M 6A-related genes characterization in hepatocellular cancer identifies prognostic relevant gene signatures

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Research

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Abstract

Background

Hepatocellular carcinoma (HCC) is among the most common types of cancers that threat the public health worldwide. N6-methyladenosine (m6A) RNA methylation, associated with cancer initiation and progression, is dynamically regulated by m6A RNA methylation associated genes. However, little is known about the expression status and the prognostic value of m6A associated genes in HCC. This study aimed to identify the expression profiling pattern and clinical significance of m6A-related genes in HCC.

Methods

The Cancer Genome Atlas (TCGA-LIHC), the Gene Expression Omnibus (GSE14520) and the Human Protein Atlas (HPA) databases were gathered for this study. Consensus clustering analysis was performed to identify the clusters of HCC with different clinical outcome. A prognostic signature built by the least absolute shrinkage and selection operator (LASSO) Cox regression model was utilized to discover subtypes correlated with different clinical outcomes of HCC patients and the differences between subgroups were characterized in terms of epigenetic dysregulation and somatic mutation frequencies.

Results

Most of the m6A-related genes were upregulated and involved with the prognosis and malignancy of HCC. A four-gene prognostic signature revealed two HCC subtypes (namely, risk-high group and risk-low group) that correlated with different clinical outcomes. Patients in risk-high group were accompanied with much more epigenetic silencing and significant mutation at TP53 and FLG, while ALB were mutated frequently for risk-low group.

Conclusion

Our characterization tightly links the expression of m6A genes with clinical outcomes of HCC, providing valuable molecular-level information that points to decoding heterogeneity, guiding personalized management and treatment of HCC patients.

Background

According to the International Agency for Research on Cancer, hepatocellular carcinoma (HCC) ranks the second leading causes of tumor-related death worldwide[1]. HCC develops in patients with chronic hepatitis such as viral hepatitis[2]. Various treatments for HCC, including resection, transplantation, and interventional therapy, have witnessed immense progress over the recent decades, but prognosis of HCC are still poor in patients with late-stage [3]. To make it worse, particular high rate of postsurgical recurrence and metastasis (50-70% at 5 years) produces a major challenge as this disease is highly refractory to conventional chemotherapy and radiation[4]. Currently, the Barcelona Clinic Liver Cancer

(BCLC) staging classification is still the most extensively used classification systems for HCC which can be applied for the assessment of prognosis and selection of best therapies [5]. However, some study has reported that the HCC patients who have the same BCLC stage may include various tumor types such as nodular or infiltrating tumors, thus resulting in differences in treatment responses and survival [6]. Therefore, to identify novel and reliable prognostic molecular signatures is critical in HCC basic and clinical research.

N6-methyladenosine (m6A), methylated at the N6 position of adenosine, is the most abundant epigenetic and evolutionarily conserved modification of mRNAs and noncoding RNAs in mammalian[7-10].Approximately 0.1–0.4% of adenosines in total RNA are modified by m6A methylation[11]. M6A methylation affects almost every aspect of RNA metabolism including, but not limited to, abundance, alternative splicing, stability, nuclear export, decay and translation[12-14], thus, negatively regulating protein expression in a post-translational manner. Identification of m6A adenosine methyltransferases ("writers"), demethylases ("erasers") and binding proteins ("readers")[15] revealed that m6A modification is reversible. Emerging evidence has indicated that the m6A modification may be involved in various physiological processes and diseases, including circadian rhythms, stem cell differentiation and maternal-to-zygotic transition (MZT)[16], and especially the carcinogenesis of several tumors, including cervical cancer[17], prostate cancer[18], breast cancer[19], pancreatic cancer[20], and hepatocellular carcinoma[21]. The characterization for m6A sparked a renewed interest in this particular RNA modification. However, the expression pattern as well as the prognosis value have not been fully elucidated in HCC.

In this study, we estimated the m6A patterns based on the twenty widely reported m6A RNA regulators and systematically characterized for the potential subtypes in a multiomics view including somatic mutation and DNA methylation. Consensus clustering analysis identified three clusters of HCC with different clinical outcome. After Cox univariate analysis and least absolute shrinkage and selection operator (LASSO) Cox regression analysis, a four-gene risk signature was built and showed good performance for predicting prognosis. More importantly, the two identified subtypes identified by the risk signature exhibited distinct DNA methylation profiling and somatic mutation spectrum.

Methods

Public data source

The TCGA-LIHC cohort data, including RNA-sequencing, mutation and clinical data, were downloaded from The National Cancer Institute Genomic Data Commons (NCI-GDC). Maftools was utilized to infer significant cancer mutated genes with default parameters. Illumina Human Methylation 450 Beadchip (450K array) was used to measure the DNA methylation data. For a gene with more than one probe mapping to its promoter, the median β value was considered. MethylMix was designed to identify gene expression that were correlated with methylation events. GSE14520 including a total of 445 samples (Affymetrix HT Human Genome U133A Array), were utilized as the validating cohort. In addition,

validation of the translation of m6A-related genes was performed using the Human Protein Atlas database.

