Investigating the dysfunctional pathogenesis of Wilms’ tumor through a multidimensional integration strategy

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Background: Wilms’ tumor (WT) is a common kidney tumor in early childhood which is characterized by multiple congenital anomalies and syndromes. With the continuous improvement of medical standards, the cure rate and survival period of WT have increased. However, its molecular mechanism is still elusive.

Methods: A comprehensive multidimensional integration strategy was used to comprehensively analyze the mechanisms of WT.

Results: By integrating the potential pathogenic genes of kidney cancer and performing co-expression analysis on the disease-related genes, 23 functional modules were obtained. All the genes were differentially expressed in WT, and were mainly involved in many biological processes and signaling pathways, such as Wnt/β-catenin, mTOR/ERK and calcineurin. Additionally, based on the relationship between transcriptional and post-transcriptional regulatory systems, in functional modules, transcription factors (TFs) including STAT3, HDAC1 and SP1 as well as non-coding RNAs (ncRNAs) such as miR-335-5p, miR-21-5p and TUG1 were identified. Finally, potential drugs for these multifactor regulated dysfunctional modules which may have certain pharmacological or toxicological effects on WT such as cisplatin, sorafenib, and zinc were predicted.

Conclusions: A multidimensional dysfunction mechanism, involving disease-related genes, TFs and ncRNAs was revealed in the pathogenesis of WT. Functional modules were used to predict potential drugs which can be used in personalized therapy and drug delivery. This study explored the pathogenesis of WT from a new perspective, and provides new candidate targets and therapeutic drugs for improving the cure rate of WT.

Keywords: Wilms’ tumor (WT); dysfunctional modules; pivot regulators; crosstalk; potential drugs

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Introduction

Epidemiological observations show that Wilms’ tumor (WT, also known as nephroblastoma) is the most common malignant kidney tumor in children (1). Moreover, 98% of cases occur in children who are under the age of 10 years and the disease is most common in children under the age of 3 years. The disease is mainly characterized by swelling of the abdomen, which may be accompanied by symptoms of digestive diseases such as abdominal pain, nausea, vomiting and loss of appetite. In addition to these core symptoms, there are problems such as divergent iris defects, urogenital abnormalities and mental retardation (2). Meanwhile, bone metastases and lung metastases occur in patients with severe prognosis and further infiltration of the inferior vena cava and right atrium could lead to sudden death (3,4). Clinicians, pathologists and radiation oncologists have taken corrective measures leading to a significant increase in the overall cure rate and long-term survival of WT patients (5). However, satisfactory results have not been achieved as poor prognosis, recurrent WT and treatment side effects are still high in some patients (6-8). Patients receiving chemotherapy exhibit some symptoms such as jaundice, ascites, hepatomegaly and pain in the right upper quadrant, causing hepatic vein occlusive disease (VOD) (9). Nevertheless, vincristine, doxorubicin (VDA), and whole lung radiotherapy (XRT) have some effects on both inflammatory myofibroblastoma (IMT) and fatal deficient blood enteritis. People with these types of diseases have low survival rate. Fortunately, researchers have focused on medical and biological aspects. Through unrelenting efforts, they have made great progress and brought out many valuable scientific results, including information on WT related genes and signal pathways, which have enhanced our understanding of the pathogenesis of this disease.

