase chain reaction (RT-PCR) assays. There were 73 nodes and 147 interactions in the pathogenic network before treatment, while 47 nodes and 66 interactions after treatment. A total of 32 candidate genes were identified in the before treatment group of ovarian cancer, of which 16 were rightly candidate genes after treatment and the others were silenced. We obtained 5 key genes (\(PIK3R2, CCNB1, IL2, IL1B\) and CDC6) for decitabine treatment that were validated by RT-PCR. In conclusion, we successfully identified 5 key genes (\(PIK3R2, CCNB1, IL2, IL1B\) and CDC6) and validated them, which provides insight into the molecular mechanisms of decitabine treatment and may be potential pathogenic biomarkers for the therapy of ovarian cancer.

Introduction

Ovarian cancer is the ninth most common cancer among women and the fifth leading cause of cancer-related death among women with recent statistics suggesting that 1 in 71 women will develop ovarian cancer (1,2). Approximately 70% of ovarian cancer cases are diagnosed at a late stage and therefore are poorly treatable (3). Although the current standard treatment for ovarian cancer involving the use of paclitaxel and carboplatin after aggressive surgical cytoreduction usually results in multyear survival, prolonged use of platinum-based chemotherapy often induces drug resistance, which causes ovarian cancer relapse and eventually the death of patients (4). Such knowledge may translate into the development of new targeted strategies. In addition, since ovarian cancer is considered to be a heterogeneous group of diseases with distinct gene expression profiles, it is likely that the focus should be towards the development of new targeted therapies capable of exploiting the molecular and genetic characteristics of ovarian cancer (5). Therefore, it is necessary to understand the pathogenesis of ovarian cancer by dissecting the components involved in the pathogenic procedure, i.e. pathogenic genes.

The pathogenic genes can be identified in the laboratory by techniques, such as gene knockout or silencing, however, the pathogenic gene list is far from complete and it is a painful process to identify pathogenic genes in the laboratory considering the genome size and time-consuming experiments (6). In contrast, computational methods can provide alternative strategies for this issue, for instance, high throughput techniques. Traditionally, studies tend to regard differentially expressed genes (DEGs) between normal and disease samples as biomarkers and pathogenic genes, but, DEGs alone may lead to false positives while identifying key genes involved in disease procedure since some genes are not involved in the pathway of pathogenic genes even though they show significant expression change (7). In the meantime, studies have shown that the most significant genes obtained from different studies for a particular cancer are typically inconsistent (8). To overcome this issue, one could evaluate pathogenic genes for disease-association using a network strategy (9).

5-Aza-2’-deoxycytidine (decitabine) is a prodrug that requires metabolic activation by deoxycytidine kinase, an active inhibitor in the triphosphate form (10). DNA polymerase catalyzes the insertion of the phosphorylated form of decitabine into DNA, and the presence of decitabine in place of the 5-methylcytosine in DNA leads to the inactivation of DNA methyltransferase inducing a re-expression of the silenced genes (11). It has been demonstrated that decitabine produces...
variable antitumor response rates in patients with solid tumors that may be leveraged clinically with identification of a predictive biomarker (12). For instance, decitabine is an effective therapy for myelodysplastic syndromes (MDS) and for acute myeloid leukemia (AML) (13). Moreover, its role in the treatment of ovarian cancer has been defined in regards to the fact that epigenetic therapy upregulates the expression of imprint tumor suppressors (14). Hence, more and more research has focused on ovarian cancer treatment with decitabine, while the molecular mechanisms of this drug remain unclear.

Therefore, in the present study, we employed a network approach to predict key genes which are potentially silenced genes for ovarian cancer before and after treatment with decitabine. The network approach was based on a pathogenic network that derived from a protein-protein interaction (PPI) network, DEGs and known pathogenic genes (seed genes), to identify candidate genes and silenced genes. Subsequently, topological properties and pathway enrichment analysis were performed for candidate genes. By combining weight values and topological properties of candidate genes and silenced genes before and after treatment with decitabine, we obtained key genes and validated key genes by reverse transcription-polymerase chain reaction (RT-PCR) assays.