PPI network construction and correlation analysis

The protein-protein interaction (PPI) among m6A RNA methylation regulators was analyzed using the STRING database. Spearman correlation analysis was employed to reveal the association among different m6A RNA methylation regulators.

Consensus clustering analysis

To identify TME patterns and classify patients for further analysis, we grouped HCC patients in TCGA cohort using the ConsensusClusterPlus package which was repeated 1000 times to ensure the stability of classification. Kaplan-Meier analysis[22] paired with log-rank test was carried out to compare patients' survival between clusters.

Screening of prognostic signatures and key prognostic genes

Univariate Cox proportional hazards regression were used to assess the independent m6A RNA methylation regulators whose expressions were significantly associated with patients'survival. Hazard ratios (HRs) were used to identify protective (HR < 1) or risky genes (HR > 1). Lasso-penalized Cox regression analysis performed by glmnet package was used to achieve variable shrinkage and selection of key independent m6A RNA methylation regulators[23]. An optimal model was determined basing on a linear combination of the expression profiles of independent prognostic m6A RNA methylation regulators weighted by the estimated regression coefficient derived from the lasso Cox regression model coefficients multiplied with its mRNA expression level. A risk score was constructed with the regression coefficients from this model. Then, we divided the TCGA HCC patients into high-risk group and low-risk group according to the optimal cut-off value of risk scores obtained from the survminer package. Cox regression analysis was used to evaluate the association between risk score and DFS/ OS, in which age, sex, TNM stage, grade was used as covariates.

Statistical analysis

Data analysis was performed with R version 3.6. All statistical tests were two-sided. $P < 0.05$ was considered statistically significant. Unless otherwise noted, continuous variables were compared with the Student's t-test, and the Kruskal–Wallis test was used for independent samples when the population could not be assumed to be normally distributed.

Results

The Landscape of m6A RNA methylation regulators in HCC

We analyzed the mRNA expression levels of the known m6A-related regulators including m6A “writers”, such as METTL3, METTL4, WTAP, ZC3H13, RBM15, RBM15B and VIRMA, m6A “readers”, such as YTHDF1, YTHDF2, YTHDF3, YTHDC1, YTHDC2, HNRNPC, HNRNPA2B1, IGF2BP1, IGF2BP2, IGF2BP3 and RBMX, m6A “erasers”, such as FTO and ALKBH5. Compared with normal liver tissue, HCC patients generally contain a higher proportion of m6A genes except ZC3H13 (Figure.1a, b). Furthermore, we validated the expression of these aberrant m6A-associated genes in HPA databases (Figure.s1).
An important detail to mention is that METTL3, WTAP, RBM15B, YTHDF1, YTHDF2, YTHDF3, HNRNPA2B1, IGF2BP1, IGF2BP2, IGF2BP3 and RBMX were absent from the HPA database. Taken together, these data confirmed the highly significant dysregulation of several m6A-related regulators in human HCC. We further systematically investigated the relationships between each individual m6A RNA methylation regulator and the pathological features of HCC, and identified that as the pathological grade and T-stage increased, the expression of HNRNPA2B1, METTL3, METTL4, RBMX, YTHDF1 and YTHDF2 increased (Figure.2, Figure.s2).

The expression characteristics of m6A RNA methylation regulators

We assessed the expression similarity of m6 regulators and clustering stability applying the ConsensusClusterPlus package, then divided the HCC patient cohort into three clusters, namely cluster 1, cluster 2 and cluster 3 (Figure.3a-c). Notably, survival analysis showed cluster 1 to be significantly associated with better DFS and cluster 2 (119 patients) to be associated with poorer DFS, cluster 3 was characterized by an intermediate prognosis (Figure.3d). A favorable prognostic trend for OS was also seen, through statistics was not significant, partly because of the limitation of the cohort size.

Moreover, the PPI network depicted a comprehensive landscape of m6A RNA methylation regulators interactions and the erasers including WTAP, VIRMA and METTL14 ranked first according to the degree of connectivity (Figure.4a). Figure.4b showed a majority of m6A RNA methylation regulators are positively correlated and the correlation between HNRNPC and RBMX is the most significant.

Prognostic value of m6A RNA methylation regulators, and Construction of the m6A signature

Next, we analyzed the possible prognostic power of m6A RNA methylation regulators in HCC by performing univariate Cox regression.. The results demonstrated that six out of twenty tested genes are significantly correlated with DFS /OS and increased expressions of HNRNPA2B1, IGF2BP3, METTL3, WTAP, YTHDF1, YTHDF2 have a poorer survival in patients with HCC.(Figure.5a). Applying LASSO analysis, in which the selected m6A RNA methylation regulators were required to appear 900 times out of 1000 repetitions, four m6A RNA methylation regulators, IGF2BP3, YTHDF1, YTHDF2 and METTL3 were selected. A risk score was generated using these genes weighted by the coefficients from the LASSO regression. To investigate the prognostic role of the four-gene risk signature, we assigned LIHC patients to groups based on high or low risk scores using the cut-off value obtained with the survminer package, and observed that high-risk group had a shorter OS/DFS than those in the low risk group (Figure.5b-c). Similarly, GEO HCC patients were divided into low- and high- risk groups and OS/DFS was significantly shorter in the high-risk group compared to that in the low-risk group (Figure.s3).