Studies have shown that WT is caused by genetic abnormalities, epigenetics, genetic mutations and environmental factors (10-12). Multiple studies have identified and confirmed that mutations in the Wilms tumor suppressor gene (WT1) are associated with the pathogenesis of WT. During embryonic development, WT1 induces differentiation of renal progenitor cells in response to Wnt signaling. In hereditary nephroblastoma, WT1 inhibits gene silencing of beta-catenin, leading to precancerous renal arrest (13). Furthermore, in the case of WT, the Wnt/beta-catenin pathway is highly activated which is influenced by AXIN2 (13-15). Additionally, Urbach et al. showed that Lin28/Let-7 pathway regulates kidney development and tumorigenesis (16). Bielen and other scientists have determined the role of PI3 and MAP kinases, and found that G1 cell cycle arrest and downstream signaling of cell death are potential drug targets. In addition, in WT, they also demonstrated the therapeutic potential of insulin-like growth factor (IGF). Based on the known kidney disease-related genes, a multi-factor regulation network was constructed through a systematic comprehensive strategy to determine the biological significance of WT dysfunction mechanism. By combining RNA-seq data, gene co-expression networks and differential expression analysis, dysfunctional co-expression modules were identified. In the modules, several genes were involved in various biological processes (BP) such as cell proliferation, differentiation, apoptosis and carcinogenesis. Moreover, the genes were also involved in WT-related signals such as Wnt/beta-catenin, mTOR/ERK and calcineurin. Consequently, based on transcriptional and post-transcriptional regulatory systems, we identified key regulators in each module. Statistical analysis showed that some regulators play an important role in the pathogenesis of WT by regulating multiple dysfunction modules. Finally, based on this multifactorial regulation network, potential drugs with potential pharmacological or toxicological effects on WT were predicted. This multi-factor co-expression network analysis not only helps to explore the relationship between gene modules and self-assembled components but also provides a valuable resource for further studies.

Methods

Data resources

RNA-seq datasets and miRNA-seq datasets of WT were downloaded from the Cancer Genome Atlas (TCGA) (17). RNA-seq datasets contained 120 primary WT patients and 6 normal individuals, while miRNA expression datasets to 126 primary WT patients and 6 normal individuals. This study was approved by the Clinical Research Ethics Committee of College of Medicine, Zhejiang University.

The NCBI Gene database contained 55 kidney cancer-related genes and 1,581 renal carcinoma-related genes (18). Additionally, there were 987 kidney cancer-related genes and 284 renal carcinoma-related genes in the OMIM database (19). After integration, a total of 2,301 disease-related genes were obtained.

STRING database (20) provides the most comprehensive
view of the protein-protein interactions (PPIs) currently available and 10,514 proteins with 405,916 interactions (score >900) in human were collected from this database. Additionally, for transcriptional and post-transcriptional regulation, from TRVUST v2, 2,492 TFs (21) with 9,396 TF-target relationships in humans were collected. In RAID v2.0, 4,331 ncRNAs in humans were associated with 431,937 ncRNA-related interactions (value >0.5) (22). Moreover, information about drugs and drug-target were downloaded from the Drugbank database (23), which was used to predict potential drugs for WT.

Identification of functional modules

In order to explore the co-expression of 2,301 WT-related genes in 120 WT patients, a weighted gene co-expression network analysis (WGCNA) was performed on their gene expression data (24), and the functional modules in WT were determined. WGCNA is a systematic biological method used to describe genetic associations between different samples, and can be used to identify gene sets with high synergistic changes at expression levels. The algorithm uses the adjacency coefficient as a measure of the correlation between any gene pairs, i.e., the power exponent weight of the nth index of the correlation coefficient. The connections between genes in the network are subjected to scale-free networks, making the algorithm more biologically meaningful. This builds a hierarchical clustering tree based on correlation coefficients between genes, whose different branches represent different modules. Among them, the selection principle of soft threshold is to make the constructed network match with scale-free network characteristics. In the data analysis process, the soft threshold power =5, and screened the gene cluster with synergistic expression behavior as a module.

Differential expression analysis

Differential expression analysis on RNA-seq datasets and miRNA-seq datasets was performed using the R language limma package (25-27). First, the readings of RNA-seq and miRNA-seq were normalized using the voom functions. Then, the differentially expressed genes and miRNAs were identified using the lmFit and eBayes functions, respectively, at default parameters.

Determination of significant crosstalk module pairs

To interpret the relationships of modules involved in the pathogenesis of WT, we performed crosstalk analysis on modules using human PPI network as background set. Initially, the original PPI network was randomized 1,000 times while maintaining the size of each node in the network. Furthermore, the significance per module pair was calculated as:

\[ P = \frac{n}{1000} \]

\( n \) is the number of random network, in which the number of interactions between module pair is more than the number in the real network. If \( P \leq 0.05 \), the module pair was considered as significant crosstalk module pair.