Materials and methods

Gene expression data. In the present study, the microarray gene expression profile of ovarian cancer with accession no. E-GEOD-25429 (15) was downloaded from ArrayExpress database. E-GEOD-25429 was comprised of 91 samples (4 normal controls, 43 ovarian cancer samples and 41 ovarian cancer samples treated with decitabine), and deposited on two platforms, A-AFFY-44-Affymetrix GeneChip Human Genome U133 Plus 2.0 [HG-U133_Plus_2] and A-AFFY-113-Affymetrix GeneChip HT Human Genome U133A [HT_HG-U133A]. When mapping the probes to genes according to the platforms, a total of 20,107 and 12,494 genes were obtained, respectively. To avoid batch effects from the different platforms, we took the intersections of two platforms as the gene expression profile which consisted of 12,493 genes for further analysis.

Identification of pathogenic network. There are some genes that have been identified as pathogenic genes of ovarian cancer in Online Mendelian Inheritance in Man (OMIM) database, an online catalog of human genes and genetic disorders (17). In the present study, a total of 87 genes were found, which were also called as known pathogenic genes. Taking the intersection with the gene expression profile, we obtained 82 intersected genes and defined them as seed genes (Table 1).

Meanwhile, we recruited human PPI from the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (18), and interactions with score >0.5 were kept as the background PPI network. Subsequently, a network was extracted from the background PPI network that included genes that interacted with seed genes, where the genes were further required to be DEGs of ovarian cancer before (condition 1) and after (condition 2) treatment with decitabine. Therefore, genes in the sub-network were more possibly pathogenic genes. Furthermore, a smaller sub-network that consisted of genes interacting with at least two seed genes was extracted from the previous network and were regarded as the pathogenic network, where the genes in the pathogenic network were believed to be correlated to pathogenesis.

Ranking of the pathogenic genes. To facilitate the biologists to select more confident pathogenic genes from our predictions, each gene was assigned a weight value based on the interactions and co-expression with seed genes, where a gene was more confident to be a pathogenic gene if it interacted and was co-expressed with more seed genes (6). The co-expression was evaluated by Pearson correlation coefficients (PCC) (19) between our predicted pathogenic and seed genes. The weight for gene $x$, $W(x)$, was calculated as following:

$$W(x) = \sum_{y \in S} \text{PCC}(x, y) \times I(x, y)$$

where $S$ is the set of seed genes, $\text{PCC}(x, y)$ is the correlation coefficient between gene $x$ and gene $y$, and $I(x, y)$ is an indication function, where $I(x, y) = 1$ if protein $x$ interacted with protein $y$ and $I(x, y) = 0$ otherwise. The weight of each predicted pathogenic gene could illustrate the correlation

$$d(i) = \frac{\bar{x}_y(i) - \bar{x}_s(i)}{s(i) + s_o}$$

where $\bar{x}_y(i)$ and $\bar{x}_s(i)$ are defined as the average levels of expression for gene $i$ in normal and ovarian cancer, respectively. $s(i)$ is the standard deviation of repeated expression measurements. The value for $s_o$ was chosen to minimize the coefficient of variation.
between this gene and the seed genes. The higher the weight of one gene, the more possible the gene was involved in the pathogenic procedure. In addition, we defined the potential pathogenic genes not seed genes as candidate genes of ovarian cancer.

Properties of the pathogenic network. For the purpose of investigating the possible roles of candidate genes, topological properties of nodes in the pathogenic network were explored, including degree, betweenness, closeness and stress. For an undirected network $G = (V, E)$, where $V$ is the set of vertices representing nodes in the network, and $E$ is the set of edges representing the relationships between the actors. A path from node $s$ to $t$ was defined as a sequence of edges and the length of a path was the sum of the weights of edges. We used $d(s, t)$ to denote the distance between $s$ and $t$ (the minimum length of any path connecting $s$ and $t$ in $G$). Let us denote the total number of shortest paths between vertices $s$ and $t$ by $\sigma_{st}$, and the number passing through node $v$ by $\sigma_{st}(v)$.

Degree. Degree is a simple local measure, based on the notion of neighborhood. It quantifies the local topology of each gene by summing up the number of its adjacent genes (20). The degree $D(v)$ of a node $v$ was defined as:

$$D(v) = \sum_j a_{vj}$$

Betweenness centrality. Betweenness centrality, $C_B(v)$, is a shortest paths enumeration-based metric in graphs for determining how the neighbors of a node are interconnected, and is considered as the ratio of the node in the shortest path between two other nodes (21), in consequence $C_B(v) \in [0, 1]$. It was calculated as follows:

$$C_B(v) = \frac{\sum_{s \neq v \neq t} \sigma_{st}(v)}{\sigma_{st}}$$

Closeness centrality. Closeness centrality, $C_C(v)$, is a measure of the average length of the shortest paths to access all other proteins in the network (22). It was defined as the reciprocal of the average shortest path length:

$$C_C(v) = \frac{1}{\sum_{s \neq v} d(s, t)}$$

Stress. This index computes the number of nodes in the shortest path between two other nodes (23). If a node was stressed, it would be traversed by a high number of shortest paths. The stress, $C_s(v)$ was defined as:

$$C_s(v) = \sum_{s \neq v} \sigma_{st}(v)$$

Pathway enrichment analysis of candidate genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis for candidate and seed genes were performed based on the Database for Annotation, Visualization and Integrated Discovery (DAVID) (24). In addition, pathways which met the criterion $P<0.01$ were selected according to Expression Analysis Systematic Explorer (EASE) test implemented in DAVID (25). The calculating formula of EASE is shown as follows:

$$p = \frac{(a+b)(e+d)}{a(a+b)(c+d)} - \frac{n}{a+c} \left( \frac{a}{a+c} \right)$$

Table I. Seed genes of ovarian cancer.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gene</th>
<th>ID</th>
<th>Gene</th>
<th>ID</th>
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<td>RNA SEL</td>
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<td>YBX1</td>
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<td>EPHX1</td>
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<td>TGFB2</td>
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pre-denaturation, followed by 35 cycles of 60 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C, and a final 10-min extension at 72˚C. Three replicates of the assay within or between runs were performed to assess reproducibility. Products of the PCR experiment were analyzed by 1.5% agarose gel electrophoresis and Quantity One software using a gel imaging analyzer (Bio-Rad, Hercules, CA, USA).

Results

Validation of candidate genes by RT-PCR. RT-PCR assays were carried out to validate key genes. Total RNA was prepared from ovarian cancer cell line A2780 before and after treatment with decitabine, and 10 ovarian cancer patient tissues prepared from ovarian cancer cell line A2780 before and after treatment of decitabine, and 10 ovarian cancer patient tissues were carried out to validate key genes. Total RNA was used for cDNA synthesis, which was then used for PCR amplification. The PCR conditions were as follows: 5 min at 95˚C for pre-denaturation, followed by 35 cycles of 60 sec at 94˚C, 30 sec at 55˚C and 30 sec at 72˚C, and a final 10-min extension at 72˚C. Three replicates of the assay within or between runs were performed to assess reproducibility. Products of the PCR experiment were analyzed by 1.5% agarose gel electrophoresis and Quantity One software using a gel imaging analyzer (Bio-Rad, Hercules, CA, USA).

Results

Detection of DEGs. Prior to the study of the DEGs between the normal controls and ovarian cancer before and after treatment with decitabine and investigation of significant genes in ovarian cancer, we designated two conditions, condition 1 (normal controls vs. ovarian cancer before treatment) and condition 2 (normal controls vs. ovarian cancer after treatment with decitabine), or in other words, condition 1 was the before treatment group and condition 2 was the after treatment group. A total of 850 and 667 DEGs were obtained from the two conditions based on SAM with Δ = 3.600 and 3.436, separately.

Identification of the pathogenic network. In the present study, interactions in the STRING database with a score >0.5 were kept as the background PPI network. With known pathogenic genes as seed genes, a network was extracted from the background PPI network, where the genes interacted with at least one seed gene. Although the genes interacting with seed genes were possibly pathogenic genes, they may also just interact with seed genes to maintain the essential biological processes for ovarian cancer. Therefore, the integration of DEGs and the network identified above helped to reduce false positives since it was believed that the expression changes of DEGs were possibly caused by the interactions with seed genes.

By mapping DEGs from condition 1 to the network extracted from background PPI network of ovarian cancer before treatment, we finally obtained a sub-network that consisted of 65 genes except 47 seed genes and 180 interactions which linked to at least one seed gene (Fig. 1). Furthermore, the genes that interacted with at least two seed genes were identified since these genes are more likely to be pathogenic genes due to their tight interactions with seed genes. As a result, 147 interactions were investigated to connect to at least two seed genes, and their interactions involved 73 genes in total, of which 41 were seed genes and the others were candidate genes; the sub-network is shown in Fig. 2 and is called pathogenic network. Notably, we found that four seed genes, KIF14, ASPM, EXO1 and RAD54L, interacted with each other and formed a clique. Therefore, these four seed genes may belong to the same complex or pathway that is involved in the pathogenic procedure.
Similarly, when changing DEGs and the background PPI network before treatment to after treatment, we obtained the sub-network (Fig. 3) and pathogenic network (Fig. 4) of ovarian cancer after treatment with decitabine. In Fig. 3, there were 83 nodes of which 39 were seed genes and 94 edges, but these genes were not entirely connected together. Discarding genes that only interacted with one seed gene, 16 candidate genes and 66 interactions were extracted from the sub-network and were formed into the pathogenic network of ovarian cancer after treatment.