The role of the risk-score subtypes in TCGA HCC cohort

The heatmap (Figure.6a) shows the expression of the four selected m6A RNA methylation regulators and clinicopathological variables in the high- and low-risk groups. Similar to their association to prognosis, low-risk group was more frequently involved with lower T stage and pathological grade (Figure.6b-c). We performed univariate and multivariate Cox regression analyses to evaluate whether the prognostic signature-based risk score was an independent factor for prognosis. When the m6A signature was evaluated as a continuous variable with the Cox regression model, the univariate and multivariate analysis validated that the stage and risk score were significantly linked with DFS/OS (Figure.6d-e). These results suggest that the risk signature is a risk factor and can independently predict the prognosis of HCC patients.

Differential somatic mutation landscape and methylation-driven genes between the HCC risk score subtypes

To identify the correlations between distributions of somatic alterations and the HCC risk score subtypes, 526 genes were shared significant mutation in the risk-high and risk-low groups. Specifically, a missense mutation on TP53 was predominantly observed in high-risk group, suggested key mutations in HCC may involve with m6A modification (Figure.7).

The methylation-driven genes are genes with different degree of methylation and expression between different groups. After downloading and processing of methylation data, we screened 569 methylation-driven genes associated with risk score subtypes via using MethylMix R package (Table.1). Of these genes, 461 genes (81.02%) were hypomethylated and the remainder of the 108 genes (18.98%) were hypermethylated. These data may provide a new perspective to study the mechanism of m6A modulation.

Discussion

The occurrence and development of HCC is a multi-step complex process that involved with genetic or epigenetic factors[24, 25]. Therefore, elucidating the underlying molecular events accounting for the tumorigenesis, diagnosis and precise individual therapy of HCC remain the greatest challenges. Recent study has demonstrated that m6A affects the epigenetic regulation of RNA, including mRNA stability[26], alternative splicing[27], and microRNA biogenesis[28]. which in turn, regulates the expression of genes. Dysregulation of m6A genes not only involves with the pathogenesis of a variety of human disease including obesity, neuronal disorders and immunological disease, but also promotes the initiation, expansion and progression of malignancies, including HCC [29-31]. Recent studies on mRNA m6A modification have linked the methylation level of m6A by the intracellular writing and erasing genes, while the regulatory functions of methylation sites in biological processes is performed by the protein molecules that read gene expression[32]. Therefore, in tumors, both m6A-related genes and protein expression levels may become a potential diagnostic marker for tumor molecular diagnosis, and potential targets for the molecular targeted therapy.

In this study, we demonstrated that the expression of m6A regulators is intimately related to the prognosis and malignancy in a large cohort of HCC patients. We observe that 11 out of 20 m6A RNA methylation regulators were promoted in the HCC samples, and further confirmed that these genes may play an important tumorigenic role and/or involve in prognosis of HCC patients. The underlying molecular mechanisms are needed to define in the future. In addition, we validated that m6A RNA methylation regulators expression alone is sufficient to separate TCGA HCC cohort into three clusters with significant differences for DFS that broadly correspond to the expression pattern of the twenty m6A RNA methylation regulators. Inspired by the findings of clustering analysis, we are interested in whether the m6A RNA methylation regulators can serve as good biomarkers for patients' prognosis. After Cox univariate analysis and LASSO Cox regression analysis, we constructed a risk signature including IGF2BP3, YTHDF2, METTL3, and YTHDF1 based on TCGA HCC dataset and the risk score was an independent prognostic marker by multivariate analyses. In terms of validity and reliability, we found that our four gene signatures performed well even in external validation data set. Furthermore, the risk-high and risk-low HCC groups also presented different significantly mutated genes (SMGs), of which P53 being significantly mutated in risk-high HCC group. It is reported a correlation of P53 pathway and the expression of YTHDF2[33], suggesting risk-high HCC group with higher P53 mutation might have a high likelihood of responding to the activation of cancer pathways. Integrative analysis by mRNA expression and promoter CGI methylation manifested a significant broad spectrum of gene silencing in risk-high HCC group compared with that in risk-low HCC group.

Insulin-like growth factor II mRNA-binding protein 3 (IGF2BP3) which is reported an oncofetal protein expressed in human fetal tissues with a time-dependent manner[34]. Breakdown the expression of IGF2BP3 restrains pluripotency genes and tumorigenesis of tumor-initiating cells [35]. In addition, this is in agreement with the results of our papers which showed that HCC patients who have high expression of IGF2BP3 have high probability undergo advanced tumor stage and poor prognosis [36].