Exploration of pivot regulators

At each module, the module’s pivot adjuster satisfied the following conditions: (I) the interaction between the governor and the module was greater than 2; (II) the interaction between the regulator and the hypergenometric test was less than 0.01. Similarly, based on drug-target information, potential drugs for WT were predicted.

Gene ontology (GO) and KEGG pathway enrichment analysis

In the enrichment analysis of genes, GO (P value cutoff =0.01, Q value cutoff =0.01) and KEGG pathway (P value cutoff =0.05, Q value cutoff =0.2) were performed with the R language Clusterprofiler package (21). In addition, the network function analysis were done by BinGO (22), a plug-in in Cytoscape.

Results

Identification of disease-related modules in WT

From the genetic perspective, functional modules represent a series of highly related genes. Meanwhile, genes in the same module may have similar biological functions and participate in the same biological pathway. At biological systems, finding a gene module with potential functions is actually a bridge to understand the differences between individual gene functions and global network characteristics. Therefore, identifying gene functional modules is important in understanding the molecular mechanisms of disease. To date, biologists have conducted extensive experiments and studies on kidney-related cancers, such as WT, and
have identified related potential pathogenic genes. To gain insights into the underlying molecular mechanisms of WT, disease-related genes were first integrated from NCBI Gene database (18) and OMIM database (19), and a total of 2,301 genes were collected. Results showed that they had co-expression behaviors based on RNA-seq datasets from 120 patients with WT, and 23 functional modules were identified (Figure 1), possibly representing the underlying molecular mechanisms of kidney cancer-associated genes in WT pathogenesis. Moreover, differential expression analysis was performed on the RNA-seq data. The results showed that all of the module genes were differentially expressed (Figure 2), suggesting that these disease-related genes in modules might be involved in the pathogenesis of nephroblastoma. Therefore, these 23 modules were identified as disease-related dysfunctional modules.

**Functions and signaling pathways of involved modular gene**

In the pathogenesis of genes involved in a disease, their function and pathway are important. Here, to further understand the biological characteristics of these modules, GO and KEGG pathway enrichment analysis on the 23 dysfunctional modules were performed. From a total of 580 cell composition (CC) entries, 355 molecular functional (MF) terms, 10,576 BP and 418 KEGG pathways (Figure S1), a number of BP were observed. These were mainly cell proliferation, migration, apoptosis, calcineurin, immune system regulation, regulation of the cell cycle and cancer signaling pathways, and various progenitor cells division. The KEGG pathway mainly includes Wnt/beta-catenin, JNK cascade and mTOR/ERK signaling pathways, which are involved in the development, progression and prognosis of WT. In addition, according to statistical analysis, up to 11 modules significantly participated in leukocyte differentiation, leukocyte migration, and protein kinase B signaling. This phenomenon indicates that WT is inextricably linked to the immune system, especially leukocyte immunity. On the other hand, according to the crosstalk relationship between modules, 146 pairs of modules with important crosstalk were identified (Figure 3). Through these crosstalk relationships, it can be considered that these disease-related genes not only have synergistic expression in the module, but also have mutual regulation between the modules, therefore forming a functional network to influence the process of WT.

