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Gene Signatures and Prognostic Values of m6A RNA Methylation Regulators in Ovarian Cancer

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Abstract

Background: N6-methyladenosine (m6A) is the most common form of mRNA modification under the field of "RNA epigenetics." However, its role in ovarian cancer (OC) development is poorly understood. In the current study, we aimed to identify gene signatures and prognostic values of m6A RNA methylation regulators.

Method: Specifically, we downloaded Mutations and Copy number variant (CNV) data from the TCGA database for 579 OC patients, then analyzed gene expression and prognosis value using integrative bioinformatics. Thereafter, we verified the related biological processes of Wilms' tumor 1-associating protein (WTAP) gene using Gene set enrichment analysis (GSEA).

Results: Results showed that almost all ovarian cancer patients (99.31%) have CNVs with at least 1 m6A regulatory gene, whereas 83.76% of cases exhibited concurrence of CNVs in more than 4 m6A regulatory genes. Additionally, alteration of m6A regulators was associated with historical grade, whereas integrative bioinformatics and Cox multivariate model analysis revealed a significant correlation between high WTAP expression and worse ovarian cancer outcomes. Moreover, GSEA revealed that high WTAP expression was associated with cell cycle regulation and MYC targets.

Conclusion: Overall, our findings demonstrate the significance of high-frequency genetic alterations of m6A RNA methylation regulators and WTAP's poor prognosis value in OC. These findings provide valuable insights into the role of m6A methylation in OC, and will be vital in guiding development of novel treatment therapies.

Keywords

bioinformatics, ovarian cancer, biomarker, N6-methyladenosine, PROGNOSIS

Introduction

Ovarian cancer (OC) is the leading cause of cancer-related deaths among all gynecological tumors.[1] A large number of OC patients are diagnosed at an advanced stage, owing to lack of biomarkers for early clinical screening, as well as occurrence of relatively non-specific symptoms. Despite advances in modern management therapies, such as cytoreductive surgery and adjuvant chemotherapy, the 5-year overall survival for more than 70% of OC patients remains less than 30%.[2] Therefore, there is an urgent need to identify novel factors that regulate tumorigenesis and new biomarkers for early diagnosis or prognosis.

Dysregulated gene expression is one of the hallmarks of cancer. N6-methyladenosine (m6A), one of the most dominant drivers of messenger RNA (mRNA) modification, provides a novel layer of post-transcriptional gene regulation.[3,4] Previous studies have demonstrated its importance in a variety of crucial biological functions, including embryogenesis, proliferation, and differentiation of stem cells, DNA damage response, integrity of the nervous system, and adaptive stress responses.[5-9] Generally, m6A regulators comprise 3 classes of components, including methylases ("writers"), demethylases ("erasers"),

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and m6A-binding proteins ("readers"),[3,10] each playing different function. For example, the methylases complex is composed of Wilms' tumor 1-associating protein (WTAP), methyltransferase-like 3 (METTL3), and methyltransferase-like 14 (METTL14), with METTL3 reported to be the key methyltransferase responsible for m6A modification. Obesity-associated protein (FTO) and AlkB homolog 5 (ALKBH5) belong to erasers, whereas YTH domain proteins (YTHDF1-3) and YTH domain-containing proteins (YTHDC1-2) constitute the readers. Recently, m6A regulators were implicated in formation and progression of specific malignant tumors, including hepatic malignant neoplasm,[11-13] glioblastoma,[14,15] acute myeloid leukemia (AML),[16] breast cancer,[17,18] lung cancer,[19] and gastric cancer.[20] However, the roles of m6A methylation in tumorigenesis and prognosis of OC remain unclear. Based on these studies, we sought to explore the gene signatures and prognostic values associated with m6A regulators in OC.

Materials and Methods

Ethics Statement

The clinicopathological information, copy number variants, mutations, gene expression microarray documents, and prognostic data were downloaded from The Cancer Genome Atlas (TCGA) project using the cBioportal website,[21] Oncomine database[22] and KM plotter database.[23] All of these are open-access public databases. In addition, all enrolled participants provided written informed consent.

TCGA Database Analysis

We enrolled a total of 579 OC patients, with Mutations and Copy number variant (CNV) data, from the Ovarian Serous Cystadenocarcinoma (TCGA, Firehose Legacy) cohort dataset (http://www.cbioportal.org).[21] Focus was given to 9 m6A regulatory genes in cbioportal, including WTAP, METTL3, METTL14, FTO, ALKBH1, ALKBH5, YTHDF1, YTHDF2 and YTHDF3. Moreover, we included the TP53 gene, which plays a critical role in the progress of OC and has an extremely high mutation rate in OC, to serve as a reference. To explore the clinical pathological and molecular parameters of diverse CNV patterns, we divided the OC cases into 2 subgroups: "With low coexisting numbers of m6A related CNV genes ($\leq$4)" and "with high coexisting numbers of m6A related CNV genes (>4)". We extracted and normalized RNA-Seq data using RSEM (RNA-Seq by Expectation-Maximization).

Kaplan-Meier Plotter Analysis

We then employed the Kaplan-Meier plotter online database (http://kmplot.com/analysis/),[23] basing on the Gene Expression Omnibus (GEO), the European Genotype Archive (EGA) and TCGA databases for analysis of the relationship between m6A regulatory genes and prognosis in OC. During the analysis, we calculated the hazard ratio (HR), 95% confidence intervals (95%CI), and *p*-value.

Oncomine Database Analysis.