YTHDF1 is identified as an m6A modified mRNA binding protein which is amplified in various types of cancers including HCC [37-39]. It is also reported that RNA m6A modification regulates anti-tumor immunity response via YTHDF1 which regulates tumorigenicity and cancer stem cell-like activity in human cancer[40] [41]. Our study verified that YTHDF1 was significantly upregulated in HCC and Kaplan-Meier analysis showed that lower YTHDF1 expression level was associated with better survival of HCC patients.

YTHDF2 (YTH-Domain Family Member 2) is the first identified and well-studied functional m6A-binding protein that mainly regulates stability of mRNA[37], and acts as a tumor inhibiting factor in HCC and Its deficiency promotes HCC growth, vasculature remodeling and metastasis, the potentially mechanism was that YTHDF2 reprogram the epi-transcriptome with the background of hypoxia[42, 43].

Methyltransferase-like 3 (METTL3) was reported as an oncogenic M6A regulator[44]. Serval study suggest that METTL3 plays a significant role in cell fate determination including promoting HCC growth and invasion [43, 45, 46]. The identified pathway METTL3/RDM1/p53/ERK may assist us to illuminate

the potential relationship of TP53 mutation and the risk signature in our study[47]. Thus, it would make sense to explore the detailed mechanism upon METTL3 manipulation in future studies.

Conclusions

Collectively, our study highlights the dramatically dysregulated m6A RNA methylation regulators between HCC and normal controls, which might play a crucial role in the initiation and progression of HCC. More importantly, a robust four-gene prognostic signature that significantly associated with the DFS/OS of HCC was constructed and validated in an independent HCC cohort, suggesting that this prognostic signature might act as a promising biomarker for monitoring HCC development. Our study provides important evidence for further study of m6A RNA methylation regulators in HCC.

List Of Abbreviations

HCC：hepatocellular carcinoma; M**6A**： N6-methyladenosine; **TCGA**： The Cancer Genome Atlas ;**GEO**： The Gene Expression Omnibus ;**HPA** ：The Human Protein Atlas; **LASSO**： The least absolute shrinkage and selection operator ;**DFS**：Disease-free survival ;**OS**：overall survival ;**BCLC**： the Barcelona Clinic Liver Cancer ;**MZT**： maternal-to-zygotic transition ;**NCI-GDC**： The National Cancer Institute Genomic Data Commons ;**PPI**：protein-protein interaction ; **HRs**： Hazard ratios

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets generated during the current study are available in the National Cancer Institute Genomic Data Commons [NCI-GDC, https://gdc.cancer. gov/], GSE14520 Gene Expression Omnibus [GEO, https://www.ncbi.nlm.nih.gov/geo/] and the Human Protein Atlas database [HPA, http://www.proteinatlas.org/].

Competing Interests

The authors declare that they have no competing interests.

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Authors contributions

QNJ, ZG designed the study. NH, CZ performed data acquisition and collected the literature. PZ and QQR carried out analysis and interpretation, and drafting of the manuscript. QNJ revised the manuscript critically for important intellectual contents. All authors approved this version of the manuscript to be submitted.

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Table

Due to technical limitations, table 1 is only available as a download in the supplemental files section.

Supplemental Information

Figure.s1 Information on the IHC staining of m6A-related genes (ALKBH5, FTO, HNRNPC, METTL4, RBM15, VIRMA, YTHDC1, YTHDC2) in HCC from The Human Protein Atlas database

Figure.s2 Expression of m6A RNA methylation regulators in HCC with different clinicopathological features. The expression levels of HNRNPA2B1, HNRNPC, IGF2BP1, IGF2BP2, IGF2BP3 and METTL4, RBM15, RBM15B, RBMX, VIRMA and YTHDC1 in HCC with different pathological grades and the expression levels of HNRNPA2B1, METTL4, RBM15B, RBMX, YTHDF1 and YTHDF3 in HCC with different T-stages.

Figure.s3 Validation of the prognostic signature in an independent GSE14520 dataset. Kaplan–Meier OS/DFS curves for patients in the high- and low-risk groups based on the risk score