**Ranking of candidate genes.** A total of 32 and 16 candidate genes (Tables III and IV) were identified by ranking the pathogenic genes based on the pathogenic network before and after treatment. To screen more reliable pathogenic genes, we assigned a weight to each candidate gene according to PCC, and ranked them in decreasing order. The higher weight of one gene, the more confident pathogenic gene of ovarian cancer was. For the candidate genes before treatment, IL2, PIK3R2, IL1B, CDC6 and CCNB1 possessed the top five rankings with a weight of 6.693, 6.027, 4.542, 3.890 and 3.643, respectively. The candidate genes of the after treatment group were part of that of before treatment, but their weights had great differences apart from PIK3R2 and CCNB1. The top five genes after treatment were PIK3R2, CDC7, TYR, E2F8 and CCNB1.

By comparing the two types of candidate genes, we found that the 16 candidate genes of the after treatment group were all involved in the 32 candidate genes, and the other 16 candidate genes before treatment were silenced after treatment. The silenced genes were: IL2, IL1B, CDC6, AURKA,
Figure 2. The pathogenic network of ovarian cancer before treatment. The red vertices denote seed genes, i.e. known pathogenic genes, the green vertices are genes that interacted with at least two seed genes, and each vertex was assigned a weight. The color bar represents the relationship between color and weight, where the deeper the color the larger is the weight.

Figure 3. The sub-network of ovarian cancer after treatment with decitabine. Nodes are genes, and the edge stand for the interaction between two genes. The red vertices denote seed genes from ovarian cancer, i.e. known pathogenic genes; the green vertices stand for genes that interacted with at least two seed genes; the yellow vertices represent genes that interacted with only one seed gene.
GINS1, BDKRB1, FBXO5, DLGAP5, NDC80, KIF18A, KIF23, HELLS, LCP2, VRK1, MCM4 and NCAPH, among which IL2 changed most. The silenced genes with weight in the top five (IL2, IL1B, CDC6, AURKA and GINS1) may be more important than others for the decitabine functional process.

Identification of key genes. In the present study, several indices were utilized to investigate topological properties of candidate genes, including degree, betweenness, closeness and stress. Among the 16 common candidate genes, we removed TRIM37, CPB2 and CYP19A1 which only interacted
with two seed genes and were not mapped main components of the pathogenic networks, and the results of the other 13 candidate genes are displayed in Fig. 5. The degree distributions for 12 candidate genes except $IGF2BP3$ in the before treatment group were the same as that in the after treatment group. As for betweenness and stress, $PIK3R2$ and $CCNB1$ were changed to a greater extent than the residual genes. The closeness for candidate genes in ovarian cancer before treatment was similar, but small differences were produced in after treatment.
The expression of one gene in ovarian cancer before and after treatment as compared to the normal controls is indicated by its P-value: *P<0.05 indicates that the gene of ovarian cancer before treatment was significantly differentially expressed compared to normal controls; **P<0.05 indicates that the gene was significantly differentially expressed in ovarian cancer after treatment compared with the normal control; and ***P<0.05 indicates that the gene was significantly differentially expressed across ovarian cancer before and after treatment.

Validation of candidate genes by RT-PCR. To study the activity and expression levels of candidate genes in ovarian cancer, we collected ovarian cancer A2780 cells before and after treatment with decitabine, and 10 ovarian cancer patient tissues to perform RT-PCR analyses. Note that the normal controls in the RT-PCR assays were para-carcinoma tissues of ovarian cancer patients. After RNA extraction, the cDNA synthesis and PCR amplification, we obtained the relative expression levels of 5 candidate genes (PIK3R2, CCNB1, IL2, IL1B and CDC6) which were taken as examples. By assessing the analysis of significance dependent on SPSS, the results are illustrated in Fig. 7. Apart from IL1B, the other four genes of ovarian cancer before treatment were significantly differentially expressed with *P<0.05 compared to normal controls and ovarian cancer after treatment (**P<0.05). Only PIK3R2 was differentially expressed between ovarian cancer after treatment and normal controls (***P<0.05).