To explore the expression patterns of the WTAP gene in OC, we employed the online tumor microarray database, Oncomine (https://www.oncomine.org/resource/login.html).[22] Specifically, this database was searched using the keyword: "WTAP," cancer type: "ovarian cancer," and analysis type: "cancer vs. normal analysis." Thereafter, we performed statistical comparisons using a 2-tailed Student's *t*-test, with data followed by $P < 0.05$ regarded statistically significant.

Gene Set Enrichment Analysis (GSEA) of RNA-Seq Data

We stratified OC cases into 4 quartiles, according to patterns of WTAP expression, from the highest (fourth quartile) to the lowest (first quartile). Then, we analyzed the enriched gene sets between the lowest and highest quartiles of WTAP expression using GSEA v3.0,[24] alongside MsigDB gene set (h.all.v6.0.symbols.gmt) as reference.[25] To determine significant enrichment, we assumed a false discovery rate (FDR) of less than 0.25 and a *p*-value less than 0.05.

Statistical Analyses

Statistical analyses were performed using SPSS 19.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 7.0 (GraphPad Software Inc, San Diego, CA, USA) software. Specifically, correlation between the clinical-pathological parameters and diverse CNV patterns of m6A regulatory genes was analyzed using the chi-square test, and Kaplan-Meier curves generated to estimate prognosis. Data followed by $P < 0.05$ was regarded statistically significant.

Results

Mutations and Copy Number Variants of m6A Regulatory Genes in OC

To elucidate the roles of 10 candidate m6A regulatory genes in OC development, we first analyzed genetic alterations of these genes using the cBioPortal TCGA dataset. Interestingly, we detected mutation events in m6A-related genes in 5 cases (TCGA-13-0920-01, TCGA-04-1342-01, TCGA-24-2271-01, TCGA-10-0938-01, TCGA-23-1117-01) among the 316 OC patients with mutation data (Supplementary Information 1). Notably, the mutation rate in the TP53 gene was relatively high in this cohort (87.66%, 277/316), which was consistent with a previous study.[26] Additionally, among all subjects with CNV data, almost all OC patients recorded CNVs with at least 1 m6A regulatory gene (575/579, 99.31%), whereas 83.76% of the cases resulted in concurrence of CNVs in more than 4 m6A regulatory genes (Figure 1A). Furthermore, the highest frequency of CNV events was recorded in the m6A "eraser" gene ALKBH5 (88.26%, 511/579), followed by the m6A "writer" gene WTAP (76.86%, 445/579) (Figure 1B and Table 1). Furthermore, analysis of CNV patterns in OC patients revealed a higher loss (2331/3398) than gain (1067/3398) of

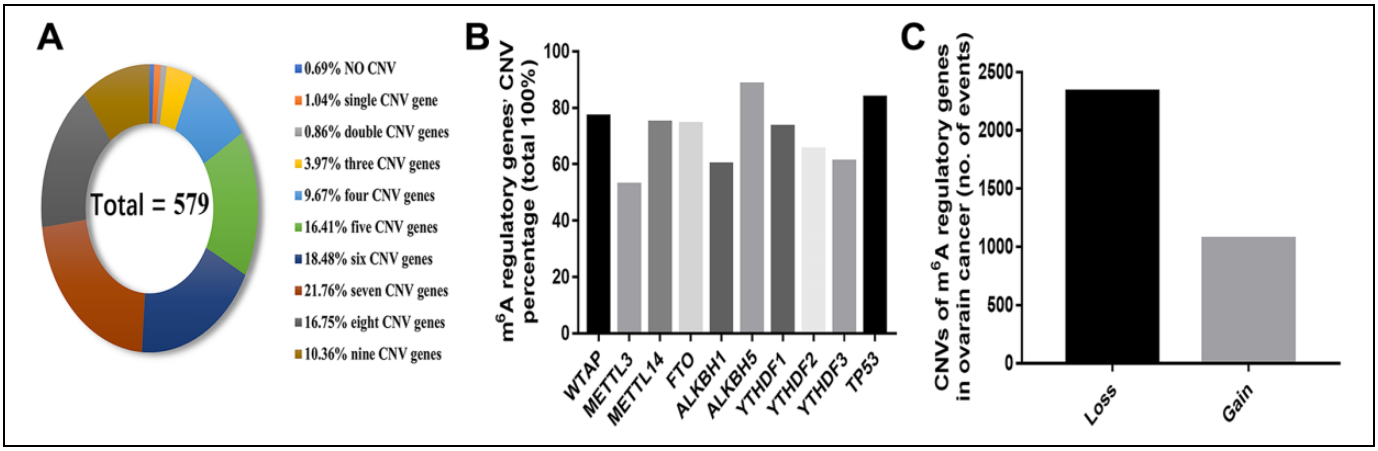

Figure 1. CNVs of m6A regulatory genes in ovarian cancer. (A) Concurrence of CNVs in specific number m6A regulatory genes in ovarian cancer samples. (B) Frequency of ovarian cancer samples with CNVs for m6A For Peer Review regulators based on data from TCGA. (C) Events of copy number gain or loss of m6A regulatory genes in ovarian cancer samples. CNV, copy number variant. m6A, N6-methyladenosine.

Table 1. Patterns of CNV Occurrence in 579 Ovarian Cancer Patients.