Figures

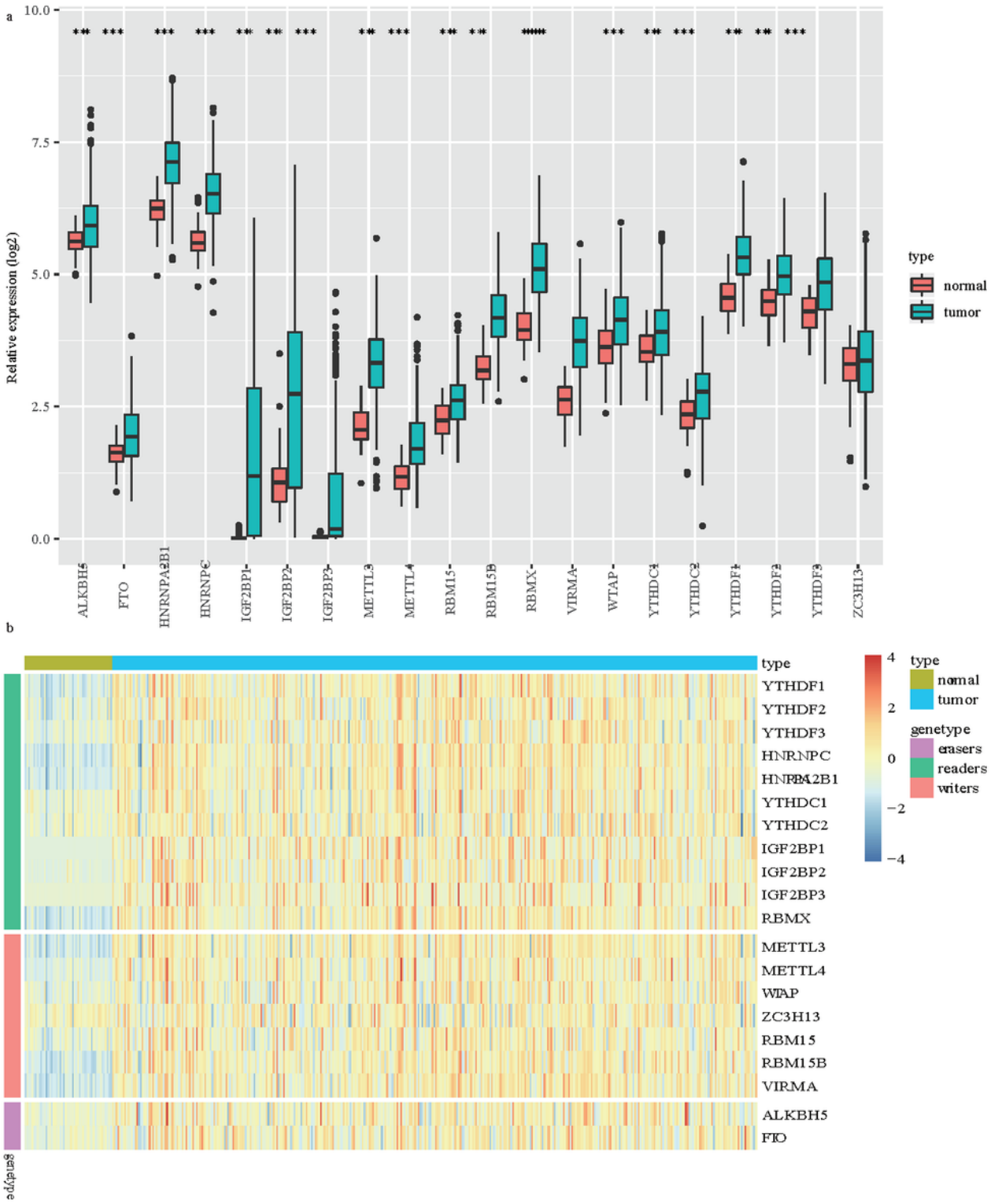

Figure 1

The landscape of m6A RNA methylation regulators in HCC. (a) Expression heatmap plotting of 20 m6A RNA methylated regulatory factors between tumor samples and normal control samples in TCGA-LIHC cohort. (b) The boxplot showed the median expression of 20 m6A RNA methylated regulatory factors in HCC samples compared with the control ones, and the thick line represented the median value of expression. * P<0.05, <0.01, <0.001, <0.0001.

