Identification of WTAP-related genes by weighted gene co-expression network analysis in ovarian cancer

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Abstract

Background: WTAP modulating other genes in the manner of transcriptional and post-transcriptional regulation, in particular, acting as a N6-methyladenosine writer or binding to the 3′-untranslated region of mRNA, promotes a variety of tumors. However, its roles and mechanisms in ovarian cancer are unknown.

Results: In this study, using univariate Cox analysis and online CPTA analysis, we found that WTAP was a poor prognostic factor for ovarian cancer and its protein expression level was higher in ovarian cancer compared to normal tissue. Subsequently, we identified a module containing 133 genes that were carefully related to WTAP expression through weighted gene co-expression network analysis. By calculating hazard ratio of these genes and comparing their differences in the WTAP high-expression group and the low-expression group, we noticed there was a significantly positive correlation between WTAP and two poor survival-related genes: FAM76A and HBS1L.

Conclusion: Our research suggests that the WTAP-HBS1L / FAM76A axis may play an essential role in ovarian cancer progression and provide new ideas for ovarian cancer research.

1. Introduction

WTAP is a regulatory gene affecting the expression of other genes both at the transcriptional and post-transcriptional levels. Under physiological conditions, WTAP has been implicated in a variety of process, including cell cycle and cell proliferation[1-3], cell survival[4], embryonic development[5]. Besides, it contributes to cancer development by disturbing the expression of cancer-promoting genes and cancer suppressor-genes[6-11]. By binding transcription factor WT1, WTAP represses transcription of WT1's target genes such as amphiregulin, Bcl-2[2] and TBL1[6]. The post-transcriptional mechanisms involve binding to the 3′-untranslated region (3'UTR) to generate increasing stability of mRNA[1, 10, 11], assisting N6-methyladenosine (m6A) modification to alter mRNA metabolism[9], acting as spliceosomes to participate in alternative splicing of pre-mRNA[12], among which the former two are more closely related to cancer development.

In the previous study, WTAP-guided m6A modification contributes to the progression of hepatocellular carcinoma via the HuR-ETS1-p21/p27 axis[9]. Besides, in pancreatic cancer, WTAP could bind to and
stabilize Fak mRNA so that activate the Fak-PI3K-AKT and Fak-Src-GRB2-Erk1/2 signal pathways, thereby promotes metastasis and chemo resistance\textsuperscript{[10]}. Moreover, WTAP may play an oncogenic role in renal cell carcinoma by binding to CDK2 transcript and enhancing its stability\textsuperscript{[11]}. Although roles and mechanisms of WTAP in several cancers have been elucidated, including colon carcinoma\textsuperscript{[6]}, cholangiocarcinoma\textsuperscript{[7]}, glioma\textsuperscript{[8]}, hepatocellular carcinoma\textsuperscript{[9]}, pancreatic carcinoma\textsuperscript{[10]}, renal cell carcinoma\textsuperscript{[11]}, it remains elusive in ovarian cancer. It was intriguing whether WTAP also contributes to cancer progression and affect the expression of tumor-related genes through post-transcriptional regulation of mRNA in ovarian cancer. Therefore\textsuperscript{[1]} bioinformatics approaches were applied to explore potential roles in ovarian cancer. By univariate Cox analysis, we identified WTAP was a poor prognostic factor for ovarian cancer. Furthermore, weighted gene co-expression network analysis was employed to determine hub genes that may be regulated by WTAP. Our results suggest that WTAP may regulate the expression of FAM76A and HBS1L\textsuperscript{[12]} which are also associated with poor prognosis. The jointly positive correlation between the expression of FAM76A, HBS1L and WTAP may provide new ideas for the study of ovarian cancer.

2. Methods
2.1 Data download
The matrix of GSE63885 containing mRNA microarray data of 101 ovarian cancer samples and corresponding clinical information were downloaded from Gene Expression Omnibus (GEO). Twenty-six samples with incomplete survival information were excluded. The matrix was normalized by the R package “limma”. RNA-seq data of 379 samples of ovarian cancer was downloaded from TCGA. The data format was HTSeq-FPKM.