		Diploid	Deep deletion	Shallow deletion	Copy number gain	Amplification	CNV sum	Percentage
Erasers	FTO	149	6	378	44	2	430	74.27%
	ALKBH1	233	0	245	88	13	346	59.76%
	ALKBH5	68	8	484	19	0	511	88.26%
Writers	METTL3	274	2	166	114	23	305	52.68%
	METTL14	146	6	387	32	8	433	74.78%
	WTAP	134	14	359	67	5	445	76.86%
Readers	YTHDF1	155	0	28	315	81	424	73.23%
	YTHDF2	201	2	220	145	11	378	65.28%
	YTHDF3	227	1	64	243	44	352	60.79%
Others	TP53	95	2	424	50	8	484	83.59%

CNV, Copy Number Variant.

Table 2. Clinical Pathological and Molecular Parameters of Ovarian Cancer Patients With Different Numbers of m6A Related CNV Genes.

		With CNV genes (≤4)	With CNV genes (>4)	*P*
Age	≤60	59	255	
	>60	34	220	0.088
Stage	I + II	8	39	
	III	68	365	
	IV	17	67	0.588
	NA	0	4	
Historical Grade	G1 + G2	20	53	
	G3 + G4	71	409	0.010
	GX	1	9	
	GB	1	1	
	NA	0	3	
Primary Tumor Site	Bilateral	25	123	
	Left or right	64	323	0.898
	NA	4	29	
TP53	No alteration	10	28	
	Alteration	34	239	0.043
	Not profiled	49	208	

CNV, Copy Number Variant. M6A, N6-Methyladenosine.

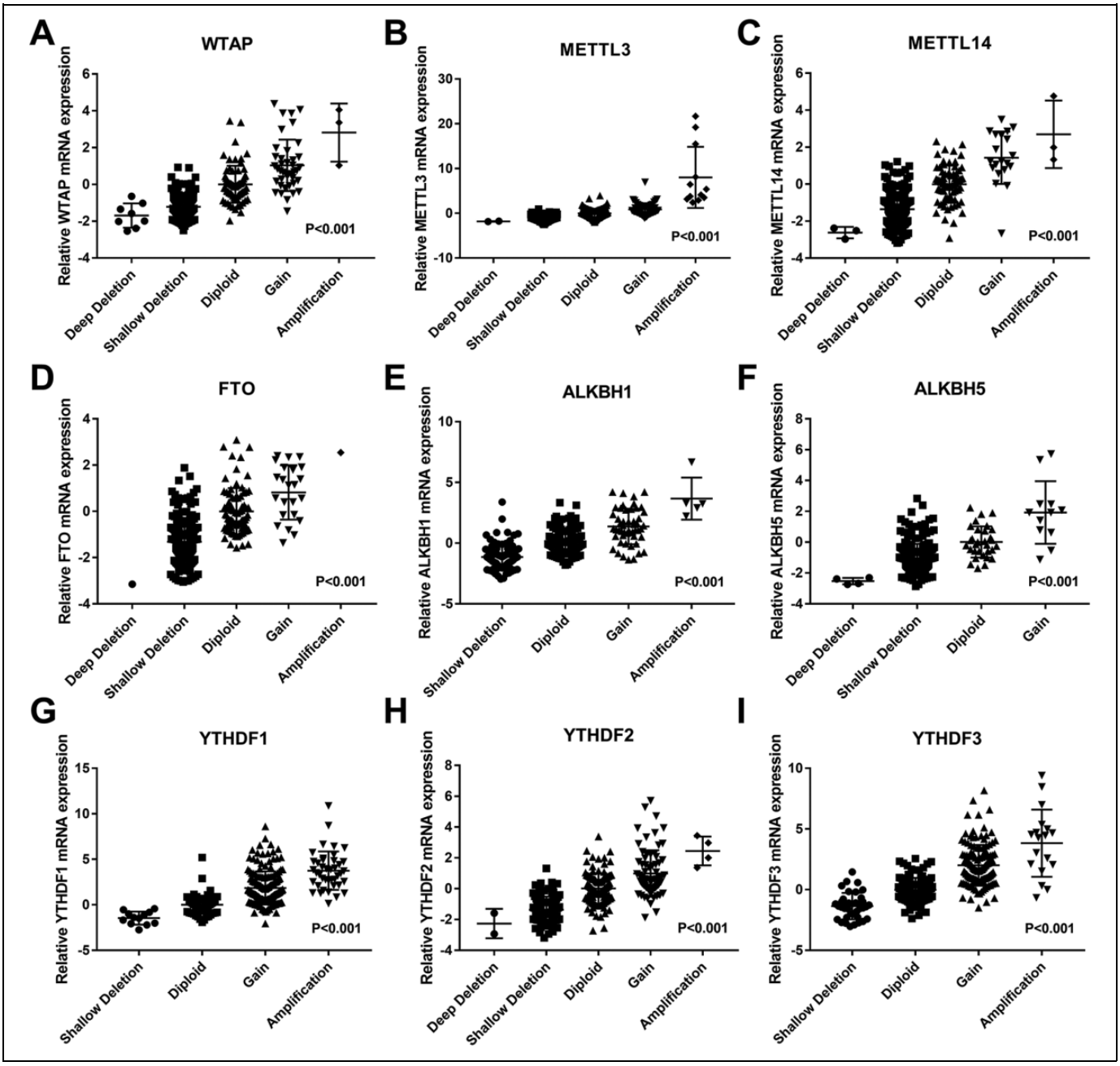

Figure 2. Relationship between diverse CNV patterns and mRNA expression for m6A regulatory genes in ovarian cancer. Copy number gains or amplification had a higher mRNA expression, but deep deletions or shallow deletions showed a lower mRNA expression in m6A regulatory genes (A-I). CNV, copy number variant. m6A, N6-methyladenosine.