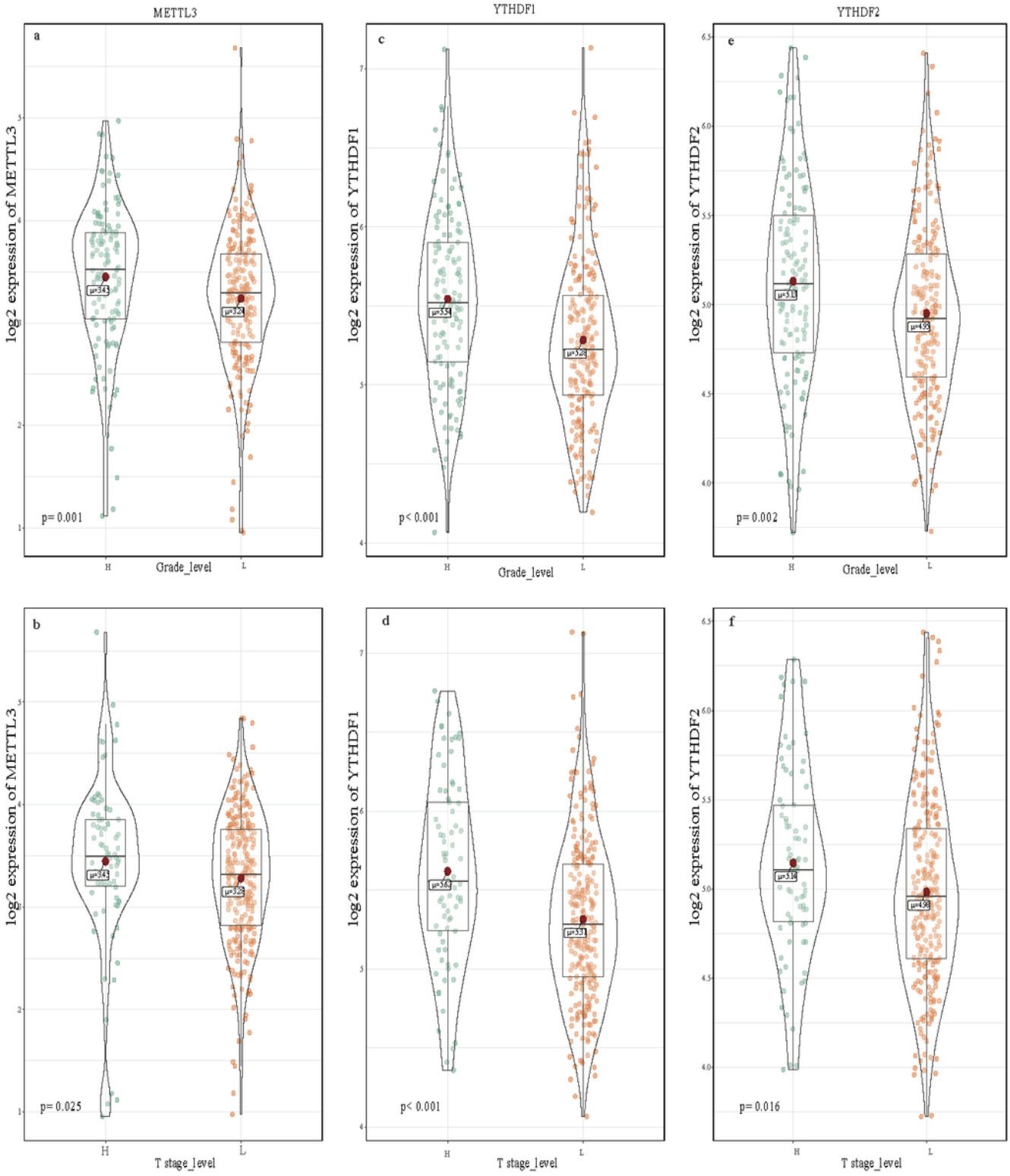

Figure 3

Relationship between m6A RNA methylation regulators and pathological grade. (a-c) The expression levels of METTL3, YTHDF1 and YTHDF2 in HCC with different pathological grades. (d-f) The expression levels of METTL3, YTHDF1 and YTHDF2 in HCC with different T-stages.

