Mining hub genes associated with late stage adrenocortical carcinoma.

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Abstract

Background: Adrenocortical carcinoma (ACC) was in poor prognosis especially the late stages. Our research tried to discover potential hub genes associated with the disease.

Methods: The GSE90713 including 5 normal and 58 ACC samples were downloaded from the GEO datasets. A total of 106 up- and 299 down-regulated DEGs were identified by BrB-ArrayTools. Then their gene ontology functions and KEGG pathways were enriched by the DAVID. The protein-protein interaction network was provided by STRING. Then, Cytohubba was used to pick out the hub genes. The Webgestalt was used to predict transcription factors and microRNAs. Finally, TCGA’s data was utilized to validate our results.

Results: The up-regulated DEGs were mainly involved in the processes of cell division and cell cycle. The down regulated DEGs were mainly involved in the processes of response to hormone and extracellular exosome. CDK1, RFC4, KIF11, TOP2A, CCNB1, MAD2L1, AURKA, NCAPG, CDKN3, TRIP13, were identified hub genes and they were closely related to the process of the mitotic cell cycle. The high expression of these genes indicated poor prognosis of the diseases (P<0.01) by Kaplan Meier test. After examination by the genome data of ACC patients in TCGA datasets, they were suggested not only associated with the metastasis, but also with the tumor staging. However, except CCNB1, KIF11, MAD2L1, and TRIP13, others didn’t perform an obvious connection with local lymph nodes invasion.

Conclusion: CCNB1, KIF11, MAD2L1, and TRIP13 were closely associated with TMN stage and prognosis. They may serve as therapeutic targets for the adrenal carcinoma.

Keywords: Adrenocortical cancer, Cytohubba, Hub genes, Late stage, Prognosis.

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Data description
The gene chip of GSE90713 was obtained from the GEO database [8]. Its platform was Affymetrix Human Gene Expression Array GPL15207. It consisted of 58 adrenocortical carcinoma samples and 5 normal adrenal samples in total. The data has been preprocessed and its distribution in GEO2R was uniform and suitable for analyzing.

DEGs identification
BRB-ArrayTools was a convenient tool for bioinformatic analysis [9,10]. We imported the expression matrix into BRB-Arraytools, exclude a gene if minimum fold change is less than 20% of an expression data value or have at least a 1.5-fold change in either direction from the gene median value or percent missing exceeds 50%. Finally, the significance threshold of univariate tests is set of 0.001 by random variance model and the groups of the normal tissue and carcinoma tissue were compared to identify the DEGs [11].

Gene ontology function annotation and pathways analysis of the DEGs
DAVID was a free online bioinformatics resource developed by the Laboratory of Immunopathogenesis and Bioinformatics [12]. All tools in the DAVID bioinformatics resources provided a functional interpretation of large lists of genes derived from genomic studies, including gene ontology and pathways analysis. Similarity, BiNGO, the plugin of Cytoscape, and Panther also provide the service of the gene ontology analysis [13,14].

PPI network construction and hub genes identification
The interaction network was obtained by uploading the DEGs to the Search Tool for the Retrieval of Interacting Genes database [15]. Then the interaction file and annotation table were input into the Cytoscape software version 3.6.0, the PPI network can be professionally visualized. The hub genes are identified by Cytohubba, which can explore important nodes and fragile motifs in an interactome network by several topological algorithms including degree-edge percolated component (EPC), maximum neighborhood component (MNC), density of maximum neighborhood component (DMNC), maximal clique centrality (MCC) and so on. In our research, the top 10 genes were identified by their MCC values [16,17].

TFs and microRNA network construction
The transcriptional factors (TFs) and microRNAs of targeted DEGs were predicted by the Webgestalt. WebGestalt supports three well-established and complementary methods for enrichment analysis, including Over-Representation Analysis (ORA), Gene Set Enrichment Analysis (GSEA), and Network Topology-based Analysis (NTA). The up and down-regulated DEGs were separately inputted into the website and their sharing TFs and microRNAs were inferred by from comparative genomic analysis and made available through MSigDB [18,19].

Hub genes function annotation
Given a set of genes to GenClip2.0, for example from high-throughput experiments, it can be helpful to know which biological functions and molecular networks may be involved, or whether genes from a given list are related to certain topics, such as various biological and pathological processes. The hub genes were then annotated by the tools [20].

Survival analysis of the obtained hub genes
The GEPIA, a portal using Genomics data from GTEx and The Cancer Genome Atlas (TCGA) project, was used to do Kaplan Meier test of these genes based on low and high expression. The hub genes acquired by Cytohubba were input into the website and their Kaplan Meier plots of overall survival and disease-free survival were obtained by the website tools [21,22].