copy number variations (Figure 1C and Table 1), which was similar to the CNV status recorded in AML and kidney malignancy.[27,28]

The Relationship Between CNVs of m6A Regulatory Genes and Clinicopathological Parameters in OC

As previously mentioned, OC patients had extremely high frequency CNVs of m6A regulatory genes, and CNVs concurrence in m6A regulatory genes was a common phenomenon. To assess CNVs' significance in OC, we explored the clinical pathological and molecular parameters in 2 groups of OC patients with different coexisting numbers of CNV genes ($\leq$4 m6A related CNV genes and >4 m6A related CNV genes). Results showed a significant association between more coexisting m6A numbers related CNV genes with higher histologic grade and TP53 alteration. However, other parameters revealed no statistical significance between the groups with regard to

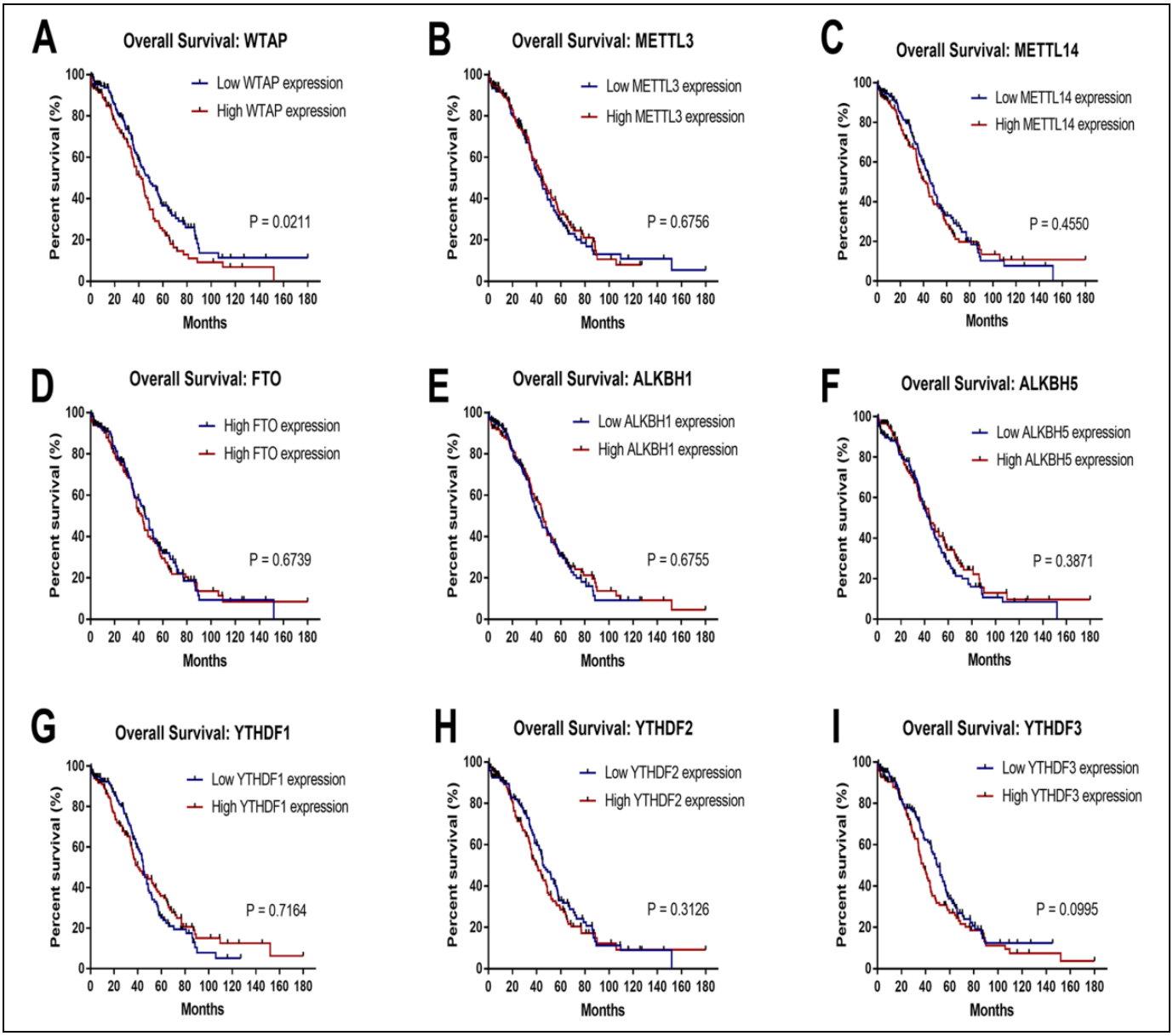

Figure 3. Relationship between different patterns of mRNA expression for m6A regulatory genes and prognosis in ovarian cancer. High mRNA expression levels of WTAP(A) was associated with worse OS, while METTL3(B), METTL14(C), FTO(D), ALKBH1(E), ALKBH5(F), YTHDF1(G), YTHDF2(H), and YTHDF3(I) had no significant effect on OS in the TCGA cohort. OS, overall survival. m6A, N6-methyladenosine.

age, stage, and primary tumor site (Table 2). We then explored CNVs' significance at the transcriptional level in 307 OC samples with RNA sequencing data, and found a significant positive correlation between mRNA expression and CNV expression patterns of m6A related genes. In all 9 genes, we found higher and lower mRNA expression levels in gain and loss of copy number variations, respectively (Figure 2).