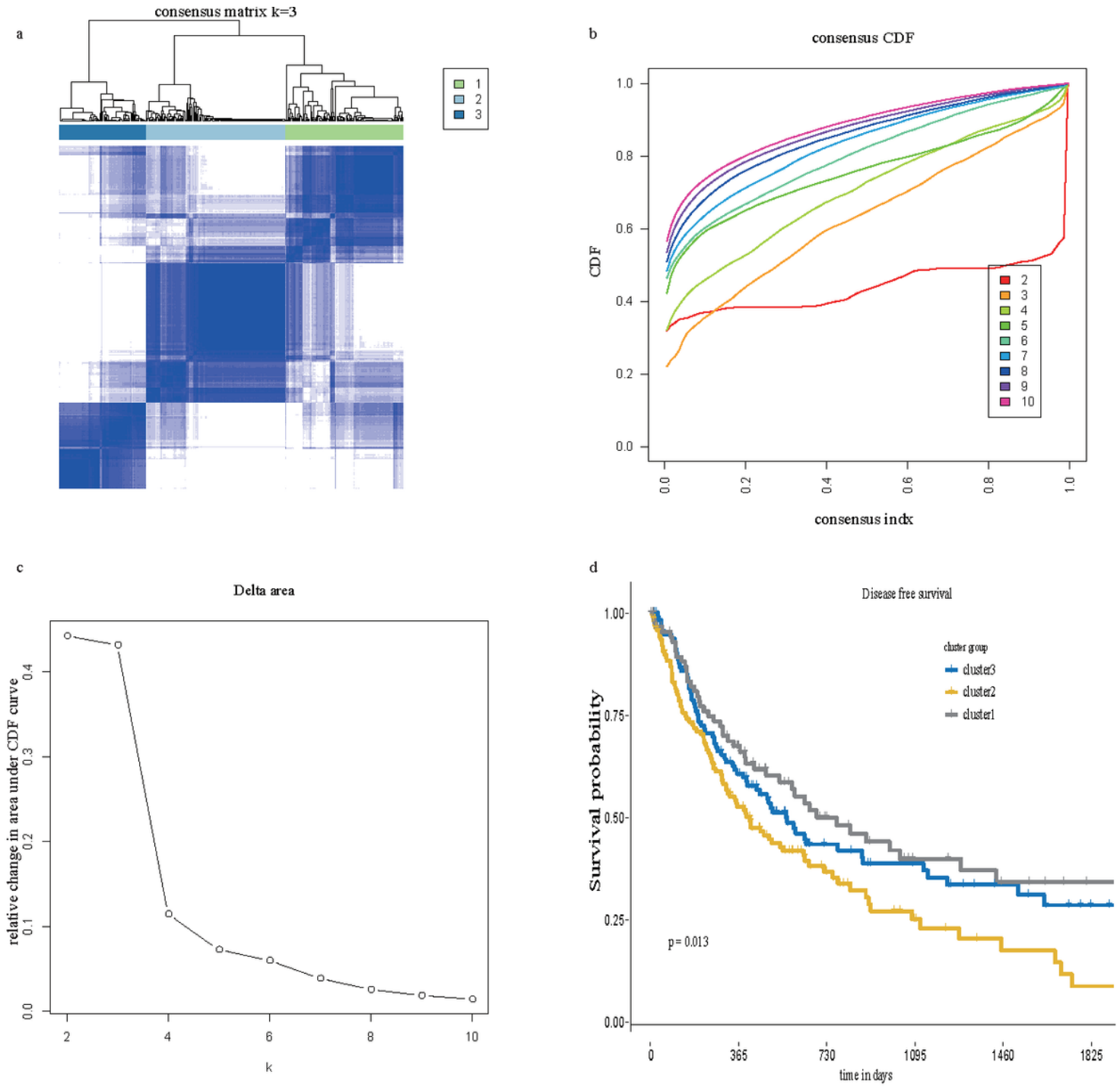

Figure 5

Differential DFS of TCGA-LIHC patients in the three different clusters. (A) Consensus clustering cumulative distribution function (CDF) for k = 2 to 10. (B) Relative change in area under CDF curve for k = 2 to 10. (c) At k=3, the correlation between groups. (d) Differential DFS of HCC in the three clusters of patients.