Validation of the obtained hub genes in TNM staging of ACCs
Genomic data from The Cancer Genome Atlas (TCGA) project had been used to validate our analysis results. As mentioned above, the ACC patient’s data was downloaded from the website. We compared these genes’ expression by Wilcoxon test which grouped in M0 (no metastasis) and M1 (metastasized to other organs and distal lymph nodes) status [21]. Then, these genes’ association with the N0 (no local lymph nodes invasion) and N1 (local lymph nodes invasion) of ACC’s patient were examined. Finally, the Kruskal–Wallis test was done to validate the genes’ expression to the tumor stage.

Results
DEGs identification
A total of 405 DEGs of ACC samples were identified by comparing to the normal samples, consisting of 106 up- and 299 down DEGs.

Gene ontology and pathway enrichment analysis results
The gene ontology (GO) categories were classified into three
groups: biological process, cellular component and molecular function. The GO terms and KEGG pathways were ranked by p-value and the top 5 were selected. The results of the upregulated DEGs were shown in Table 1 and the results of the down regulated DEGs were shown in Table 2. The upregulated DEGs were mainly involved in the processes of cell division, G1/S transition of the mitotic cell cycle, anaphase-promoting complex-dependent catabolic process, mitotic spindle organization, and DNA repair. The down regulated DEGs were mainly involved in the processes of response to estrogen and the peptide’s hormone, outflow tract septum morphogenesis, regulation of cell growth and erythrocyte homeostasis. Most of the up-regulated DEGs were involved in the cell cycle, Oocyte meiosis, p53 signaling, DNA replication, and progestereone-mediated oocyte maturation pathways. Most of the down regulated DEGs were involved in fatty acid degradation metabolic, metabolic, carbon metabolism, seleno-compound metabolism, and mineral absorption pathways.

**PPI network construction and analysis**

The PPI network constructed by the Cytoscape, as shown in Figure 1A. The PPI network included 230 nodes and 841 interactions, involving 70 up and 160 downregulated genes. The hub genes of the network were identified by Cytohubba, a plugin in the Cytoscape. The results were shown in Figure 1B. As ordered by MCC values, the top 10 hub genes were CDK1, RFC4, KIF11, TOP2A, CCNB1, MAD2L1, AURKA, NCAPG, CDKN3, TRIP13.

**TFs and microRNA network construction**

The TFs and microRNAs were inferred by Webgestalt tools with the overrepresentation enrichment analysis method. The FDR threshold was set of 0.01. After screening and filtering,

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**Table 1.** The top 5 enriched gene ontology functions and KEGG pathways of the up-regulated DEGs, *The terms are ranked by p-value.*

<table>
<thead>
<tr>
<th>Term</th>
<th>BP</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0051301~cell division</td>
<td>BP</td>
<td>1.38E-09</td>
</tr>
<tr>
<td>GO:0000822~G1/S transition of mitotic cell cycle</td>
<td>BP</td>
<td>6.49E-07</td>
</tr>
<tr>
<td>GO:0031145~anaphase-promoting complex-dependent catabolic process</td>
<td>BP</td>
<td>2.36E-06</td>
</tr>
<tr>
<td>GO:0007052~mitotic spindle organization</td>
<td>BP</td>
<td>1.31E-05</td>
</tr>
<tr>
<td>GO:0006281~DNA repair</td>
<td>BP</td>
<td>1.91E-05</td>
</tr>
<tr>
<td>GO:0005654~nucleoplasm</td>
<td>CC</td>
<td>2.19E-10</td>
</tr>
<tr>
<td>GO:0005634~nucleus</td>
<td>CC</td>
<td>5.47E-09</td>
</tr>
<tr>
<td>GO:0005876~spindle microtubule</td>
<td>CC</td>
<td>6.07E-08</td>
</tr>
<tr>
<td>GO:0005829~cytosol</td>
<td>CC</td>
<td>8.27E-05</td>
</tr>
<tr>
<td>GO:0005737~cytoplasm</td>
<td>CC</td>
<td>9.38E-04</td>
</tr>
</tbody>
</table>

**Table 2.** The top 5 enriched gene ontology functions and KEGG pathways of the down-regulated DEGs, *The terms are ranked by p-value.*

<table>
<thead>
<tr>
<th>Term</th>
<th>BP</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0043627~response to estrogen</td>
<td>BP</td>
<td>0.00742</td>
</tr>
<tr>
<td>GO:0043434~response to peptide hormone</td>
<td>BP</td>
<td>0.00275</td>
</tr>
<tr>
<td>GO:0003148~outflow tract septum morphogenesis</td>
<td>BP</td>
<td>0.0034</td>
</tr>
<tr>
<td>GO:0001558~regulation of cell growth</td>
<td>BP</td>
<td>0.00431</td>
</tr>
<tr>
<td>GO:0034101~erythrocyte homeostasis</td>
<td>CC</td>
<td>0.00599</td>
</tr>
<tr>
<td>GO:0070062~extracellular exosome</td>
<td>CC</td>
<td>1.30E-07</td>
</tr>
<tr>
<td>GO:0005789~endoplasmic reticulum membrane</td>
<td>BP</td>
<td>3.81E-04</td>
</tr>
<tr>
<td>GO:0005739~mitochondrion</td>
<td>BP</td>
<td>5.38E-04</td>
</tr>
<tr>
<td>GO:0005615~extracellular space</td>
<td>CC</td>
<td>0.001376</td>
</tr>
<tr>
<td>GO:0005576~extracellular region</td>
<td>CC</td>
<td>0.003318</td>
</tr>
</tbody>
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**BP:** Biological Process; **CC:** Cellular Components; **MF:** Molecular Functions.