Prognostic Values of m6A Regulatory Genes in OC

We investigated whether different CNVs patterns in m6A regulatory genes were correlated with prognosis of OC patients, and found no significant prognostic values (Supplementary Information 2). We then explored the relationship between profiles of mRNA expression across all 9 m6A regulatory genes and prognosis in OC patients using the TCGA database. Notably, high WTAP expression level was significantly correlated with the worst overall survival (OS) ($p = 0.021$), whereas expression patterns of the other m6A regulatory genes had no effect on OC (Figure 3). Furthermore, we used the online Kaplan-Meier plotter database to examine the prognostic value of m6A regulatory genes in OC, and found an association between high mRNA expression levels of WTAP, FTO, ALKBH1, ALKBH5, YTHDF1, YTHDF2, and

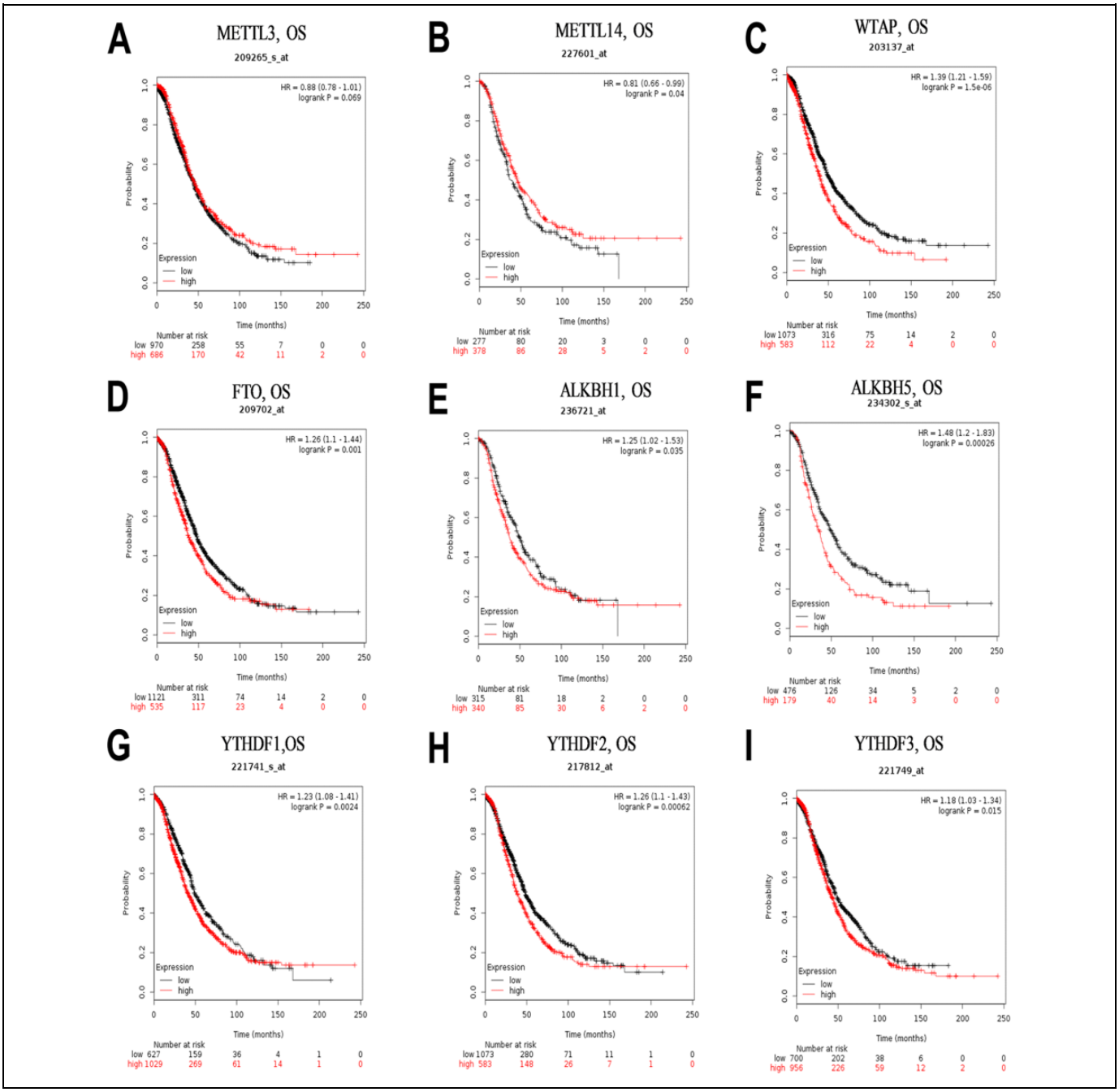

Figure 4. Kaplan–Meier survival curves for m6A related genes expression in ovarian cancer (A–I). High mRNA expression levels of WTAP (C), FTO (D), ALKBH1 (E), ALKBH5 (F), YTHDF1 (G), YTHDF2 (H), and YTHDF3 (I) and low mRNA expression levels of METTL14 (B) were associated with worse OS, whereas METTL3 (A) had no significant effect on OS in the KM cohort. OS, overall survival. m6A, N6-methyladenosine.

YTHDF3, as well as low levels of METTL14, with poor prognosis (Figure 4). Conversely, METTL3 was not associated with prognosis in ovarian cancer patients. Although the high and low levels of m6A regulatory gene expression demonstrated distinct prognostic value depending on the different databases, it was evident that WTAP is a potential prognostic factor for OC.