2.2 Identification of prognostic m6A regulators
In this study, we included m6A regulators including writers: METTL3, METTL14, WTAP, VIRMA, RBM15, ZC3H13; eraser: ALKBH5, FTO; reader: HNRNPA2B1, HNRNPC, IGF2BP1, IGF2BP2, IGF2BP3, YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3. For GEO data, we applied univariate Cox analysis to identify regulators related to ovarian cancer prognosis. Genes with p-values less than 0.05 were considered statistically significant.
2.3 Construction of co-expression network

The top 5000 genes with a significant median absolute deviation (MAD) in 75 ovarian cancer samples in GSE63885 were selected and qualified as expression matrix to establish a co-expression network by the “WGCNA” package in R[13]. The clinical traits table contained the sample's overall survival(OS), WTAP expression, grade, and stage information, which were all converted into numerical values: Figo stage (IIIB,IIIC: 2; IIA, IIIB, IIIC: 3; IV:4); grade (G2: 1; G3: 2; G4: 3). For the WTAP expression, we used the function “surv_cutpoint” in the R package “survminer” to determine the best cutoff value most relevant to survival. The WTAP expression higher than the best cutoff value was marked as “2”, and the WTAP expression of samples less than the best cutoff value was marked as “1”. Another method was to use its expression values as clinical information directly.

In the beginning, a hierarchical clustering tree was constructed using the expression matrix to detect outliers. Then we calculated the correlation matrix by Pearson's correlation analysis. Subsequently, the function “pickSoftThreshold” was used to select the optimal soft threshold $\beta$ on which the conversion from the correlation matrix into an adjacency matrix is reliant on to make a scale-free co-expression network. In order to divide genes with similar expression patterns into different modules, the topological overlap dissimilarity measure (TOM) was used to calculate the degree of association between genes. Then the topological overlap matrix and corresponding dissimilarity matrix were obtained. Based on the high topological overlap of genes in the module, the hierarchical clustering dendrograms were constructed and the adaptive branch pruning method was used to divide and merge modules with a similar module eigengene (ME), the first principal component of a module. A minimum of 30 genes per module was required.

2.4 Identification of hub module and function annotation

Correlation analysis was performed using the ME of each module and numerical clinical information, and the association was represented by the Person coefficient. The module highly relevant to a given clinical feature was selected for further analysis. As for function annotation, we used the R package “clusterProfiler” [14] to conduct Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. P-values <0.05 were considered statistically significant.

2.5 Identification of hub genes
For genes in the hub module, we used univariate Cox analysis to screen genes that were significantly associated with prognosis. Genes with p-values less than 0.01 were seemed as module’s prognostic genes. According to the expression level of WTAP, we used the quartile method to divide the sample into two groups: WTAP low-expression group (0-25%) and WTAP high-expression group (75% -100%). The Wilcoxon test was used to compare the expression of the module’s prognostic genes between the two groups. P-values <0.01 were considered statistically significant.

2.6 Gene-set enrichment analysis
Gene-set enrichment analysis (GSEA) software was downloaded from https://www.gsea-msigdb.org/gsea/datasets.jsp (Accessed 22 February 2020). Based on the median gene expression, we divided the samples into two groups and selected the C2 (c2.cp.kegg.v6.1.symbols.gmt) sub-collection downloaded from the Molecular Signatures Database https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp#C2. (Accessed 22 February 2020) as the reference gene set to perform GSEA [15, 16].

2.7 Online analysis
For online survival analysis, Kaplan-Meier Plotter was used ( https://kmplot.com/analysis/index.php?p=service&cancer=ovar, accessed 20 February 2020. ) [17]. For protein analysis, we compare Clinical Proteomic Tumor Analysis Consortium (CPTAC) samples in UALCAN ( http://ualcan.path.uab.edu/ accessed 18 February 2020). [18]

3. Result
3.1 WTAP was a poor prognostic factor for ovarian cancer
To investigate the effect of WTAP and other m6A regulators on the prognosis of ovarian cancer, we performed univariate Cox analysis on 18 m6A regulators. The results showed that WTAP with the hazard ratio (HR)> 1, p <0.01 in the GSE63885 dataset was a risk factor for ovarian cancer (Fig. 1A). At the same time, patients with high WTAP expression had a dismal OS and progression-free survival (PFS) when compared with patients who had low WTAP expression in online Kaplan-Meier Plotter (Fig.1B,1D). Besides, we noticed that protein expression of WTAP were elevated in ovarian cancer and others including breast cancer, colon cancer, clear cell renal cell carcinoma, uterine corpus endometrial carcinoma compared with corresponding normal tissues (Fig. 1C). We concluded WTAP
was a risk factor that negatively affects the prognosis of patients with ovarian cancer.

3.2 Construction of co-expression network
As mentioned above, we selected the top 5000 genes with a significant MAD in 75 ovarian cancer samples as the expression matrix to establish a hierarchical clustering tree, and no outliers were removed (Fig. 2A). We chose $\beta$ of 3 (scale-free $R^2 = 0.93$) as the appropriate soft-thresholding value to ensure a scale-free network (Fig 2C,2D). Finally, total of 9 modules were identified(Fig. 2B).