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Validation of the obtained hub genes in the TNM staging of ACCs.

The DEGs of metastatic ACCs were acquired by comparing normal adrenal tissue and primary adrenal cortical carcinoma tissue, and the hub genes were identified by Cytohubba. Those hub genes were then examined by the TCGA datasets. First, these genes expression in M1 and M0 patients were showed in Figure 5. All of them were significant in distinguishing M0 and M1 staging patients. (Wilcoxon test P-value<0.01). They were higher expressed in M1 than that in M0 patients. CDK1, RFC4, and KIF11 were most outstanding among them (P<0.0001). This outcome suggested our analysis was correct and reliable. Then, the N stage was also tested by Wilcoxon test while the T stage was tested by the Kruskal−Wallis test. Their boxplots of these genes were separately shown in Figure 6 and Figure 7. CCNB1, KIF11, MAD2L1, and TRIP13 were statistical meaningful compared in N0 and N1 groups. Others were not related to the N stage. Moreover, the medium expression of most genes grows with the T stage increases. All of them were linked to tumor stages (P<0.05). CDK1, TOP2A and TRIP13 were most significant in different T stage groups (P<0.0001).

Discussion

As mentioned above, adrenal cortical carcinoma was a rare disease. It was caused by a cancerous growth in the outer layer of the adrenal glands [23]. Surgery removing the adrenal gland and surrounding tissue was the main method to the disease, and its spreading to other organs and tissues was often lack of therapy methods accompanied with poor
Figure 2. (A) The network of the DEGs and TFs generated by Webgestalt. It contained 111 nodes and 162 edges. The red indicated upregulated; the green indicated downregulated. The diamond indicates the TFs, the rectangle indicates DEGs. (B) The microRNA network of the DEGs. It contains 77 nodes and 79 edges. The red indicates upregulated, the green indicates downregulated. The “V” shape indicates the microRNAs, the rectangle indicates the DEGs. MIR: microRNA; TFs: transcriptional factors; DEGs, differential expressed genes.

Figure 3. Gene function annotation by Genclip2.0. Green indicated that the corresponding gene-term association was positively reported. Black represented the corresponding gene-term association was not yet reported. The outcomes suggested that the genes were mainly associated with cell cycle, DNA replication checkpoint, double-strand break, histone deacetylase and so on.
Mining hub genes associated with late stage adrenocortical carcinoma. Discovering the hub biomarkers becomes meaningful for its accuracy therapy. In our research, the microarray GSE90713 including 5 normal and 58 tumor samples were downloaded from the GEO datasets. After being analyzed by BrB-arraytools, a total of 405 DEGs were identified. The upregulated DEGs were mainly involved