The Relationship Between Clinicopathological Characteristics and Gene Expression With Survival

We used the multivariate Cox proportional hazards model to explore the effect of m6A regulatory gene expression and clinicopathological characteristics on survival. Results showed that age ($p = 0.027$) and WTAP ($p = 0.024$) mRNA expression

Table 3. Effect of Expression Profiles of m6A Regulatory Genes and Clinicopathological Characteristics on Survival Based on Multivariate Cox Proportional Hazards Model.

Ovarian cancer (N = 231)	Variable	Coef	HR	95%CI_l	95%CI_u	*P*-value
	Age	0.364	1.439	1.042	1.988	0.027
	Race	-0.223	0.800	0.465	1.377	0.421
	Stage	0.335	1.397	0.632	3.090	0.409
	Grade	0.423	1.527	0.934	2.497	0.092
	WTAP	0.174	1.191	1.023	1.385	0.024
	METTL3	0.046	1.047	0.874	1.255	0.618
	METTL14	-0.001	0.999	0.851	1.173	0.995
	FTO	0.149	1.161	0.991	1.361	0.065
	ALKBH1	-0.091	0.913	0.770	1.082	0.293
	ALKBH5	-0.035	0.965	0.831	1.121	0.642
	YTHDF1	-0.011	0.989	0.837	1.170	0.900
	YTHDF2	0.056	1.058	0.898	1.246	0.504
	YTHDF3	0.045	1.046	0.900	1.215	0.561

M6A, N6-Methyladenosine. Coef, Regression Coefficient; HR, Hazard Ratio; 95%CI_l, 95% Confidence Interval Lower Limit; 95%CI_u, 95% Confidence Interval Upper Limit.

were independent prognostic factors (Table 3), whereas race, stage, grade, as well as expression of METTL3, METTL14, FTO, ALKBH1, ALKBH5, YTHDF1, YTHDF2, and YTHDF3 were not independent prognostic factors in OC.

Levels of WTAP Expression in OC

Screening of the Oncomine microarray database revealed significantly higher RNA levels of WTAP in OC tissues than healthy controls across 3 representative datasets (Figure 5A), including the Lu, Hendrix, and Bonome Ovarian dataset[29-31] (Figure 5B-5D).

Enrichment Analysis of the WTAP Gene

Considering WTAP's vital role in regulating a variety of crucial biological functions (especially during the m6A modification of mRNA), we determined the biological processes in which this factor is involved in OC. Firstly, we divided patterns of WTAP's RNA expression into 2 groups, then performed Gene set enrichment analysis (GSEA). Finally, we identified 4 significant high-scoring sets, including E2F_TARGETS, MYC_TARGETS_V2, MYC_TARGETS_V1, and G2M_CHECKPOINT (see Table 4 and Figure 6). These results provide new insights into the role of epigenetic regulation in OC.

Discussion

Generally, m6A writers, erasers, and readers might show diverse patterns across distinct malignancies or independent databases. Results from the present study have shown that a higher frequency of both CNVs in single m6A related genes as well as concurrent CNVs in 2 or more genes, relative to those previously reported in kidney malignancy[27] and AML.[28] To some extent, our findings suggest that regulation disorder of m6A might have a more significant impact on development and progression of ovarian cancer. Previous studies have demonstrated the synergistic roles played by multiple m6A related genes in formation of complexes.[5] In the present study, it was evident that WTAP, the m6A "writer" gene, was more predisposed to CNVs and had a significantly higher prognostic value than other m6A related genes in OC. However, other genes, such as METTL3, FTO, and ALKBH5, have been previously shown to be more crucial in kidney malignancy,[27] breast cancer,[18] glioblastoma,[15] and AML.[32] Analysis of the TCGA databases revealed that only high WTAP expression was significantly associated with more inferior OS. Additionally, results from the Kaplan-Meier plotter database also showed that high expression of WTAP, FTO, ALKBH1, ALKBH5, YTHDF1, YTHDF2, and YTHDF3, as well as low expression of METTL14, were all associated with poor OS. These differences might be attributed to the diverse methods of data detection and analysis as well as different mechanisms of inherent biological characteristics. Despite the inconsistency, we still found a consistent prognostic correlation between WTAP mRNA expression and OC, based on an integrative bioinformatics analysis. The resulting diverse expression patterns of m6A regulatory genes across different tumor types or in independent databases affirmed the complexity of the m6A's post-transcriptional regulation mechanism, and suggested presence of tumor specificity of the m6A regulators.

WTAP, located in the nucleus, was first identified as a partner of Wilms' tumor 1 protein.[33] It was then mapped to human chromosome 6q25-27, and an allele loss detected in all histological OC types.[34] Interestingly, our research also found that a shallow deletion in the WTAP gene was the high-frequency pattern of CNVs. Previous studies have described WTAP in the context of cell proliferation and apoptosis of vascular smooth muscle cells before.[35-37] Recently, the gene was found to play a crucial role in the tumorigenesis of various malignancies, such as AML, cholangiocarcinoma, endometrial cancer, and