3.3 Identification of hub module associated with WTAP expression and function annotation
The association between ME of each module and numerical clinical information was represented by the Pearson coefficient (fig. 3A), among which the coefficient between the red module and the WTAP expression was the highest and positive (Pearson cor = 0.6, $p = 1e-8$). At the same time, the red module was also negatively correlated with OS (Pearson cor = -0.34, $p = 0.003$). Therefore we chose 133 genes in the red module for further analysis. The scatter plot showed the correlation between module membership (MM) (association between genes and modules) and gene significance (GS) association between genes and clinical trait for WTAP expression or OS respectively (Fig 3B,3C). To explore the function of genes in the red module, we performed GO analysis. Enriched biological processes (BPs) mainly involved “positive regulation of cellular protein localization”, “regulation of chromosome organization”. The cellular components (CCs) were primarily enriched in “focal adhesion”, “cell-substrate adherens junction” and “cell-substrate junction”. Enriched molecular functions (MFs) chiefly comprised “S-adenosylmethionine-dependent methyltransferase activity”(Fig 3D). KEGG pathway analysis showed that the “AMPK signaling pathway”, “Insulin signaling pathway”, “Tight junction” and “Human T-cell leukemia virus 1 infection” pathway were the most enriched (Fig 3E).

3.4 Identification of hub genes that may be regulated by WTAP
In order to further determine the prognosis related genes that may be regulated by WTAP, we performed univariate Cox analysis of all genes in the red module, and the screening threshold was $p < 0.01$. A total of 20 genes were selected as prognostic genes in red module for subsequent analysis (Table 1). According to the expression level of WTAP, we divided samples into two groups: WTAP high-
expression group (WTAP expression is higher than Q4) and WTAP low-expression group (WTAP expression is lower than Q1). Then, we compare the expression difference of 20 prognostic genes in the red module. Genes with significant differences (p<0.01) are: FAM76A, HBS1L, MPDU1, UBE2M, PSENEN, NRB1 (Table 2). Among them, FAM76A and HBS1L highly expressed in the WTAP high-expression group, and also lowly expressed in the WTAP low-expression group (Fig 4A,4C), which can be confirmed in TCGA data (Fig 4E,4G). At the same time, the high expression of FAM76A and HBS1L also suggested poor survival in ovarian cancer patients (Fig 4B,4D,4F,4H). As the close relationship and their prognostic effect, we speculated the mRNA of FAM76A and HBS1L may be regulated by WTAP mediated m6A modification and were affected by the positive-reader effect to promote their stability. Apart from that, the binding of WTAP to the 3'UTR may also play a role.

3.5 Gene-set enrichment analysis of real hub genes
GSEA was performed to determine a potential mechanism for HBS1L and FAM76A involved in ovarian cancer. Three gene sets enriched in high HBS1L expression group: KEGG_Olfactory_Transduction (NES=2.11, NOM p-value=0.002, FDR q-value=0.007), KEGG_Regulation_of_Autophagy (NES=1.78, NOM p-value=0.006, FDR q-value=0.132), KEGG_SNARE_INTERACTIONS_IN_VESICULAR_TRANSPORT (NES=1.73, NOM p-value=0.008, FDR q-value=0.152)(Fig 5A). In high FAM76A expression group, enriched gene sets included KEGG_Ribosome (NES=1.62, NOM p-value=0.04, FDR q-value=0.152), KEGG_PROTEIN_EXPORT (NES=1.62, NOM p-value=0.018, FDR q-value=0.076), KEGG_Lysine_Degradation (NES=1.6, NOM p-value=0.016, FDR q-value=0.078) (Fig 5B).