**Pivot regulators and potential drugs correlated with disease-related modules**

WT is a complex cancer, and the dysfunctional modules of WT are regulated by many factors. Based on the transcription and post-transcriptional regulation, a pivotal regulatory factor that significantly regulates dysfunction were identified. This included 157 transcription factors (TFs) with 2,569 TF-target relationships and 1,409 ncRNAs with 2,007 ncRNA-protein interactions (Figure 4). In addition, differentially expressed TFs and miRNAs in WT were identified based on RNA-seq and miRNA-seq datasets. Statistical analysis showed that the TF STAT3 significantly regulated six modules, and HDAC1, SP1, NFKB1 and RELA regulated five modules. Whereas miR-335-5p significantly regulates 7 modules, miR-21-5p, miR-25-3p, miR-9-5p, miR-27b-3p and TUG1 regulate 5 modules. By up-regulating miR-370, IL-6-activated STAT3 can inhibit the expression of WTX, which promotes the occurrence, proliferation and invasion of WT (24,28). SP1, a TF of WT1, mediates the incidence of WT (29). HDAC1 inhibits the expression of augWT1 and the corresponding target and this plays an important role in WT. Additionally, NFKB/Rel which up-regulates WT1 expression activates WT1 upstream regulatory cascade, promoting the development and progression of WT (30). MiR-21-5p was experimentally confirmed to be overexpressed in WT patients, and it promotes the migration and invasion of WT cells (31). Overall, these pivot regulators with high degree of certainty may have key effects on the regulation of the pathogenesis of WT.

Based on the dysfunction mechanism of WT, it is desirable to explore potential therapeutic mechanisms for clinical and drug development, and to study effective treatments for reference. Here, based on the drug-target information from Drugbank (23), potential drugs for these dysfunctional modules were predicted. A total of 550 drugs with 721 drug-target interaction pairs were identified (Figure 5). These drugs have potential pharmacological or toxic effects on WT. Among them, cisplatin, sorafenib, copper and zinc have a significant regulation relationship with five modules, which may produce more significant effects or toxicity. Cisplatin has been used as a chemotherapeutic agent for WT. Sorafenib is an oral small molecule inhibitor, helpful in the treatment of rhabdomyosarcoma, nephroblastoma and hepatocellular carcinoma (HCC) (32,33). Studies have confirmed that
Figure 1 Co-expression of WT-related genes. (A) Clustering dendrogram of genes. Each color represents one module, and there are 23 modules in total. Y-axis represents the height of the gene tree. (B) Heatmap plot of gene network. The heatmap depicts the Topological Overlap Matrix (TOM) among all genes in the analysis. (C) Cluster tree relationship between modules. Circles of different colors represent different modules and there are 23 modules in total. Modules with similar expressions are grouped together to characterize the modular relationship in terms of collaborative expression.
ZFP346, ZNF224 and other zinc finger proteins act as transcriptional cofactors of WT1 in WT, leukemia and other cancers, and affect cancer progression (34,35). In addition, copper-bound monoamine oxidase (AOC1) is activated by WT1 and mediates polyamine breakdown, playing a key role in cell growth and proliferation of embryonic kidney and gonads (36). Exploring disease treatment mechanisms is the goal of most biomedical researchers. This study predicts that WT’s potential drugs lay a strong theoretical foundation for further exploration.

**Discussion**

Wilms tumor (WT) can be associated to the loss and mutation of the WT1 gene (located at 11p13) on chromosome 11. It may also be associated with proliferation and differentiation of mesenchymal cells in the posterior of kidney tissues (37,38). Although researchers have explored the etiology of WT at different aspects, the underlying pathogenesis is still unclear. In this study, 23 functional modules combined were identified using the WT patient’s
RNA-seq data and 2,301 known kidney cancer-associated genes, and these genes were differentially expressed in WT. Enrichment analysis showed that these genes were involved in other validated pathways in addition to the common WT-related signaling pathways such as Wnt/β-catenin/Ras protein signal transduction, mTOR signaling pathway/positive regulation of ERK1 and ERK2 cascade (39-41). Studies have shown that in WT1, low expression, deletion and mutation could lead to increased expression of the IGF-1 receptor (IGF-IR), hence it is necessary to regulate the synthesis and secretion of IGF-II (42,43). Additionally, several studies have demonstrated that the phosphatidylinositol-3-kinase/Akt pathway is regulated by miR-19b, which mediates cell proliferation, cell cycle progression, migration and invasion, and participates in induction of glomerular proteinuria disease with activated macrophages in WT (44-46). Subsequently, 146 significant crosstalk relationships between modules were also observed, which bridged the gap between each potential dysfunctional mechanism. Moreover, by integrating TF targets and ncRNA-mRNA interactions, key regulators that significantly regulate dysfunction modules were identified. It highlights TFs STAT3, SP1, HDAC1, NFKB1, RELA, miR-335-5p, miR-21-5p, miR-23-3p, miR-9-5p, miR-27b-3p and lncRNA TUG1 and lncRNA TUG1 with higher levels. This study reveals the multidimensional effects of

Figure 3 The significant crosstalk module pairs. The size of node represents the degree, and the thickness of edge represents the number of crosstalk interaction between two modules.