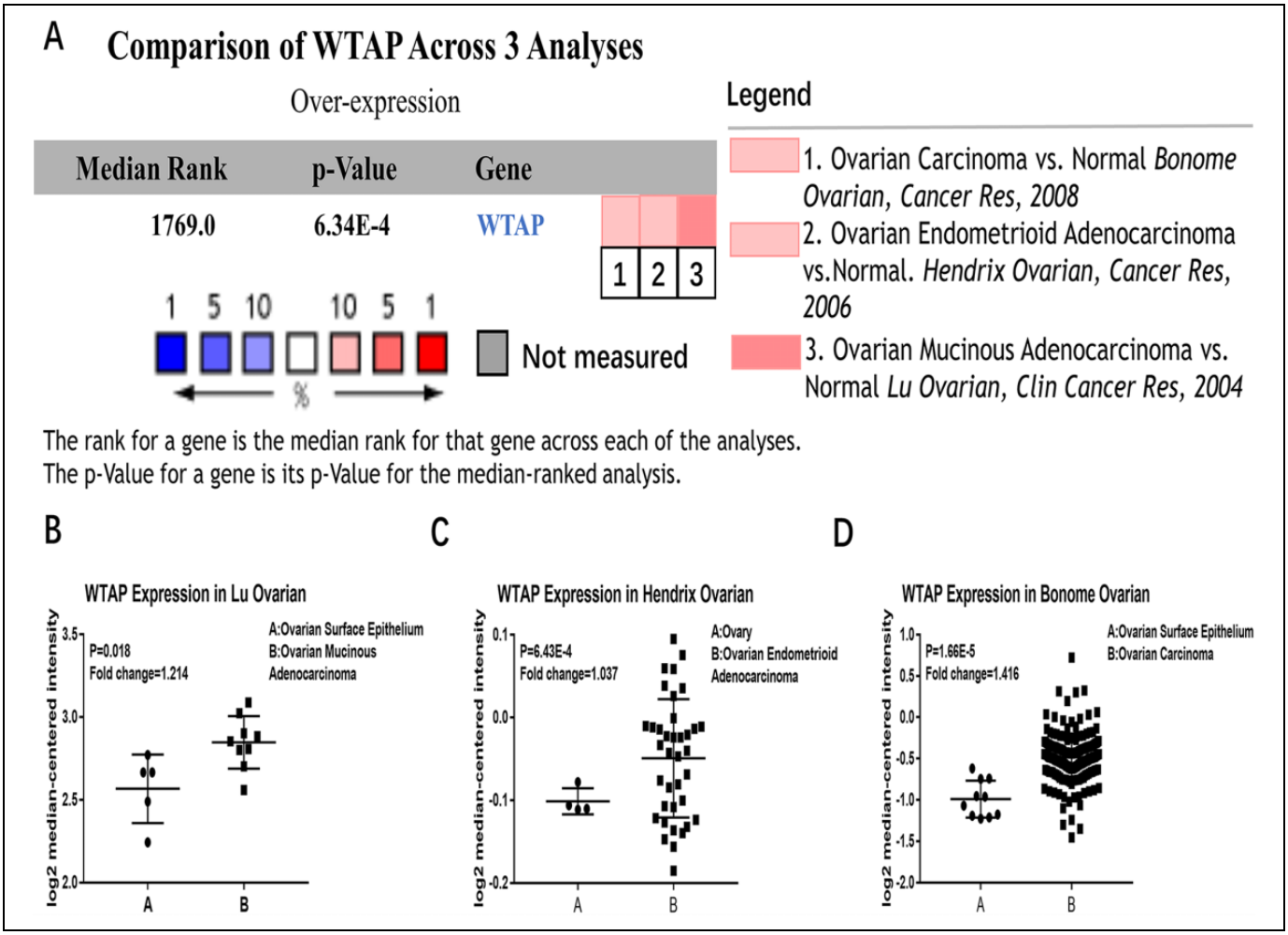

Figure 5. Expression of WTAP in the Oncomine database. (A) Comparisons of the expression of WTAP in OC in 3 independent analyses from the Oncomine database. Validation of WTAP expression in ovarian cancer in the Lu Ovarian dataset (B), Hendrix Ovarian dataset (C), and Bonome Ovarian dataset (D). (*P < 0.05; **P < 0.01; ***P < 0.001).

Table 4. Four High-Scoring Sets are Identified by GSEA Analysis.

GS DETAILS	SIZE	ES	NES	NOM *p*-val	FDR *q*-val
HALLMARK_E2F_TARGETS	162	0.61	1.83	0.018	0.082
HALLMARK_MYC_TARGETS_V2	50	0.62	1.81	0.006	0.043
HALLMARK_MYC_TARGETS_V1	165	0.55	1.78	0.016	0.039
HALLMARK_G2M_CHECKPOINT	158	0.55	1.73	0.032	0.044

GSEA, Gene Set Enrichment Analysis.

glioblastoma.[38-41] Besides, accumulating evidence suggests that WTAP is a new constituent of the human m6A multiprotein writer complex, and plays a role in facilitating recruitment of the m6A complex to a target site.[42] In fact, some studies have demonstrated that the WTAP complex plays a crucial role in cell cycle regulation, including G2/M transition, by stabilizing cyclin A2 mRNA[43] and CDK2 mRNA,[44,45] which is consistent with our hypothesis. Wilms tumor 1 gene, a partner of WTAP, has also been suggested to be an oncogene that induces expression of MYC.[46,47] Besides, previous studies have suggested that some key transcription factors, such as FOXO1 and IRF1, might be involved in regulation of WTAP promoter, and are also be closely related to tumorigenesis, tumor growth, and invasion.[48] WTAP has also been found to be highly expressed in ovarian cancer[49,50] and associated with worse survival outcomes in high-grade serous ovarian carcinoma.[49] However, this evidence is still insufficient, while the underlying molecular mechanism of WTAP in OC remains unknown.