4. Discussion
In this study, we first evaluated the effects of 18 regulators for m6A modification on the prognosis of ovarian cancer and found that high expression of WTAP was a risk factor for the prognosis of ovarian cancer. Subsequently, with the help of WGCNA, we identified two hub genes: FAM76A and HBS1L, which may be regulated by WTAP. The mechanism of WTAP regulating other genes includes transcriptional and post-transcriptional level. In cancers, the latter one, especially m6A modification and binding to the 3'UTR of mRNA, matters.
N6-methyladenosine (m6A) modification, most common reversible post-transcriptional modification for RNA, is installed by methyltransferases (writer), demethylases (eraser), and m6A binding proteins (reader)\(^{(19)}\). As a m6A writer, WTAP contribute to the localization of methyltransferase METTL3-METTL14 to the nuclear speckle\(^{(20)}\). Mechanisms of m6A modification disorder aggravating cancer can be attributed to the following factors: Is the target gene of m6A a cancer-promoting gene or cancer-suppressing gene? Is the methylation modification level of the target mRNA methylated or demethylated? Is the role of m6A reader positive or negative? For example, IGF2BP1, a positive-reader which enhance mRNA stability and storage, promotes the expression of oncogene SRF in an m6A-dependent manner in ovarian cancer\(^{(21)}\). In endometrial cancer, METTL14 mutation or METTL3 down-regulation leads to a decrease of mTOR m6A levels, which is a regulator of oncogene AKT. YTHDF2, a negative reader promoting m6A mRNA degradation, thus reduces degradation of mTOR and activates AKT pathway\(^{(22)}\). As for 3'UTR, adenosine and uridine rich elements (AREs) is one of the important cis-element in it. RNA-binding proteins (RBPs) combined with AREs can accelerate mRNA decay or improve its stability\(^{(23)}\). Similar to the mechanism mentioned above, WTAP can bind to the 3’UTR of cyclin A2 mRNA to improve its stability in umbilical vein endothelial cells. The nine-base sequence ACAAAAUUAU, which is also rich in A and U, is necessary for its binding\(^{(1)}\).

The significantly positive correlation between WTAP expression and FAM76A and HBS1L in our study lead us to speculate the possibility that WTAP mediate m6A modification on the mRNA of FAM76A and HBS1L as well as coordinate with positive-reader, or WTAP bind to the 3’UTR of FAM76A and HBS1L mRNA promoting their stability. However, how the WTAP-HBS1L / FAM76A axis plays a role in ovarian cancer progression needs further experimental verification. Another question needs to be considered is how is the high expression of WTAP modulated? WTAP can regulate the expression of other genes, and WTAP may also be adjusted. CA4 is a tumor suppressor gene for colon cancer and inhibits Wnt pathway by attenuating WTAP-WT1-TBL1 axis, among which WTAP is degraded because CA4 is stimulating its polyubiquitination\(^{(6)}\). In addition, the expression of METTL3, “write” same as WTAP, is higher in gastric cancer due to p300-mediated H3K27 acetylation of METTL3 promoter. METTL3
regulates HDGF mRNA in the way of m6A modification, and positive reader IGF2BP3 recognize it promoting its stability. Therefore, HDGF promotes glycolysis and angiogenesis, leading to tumor growth and metastasis.[24]. Is there a similar regulatory mechanism for WTAP expression in ovarian cancer? This problem requires more researches to explain and is also our interest which will be explored in our future work.

Conclusions
In this study, we found that WTAP functions as an oncogenic factor promoting the progression of ovarian cancer. Meanwhile, upregulating the expression levels of HBS1L and FAM76A may well be involved in the underlying mechanism of WTAP. It may be a promising prognostic biomarker and therapeutic target for ovarian cancer.

Abbreviations
WGCAN: weighted gene co-expression network analysis; 3’UTR: the 3’-untranslated region; m6A: N6-methyladenosine; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; OS: overall survival; PFS: Progression-free survival; ME: module eigengene; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: Gene-set enrichment analysis; CPTAC: Clinical Proteomic Tumor Analysis Consortium; MM: module membership; GS: gene significance; AREs: adenosine and uridine rich elements.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Availability of data and materials
The datasets used during the current study were available from TCGA, GEO, Kaplan-Meier plotter, UALCAN that provide free online tools or open resources.

Competing interests
The authors declare that they have no competing interests.

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Authors’ contributions
YK designed and supervised the study. JW and JX collected and analyzed the data. JW, JX and YH drafted the first version of the manuscript. YK and KL reviewed and revised the manuscript. KH and YD edited the pictures and tables of the article. All authors read and approved the final manuscript.