Figure 4. The Kaplan Meier survival curve of the top ten genes by high and low expression in overall survival and disease free survival. (A) TRIP13 (****, ****). (B) KIF11 (****, ****). (C) MAD2L1 (***, **). (D) CDKN3 (****, **). (E) RFC4 (***, ***). (F) CDK1 (***, ***). (G) TOP2A (**, **). (H) CCNB1 (***, **). (I) NCA2G (****, ***). (J) AURKA (***, **); *, P-value<0.05; **, P-value<0.01; ***, P-value<0.001; ****, P-value<0.0001. The * in the brackets are separately representing the log-rank p-value of overall survival and disease-free survival.
Figure 5. Validation of the ten hub genes expression associated with M stage in TCGA patients samples of ACCs. (A) AURKA. (B) CCNB1. (C) CDK1. (D) CDKN3. (E) KIF11. (F) MAD2L1. (G) NCAPG. (H) RFC4. (I) TOP2A. (J) TRIP13. *, P-value<0.05; **, P-value<0.01; ***, P-value<0.001; ****, P-value<0.0001.
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Figure 6. Validation of the ten hub genes expression associated with N stage in TCGA patients samples of ACCs. (A) AURKA. (B) CCNB1. (C) CDK1. (D) CDKN3. (E) KIF11. (F) MAD2L1. (G) NCAPG. (H) RFC4. (I) TOP2A. (J) TRIP13. *, P-value<0.05; **, P-value<0.01; ***, P-value<0.001; ****, P-value<0.0001.
Figure 7. Validation of the ten hub genes expression associated with T stage in TCGA patients samples of adrenal cortical carcinoma. (A) AURKA. (B) CCNB1. (C) CDK1. (D) CDKN3. (E) KIF11. (F) MAD2L1. (G) NCAPG. (H) RFC4. (I) TOP2A. (J) TRIP13. *, P-value<0.05; **, P-value<0.01; ***, P-value<0.001; ****, P-value<0.0001.
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in the processes of cell division and cell cycle. The downregulated DEGs were mainly involved in the processes of response to hormone and extracellular exosome. The DEGs were inputted into the STRING website, the PPI network was visualized by Cytoscape. The hub genes were identified by Cytohubba, and top 10 genes were obtained: CDK1, RFC4, KIF11, TOP2A, CCNB1, MAD2L1, AURKA, NCAPG, CDKN3, TRIP13. These genes are upregulated in the metastasis ACCs and associated with mitotic cell cycle and so on. In addition, the microRNAs and TFs were also inferred by Webgestalt and their network was shown in Fig 4, including 12TFs and 18 microRNAs. The TFs contained E2F, E2F4DP1, MYCMAK, NFIY and so on. MicroRNAs included hsa-mir-492, hsa-mir-488, hsa-mir-34b, hsa-mir-520d, hsa-mir-517, hsa-mir-501, hsa-mir-190, hsa-mir-186 and so on. The top ten genes were then validated by the TCGA datasets, which indicated these genes were relative highly expressed in metastasized tumors. Their high expression of these genes indicated poor prognosis of the patients. RFC4 and PCNA were necessary for elongation of DNA templates by DNA polymerase; RFC possessing DNA-dependent ATPase activity were biologically active in various malignant tumors [26,27]. KIF11 was a kinesin-like protein involved in spindle dynamic activity, which had been reported in oral cancer [28,29]. TOP2A was a DNA topoisomerase, regulating the DNA topologic states during transcription. It also amplified in several cancers and its mutation had been connected to the drug resistance [30-32]. CCNB1 was a cell cycle-regulated protein participating in mitosis and formation of MPF [33]. MAD2L1 was involved in the accuracy of chromosome segregation and stop the attack until all chromosomes were correctly positioned at the metaphase plate [34]. CDKN3 was a cyclin-dependent kinase inhibitor and dephosphorylated CDK2 kinase to prevent its activation [35]. CDK1 activated cell transcription factor Hcm1 and targeted it for degradation through phosphorylation of distinct sites, it also played crucial roles in regulating cell cycle progression from G1 to S, through S, and G2 to M phase [36,37]. AURKA was also a cell cycle-regulated kinase participating in microtubule construction and structure steady in mitosis anaphoresis. AURKA was positive in centrosome and spindle poles of different cell mitotic phases [38,39]. NCAPG probably helped introduce positive supercoils into relaxed DNA, Zhang et al. reported that knockdown of NCAPG induces HCC cell mitosis and inhibits cell growth, proliferation, and migration in vitro. TRIP13 interacted specifically with the ligand binding domain and may play a role in early-stage non-small cell lung cancer [40,41]. It was also involved in controlling G2/prophase processes such as DNA break formation and recombination, checkpoint signaling, and chromosome synopsis; it was a conserved member of ATPases associated with diverse cellular activities [42]. These genes of CCNB1, KIF11, MAD2L1, and TRIP13 were closely associated with tumor stage and prognosis, as well as high HR. They may serve as therapeutic targets for the adrenal carcinoma. The Drugbank and upertargets may be used for searching potential drugs for these genes. Their actuals effects on metastasis ACCs need further experimental validations.

Conclusion
In conclusion, CDK1, RFC4, KIF11, TOP2A, CCNB1, MAD2L1, AURKA, NCAPG, CDKN3, TRIP13 have been identified as hub genes in metastasized ACCs. They were not only associated with tumor’s metastasis but also tumor’s staging and patients’ prognosis, except for CCNB1, KIF11, MAD2L1, and TRIP13, others were not meaningful in local lymph node invasion. These genes of CCNB1, KIF11, MAD2L1, and TRIP13 were closely associated with tumor TMN stage and prognosis; they may serve as therapeutic targets for the adrenal carcinoma.

Competing Interests
The authors declare that the research was conducted in the absence of any commercial that could be construed as a potential conflict of interest.

Availability of Data and Materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request. The gene chip GSE90713 is available online.

Consent for Publication
All the authors have consented for the publication.

Authors’ Contributions
Di Yu contributed to figure construction and article writing while Chen Dongshan, and YuWei helped with drafting and proofreading, and Yan Lei provided funding and technical support and monitored the whole processes.

Ethics Approval and Consent to Participate
Not applicable.

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