**Discussion**

In the present study, we predicted key genes associated with ovarian cancer following treatment with decitabine utilizing a pathogenic network method. The results identified 5 key genes, PIK3R2, CCNB1, IL2, IL1B and CDC6, which had high weight and good topological properties (degree, betweenness, closeness and stress) in the pathogenic network before and after treatment. In addition, these genes were validated by RT-PCR assays.

The phosphatidylinositol 3-kinase (PI3K) enzyme is an obligate heterodimer composed of a regulatory subunit (PIK3R) and a catalytic subunit (PIK3C) (26). Once the interaction of PIK3R with a variety of receptors is recruited, PIK3C is activated through a conformational switch and produces phos-

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**Table V. Pathways enriched by seed genes and candidate genes with P<0.01.**

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Count</th>
<th>P-value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurotrophin signaling pathway</td>
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<td>3.14E-04</td>
<td>KRAS, JUN, NTRK1, SHC1, AKT3, PIK3R2, NGF</td>
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<tr>
<td>Cell cycle</td>
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<td>T cell receptor signaling pathway</td>
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<tr>
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phosphatidylinositol-3,4,5-trisphosphate (PI(3)P), which functions as a cellular second messenger (27). PI(3)P encodes kinases, of which the most important is AKT that control a multitude of pathways, including cell growth, survival and metabolism (28).

As a consequence, there is a close relationship between PI3K and AKT. It has been reported that alterations to the PI3K-AKT signaling pathway are common in human cancer, for example, in ovarian cancer (29). We discovered that phosphoinositide-3-kinase, regulatory subunit 2 (PIK3R2) and v-akt murine thymoma viral oncogene homolog 3 (AKT3) co-function in several pathways which also play significant roles in the process of ovarian cancer, such as neurotrophin signaling pathway and ErbB signaling pathway (30,31). Cheung et al (32) demonstrated PIK3R2 mutations on PI3K signaling in endometrial cancer, thus we may infer that PIK3R2 mutations also exist in ovarian cancer.

Cyclin B1 (CCNB1) is a regulatory protein involved in mitosis and the product complexes to form the maturation-promoting factor. Its transcription leading to aberrantly high levels of CCNB1 throughout the cell cycle is associated with excessive hyperplasia in several human cancers (33). For example, CCNB1 was found to have significant predictive power in distant metastasis-, disease- and recurrence-free survival, and overall survival of breast cancer patients (34). We found that CCNB1 was overexpressed in an ovarian cancer cell line, but after decitabine treatment, its level decreased to some extent.

Interleukin 2 (IL2) is a pleiotropic cytokine produced after antigen activation and has roles in key functions of the immune system, tolerance and immunity, primarily via its direct effects on T cells in regards to the mediation of T cell growth and proliferation (35). In the present study, we found that IL2 was enriched in the T cell receptor signaling pathway. In ovarian tumors, myeloid cells are one of the major determinants of immune suppression, and the accumulation of these immunosuppressive activities may lead to further worsen cancer (36). Duraiswamy et al demonstrated that therapeutic pathway blockade augments other modalities of immunotherapy T cell function preventing immune decline in ovarian cancer (37). We may infer that IL2 had a potential role in decitabine-treated ovarian cancer patients through the medium of T cell.

Cell division cycle 6 (CDC6) is an essential regulator of DNA replication and plays important roles in the activation and maintenance of the checkpoint mechanisms in the cell cycle (38). Deregulation of CDC6 leads to aberrant DNA replication, DNA damage and genomic instability, and may even contribute to tumorigenesis (39). CDC6 has been associated with the oncogenic activities in human types of cancers, such as lung (38), breast (40) and ovarian cancer (41). For instance, Deng et al found that CDC6 was upregulated, discovered a novel regulatory signaling pathway of CDC6 and provided a new potential therapeutic target for ovarian cancer patients (41). In addition, it has been suggested that a number of genes are inversely correlated with CDC6 in functional models of the ovarian cancer cell line HEYA8 (42). In the present study, we also found that CDC6 was upregulated in ovarian cancer samples.

In conclusion, we have successfully identified 5 key genes (PIK3R2, CCNB1, IL2, IL1B and CDC6) and validated them by RT-PCR. Our findings provide insight into the molecular mechanisms of decitabine treatment and may be potential pathogenic biomarkers for the therapy of ovarian cancer.

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References