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Tables
Table1
| Gene     | HR    | HR.95L   | HR.95H   | pValue     |
|----------|-------|----------|----------|------------|
| AGO2     | 2.025000718 | 1.374608716 | 2.98312375 | 0.000357437 |
| UNC93B1  | 1.502221016  | 1.184893867  | 1.904531742 | 0.000775895 |
| FAM76A   | 1.909579712  | 1.304432604  | 2.795464223 | 0.000878707 |
| ADAM15   | 2.82313392   | 1.49392582   | 5.335112594 | 0.001393395 |
| CLDN3    | 1.422241764  | 1.144704285  | 1.767069157 | 0.001472124 |
| HBS1L    | 2.06855213   | 1.302189037  | 3.285934524 | 0.002082551 |
| UBE2M    | 1.978033509  | 1.273960875  | 3.071221918 | 0.002376843 |
| PSENEN   | 1.513470421  | 1.152289873  | 1.987861536 | 0.002892504 |
| CLN6     | 2.184416433  | 1.29885511   | 3.673754769 | 0.003221297 |
| HES4     | 1.454077184  | 1.122018693  | 1.884407516 | 0.004649206 |
| MFSD14C  | 2.419201169  | 1.301407526  | 4.497080415 | 0.005225539 |
| NRBP1    | 1.804336165  | 1.176882937  | 2.766315064 | 0.006789802 |
| AGPAT2   | 1.781321775  | 1.16794261   | 2.71634921  | 0.007344708 |
| MARK2    | 1.619059052  | 1.137150146  | 2.305194459 | 0.007519206 |
| EXPH5    | 1.639797715  | 1.140602095  | 2.357471161 | 0.007579483 |
| OAZ2     | 2.090922206  | 1.212291813  | 3.606355851 | 0.007997563 |
| NBEAL2   | 1.838287134  | 1.166020595  | 2.898147426 | 0.008760691 |
| MPDU1    | 1.765161875  | 1.152584527  | 2.703312748 | 0.008976294 |
| FASN     | 1.581298787  | 1.121306047  | 2.229994088 | 0.008981118 |
| GNB2     | 1.559006501  | 1.115853534  | 2.178154388 | 0.009257287 |

Prognostic genes in the red module. P < 0.01 was considered statistically significant.

Table 2

| Gene     | WTAP low-expression group | WTAP high-expression group | logFC     | pValue     |
|----------|---------------------------|---------------------------|-----------|------------|
| FAM76A   | 8.516567684               | 9.489020421               | 0.159987043 | 2.30E-05  |
| HBS1L    | 8.787158947               | 9.413139105               | 0.099279124 | 0.00222178 |
| MPDU1    | 8.993703316               | 9.516729947               | 0.08155064  | 0.004175536 |
| UBE2M    | 9.348555211               | 9.949933684               | 0.089943492  | 0.004618308 |
| PSENEN   | 9.207348579               | 10.10675216               | 0.134461785  | 0.005629317 |
| NRBP1    | 8.574702737               | 9.044152947               | 0.076898732  | 0.005629317 |
Significant genes among prognostic genes in the red module when comparing expression difference in WTAP low-expression group and high-expression group. P < 0.01 was considered statistically significant.

Figures

A Survival prognosis forest map of univariate Cox analysis in GSE63885. Each point in the forest plot represents the HR of the gene, and the line on both sides of the point represents the 95% confidence interval (95% CI) BD OS and PFS curves constructed by the online Kaplan-Meier plotter for ovarian cancer based on the low and high expression of WTAP. Log-rank P < 0.05 was considered statistically significant.

B Protein expression of WTAP across cancers (with tumor and normal samples)

C D Protein expression of WTAP in different tumors and corresponding normal tissues obtained through CPTAC analysis in UALCAN.
A Clustering of 75 ovarian cancer samples and corresponding clinical information where the numeric information is larger, the darker color is shown. B Cluster dendrogram in which branches correspond to gene modules based on topological overlaps and each piece of the leaves represents a gene. C The scale-free index and the average connectivity calculated under different $\beta$. The approximate scale-free topology can be achieved at a soft threshold power of 3. D Histogram of connectivity distribution and checking the scale-free topology when $\beta = 3$. 

Figure 2
A Heatmap of the correlation between MEs and clinical traits. The numbers in brackets represent the P-values, and the numbers without the brackets indicate the correlation. BC Correlation between MM and GS for OS or WTAP expression. The former indicates the correlation between genes and MEs, and the latter indicates correlation between genes and clinical trait. DE GO and KEGG enrichment analysis of 133 selected genes. P < 0.05 was considered statistically significant.
Figure 3

AC Difference of FAM76A and HBS1L expression between WTAP high-expression and low-expression expression groups. Wilcoxon P < 0.05 was considered statistically significant. BD OS curves of FAM76A and HBS1L in GSE63885. EG Difference of FAM76A and HBS1L expression between WTAP high-expression and low-expression groups in TCGA. FH PFS curves of FAM76A and HBS1L from the online Kaplan-Meier plotter for ovarian cancer.
Figure 5

A Pathways highly enriched in HBS1L high-expression group through GSEA. B Pathways profoundly enriched in FAM76A high-expression group through GSEA.