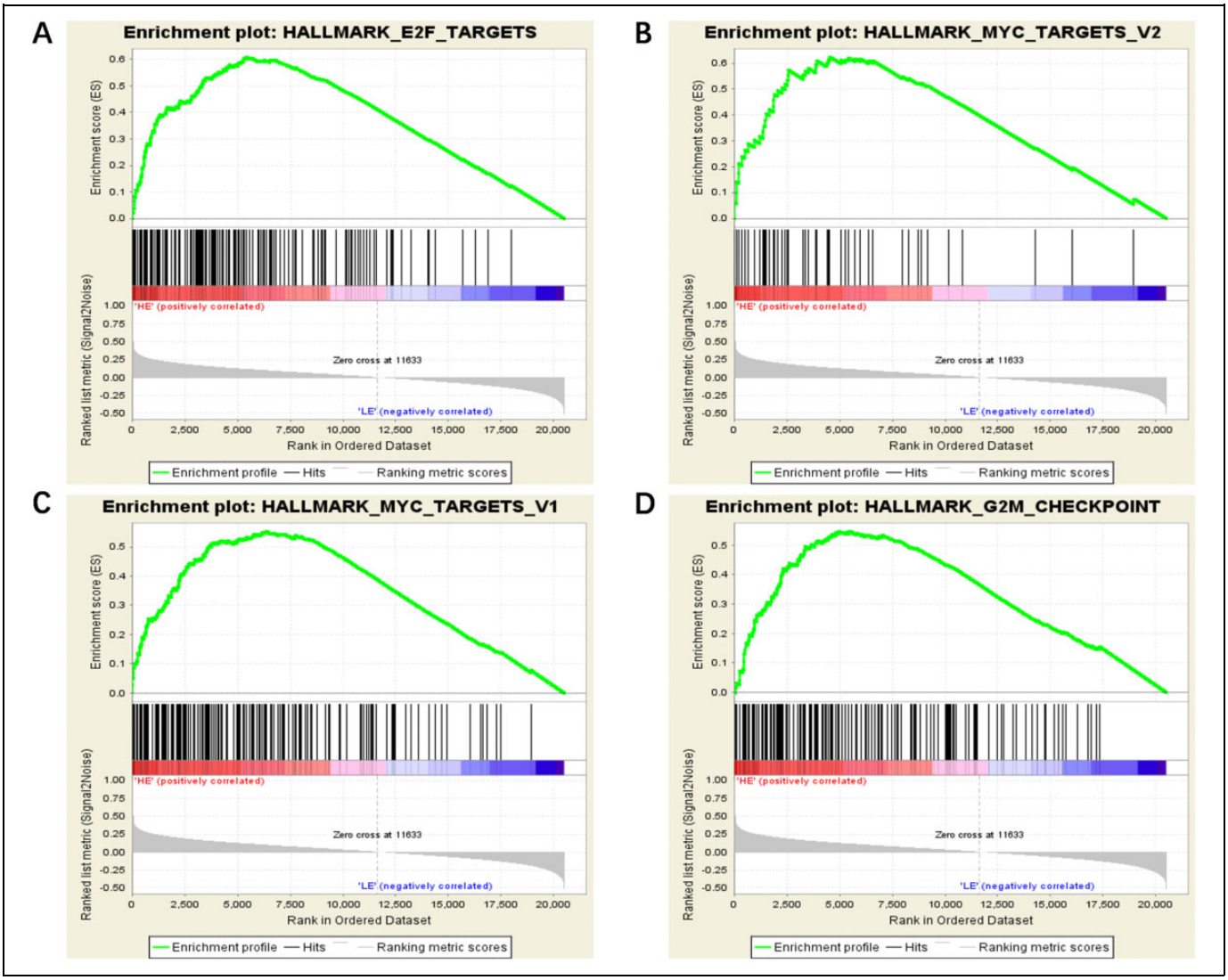

Figure 6. GSEA results for WTAP in OC patients. Four high-scoring sets, including E2F TARGETS (A), MYC TARGETS V2 (B), MYC TARGETS V1(C), and G2M CHECKPOINT (D), are identified by GSEA analysis. GSEA, Gene set enrichment analysis.

Our study had some limitations. Firstly, this was a retrospective analysis, based on publicly available popular datasets. Therefore, the number of included patients was limited, and the results may not be as reliable as those from prospective studies. Secondly, more *in vivo* and *in vitro* experiments are required for functional and clinical validation.

In some ways, the function of m6A RNA methylation regulators in tumorigenesis may be confusing. For instance, WTAP is reportedly an overexpressed oncogene, with its high expression strongly correlated with poor survival in bladder cancer,[51] renal cell carcinoma,[44] pancreatic ductal adenocarcinoma,[52] and Malignant Glioma,[53] which corroborates our findings from OC. Conversely, we found that shallow deletion was WTAP's main CNV pattern, with loss of copy number events significantly associated with lower WTAP mRNA expression. This contradiction is puzzling and may be attributed to the distinctively dynamic process of m6A regulation or the heterogeneity of the tumor. This affirms WTAP's function as a multi-dimensional oncogene.[54]

Conclusion

Overall our findings demonstrate the significance of high-frequency genetic alterations of m6A RNA methylation regulators and WTAP's poor prognosis value in OC. These findings provide new insights into the role of m6A methylation in OC, and will be vital in guiding development of novel treatment therapies.

Authors' Note

All patient-related raw data was downloaded from open-access public databases and it is confirmed that all participants involved have gave written informed consent.

Declaration of Conflicting Interests

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Supplemental Material

Supplemental material for this article is available online.

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