Figure 4 The regulation network mediated by pivot regulators. (A) The regulation network mediated by pivot TFs. The blue arrows represent module and the circles represent pivot TFs, whose size and color represent the differential expression. The larger the node, the smaller P value and the more the significance. The color arranged from red to blue represents up-regulation to down-regulation. (B) The regulation network mediated by pivot ncRNAs. Circles represent module and diamonds represent pivot ncRNAs. The larger the circle, the more the association with miRNA. TF, transcription factor; ncRNA, non-coding RNA.
STAT3 in WT, and for instance, IL-6-activated STAT3 can inhibit expression of WTX. In addition, it is possible to up-regulate the expression of hypoxia-inducible factor-1alpha (HIF-1alpha) and vascular endothelial growth factor (VEGF). This promotes the development of WT, angiogenesis and the metastasis of cancer cells (24,28). SP1 has also been shown to be highly expressed in mesenchymal, early tubules, developing podocytes and mature glomeruli, and is a TF of WT1 which mediates WT (29,47). HDAC1 is recruited by the expressed WT1, which inhibits the expression of the corresponding target gene and plays an important role in WT (48). Moreover, a member of NFKB/Rel family up-regulates WT1 expression and activates WT1 upstream of the regulatory cascade, promoting the development and progression of WT (30). On the other hand, in various cancers including osteosarcoma, gastric cancer, non-small cell lung cancer, miR-335-5p was found to mediate tumor proliferation, migration and invasion. However, this has not been reported to play a role in WT (31,49). The predictive analysis of this study indicates its potential regulatory role in WT, but its basic molecular mechanism needs to be studied extensively. In 5 modules, cisplatin, sorafenib, zinc, and copper were found. However, cisplatin as a chemotherapeutic agent for WT, not only inhibits the growth of WT cells by inducing apoptosis, but also inhibits WT growth and invasion by promoting the binding of BAX protein to transcriptional activator 3 (STAT3) to target glucose regulatory protein 78 (GRP78). Sorafenib is an oral small molecule inhibitor that controls multiple kinases for tumor growth and angiogenesis. It can treat rhabdomyosarcoma, nephroblastoma and HCC that are unresponsive or intolerant to standard therapies. It has been established that Zinc can bind to certain proteins to form zinc finger proteins. Studies have confirmed that ZFP346, ZNF224 and other zinc finger proteins act as transcriptional cofactors of WT1 in WT, leukemia and other cancers, and they could have significant influence on cancer progression (34,35). Furthermore, it was observed that red blood cells from children with nephroblastoma have higher concentration of zinc and this is associated with zinc superoxide dismutase activity. On the other hand, AOC1 is activated by WT1 and mediates polyamine breakdown, playing a key role in cell growth and proliferation of embryonic kidney and gonads (36). Although the direct role of zinc and copper in WT has not been fully established, the results of this study suggest their potential roles.

In summary, this work reveals the multifactor-mediated dysfunctional network and characterizes the potential molecular mechanisms of WT. This not only clarifies the overall effect of related genes at a global level, but also
improves the potential pathogenesis of WT, providing numerous candidate resources for future experimental verification. The prediction of potential drugs revealed the therapeutic mechanism of WT and as well provides candidate molecules for drug design and drug relocation studies of WT.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References


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Figure S1. GO and KEGG pathway enrichment analysis. (A) GO enrichment analysis of genes per module; (B) KEGG pathway enrichment analysis of genes as per module; (C) function analysis of all genes in modules. Colors from white to yellow indicate significance level enhancements. The size of the node represents the number of functional/pathway entry genes involved. GO, Gene ontology.