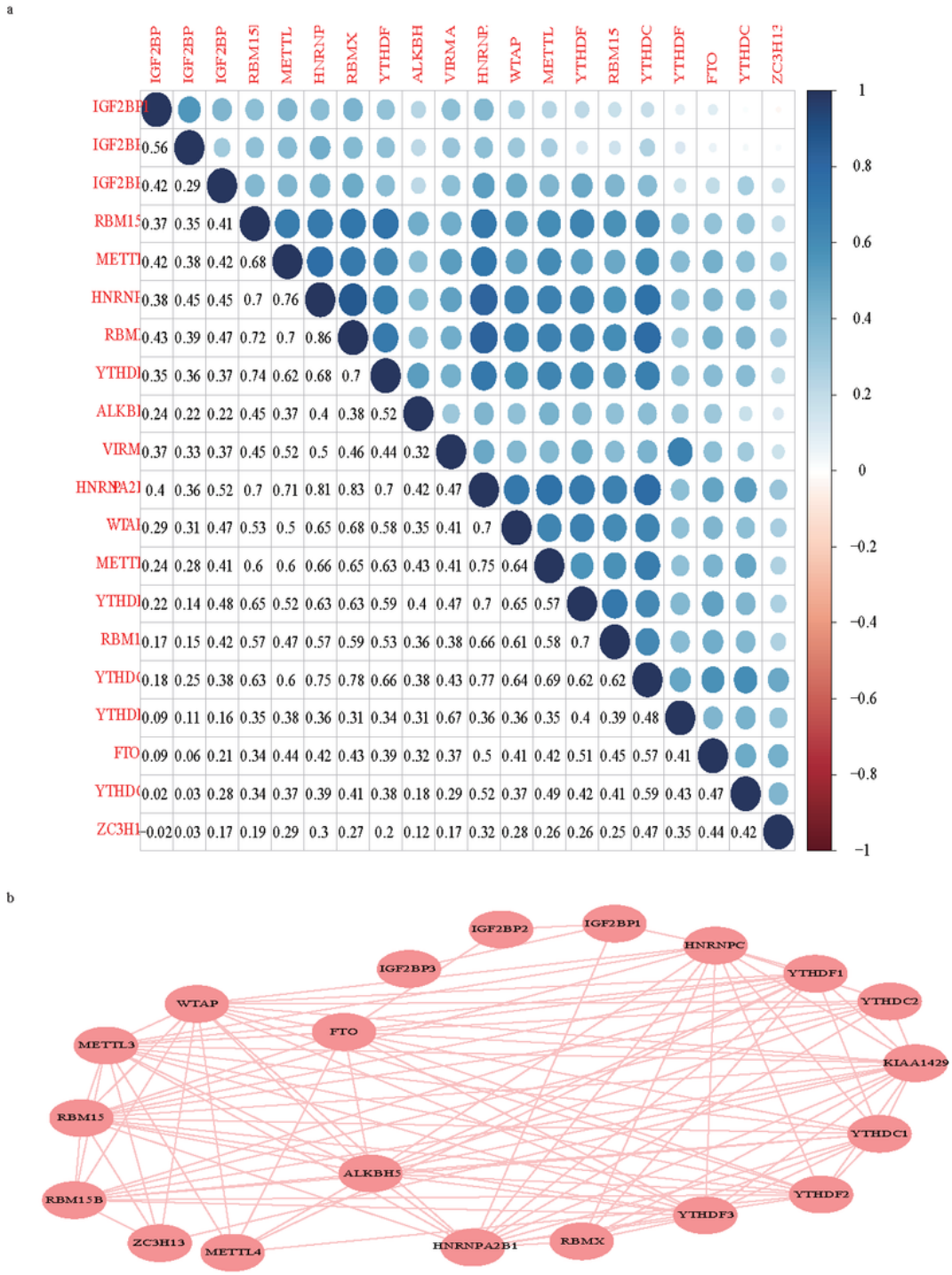

Figure 7

Relationships among m6A RNA methylation regulators (a) PPI network of RNA methylation regulators. (b) The correlation of the 20 m6A modification regulators estimated by spearman correlation analysis.

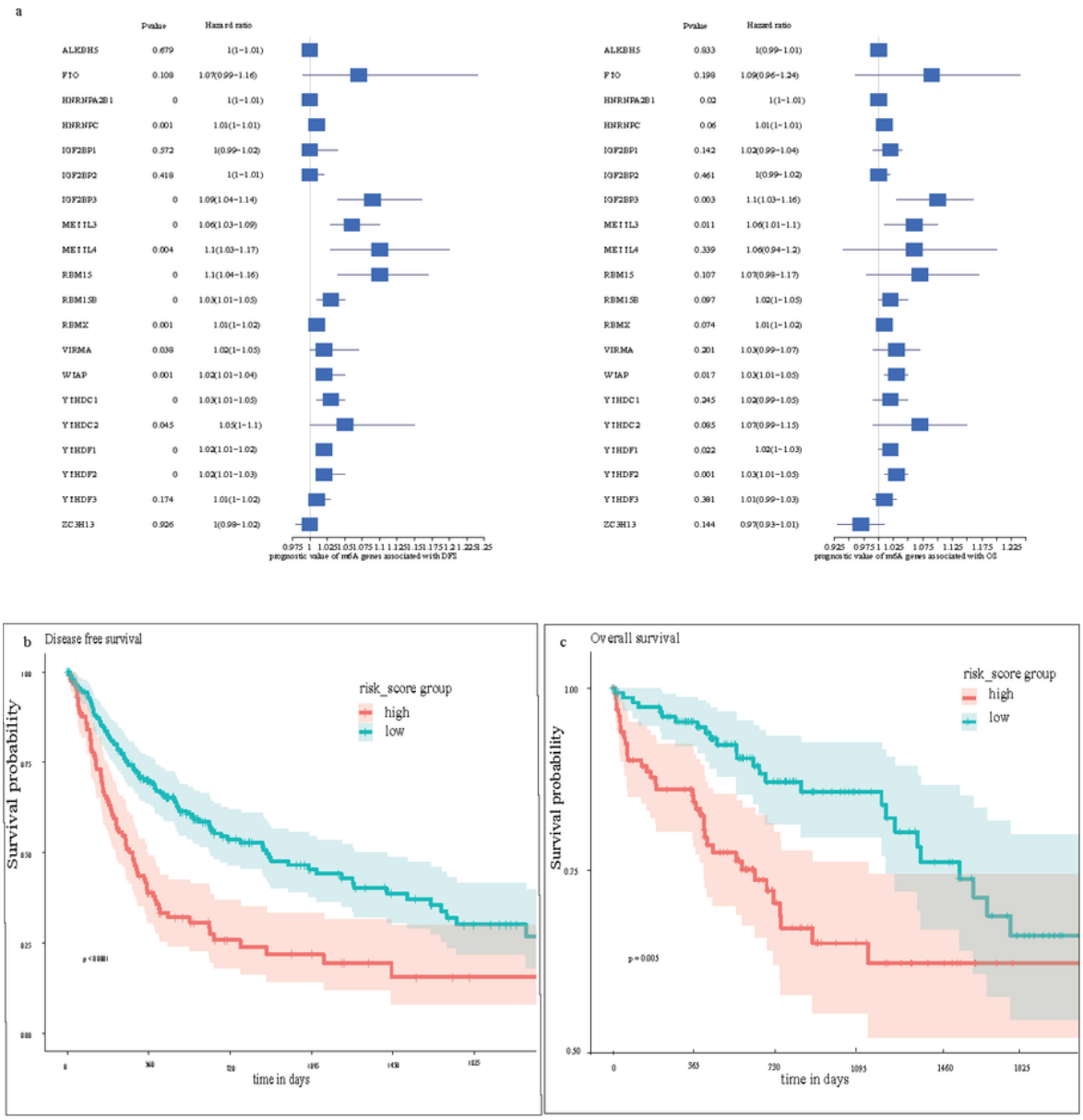

Figure 10

Prognostic analysis of m6A genes and risk-signature. (a)Univariate and multivariate regression analysis of the m6A regulators regarding prognostic value. (b-c) Kaplan-Meier curve showing the difference in (b) disease-free survival and (c) overall survival between the low- and high-risk groups.

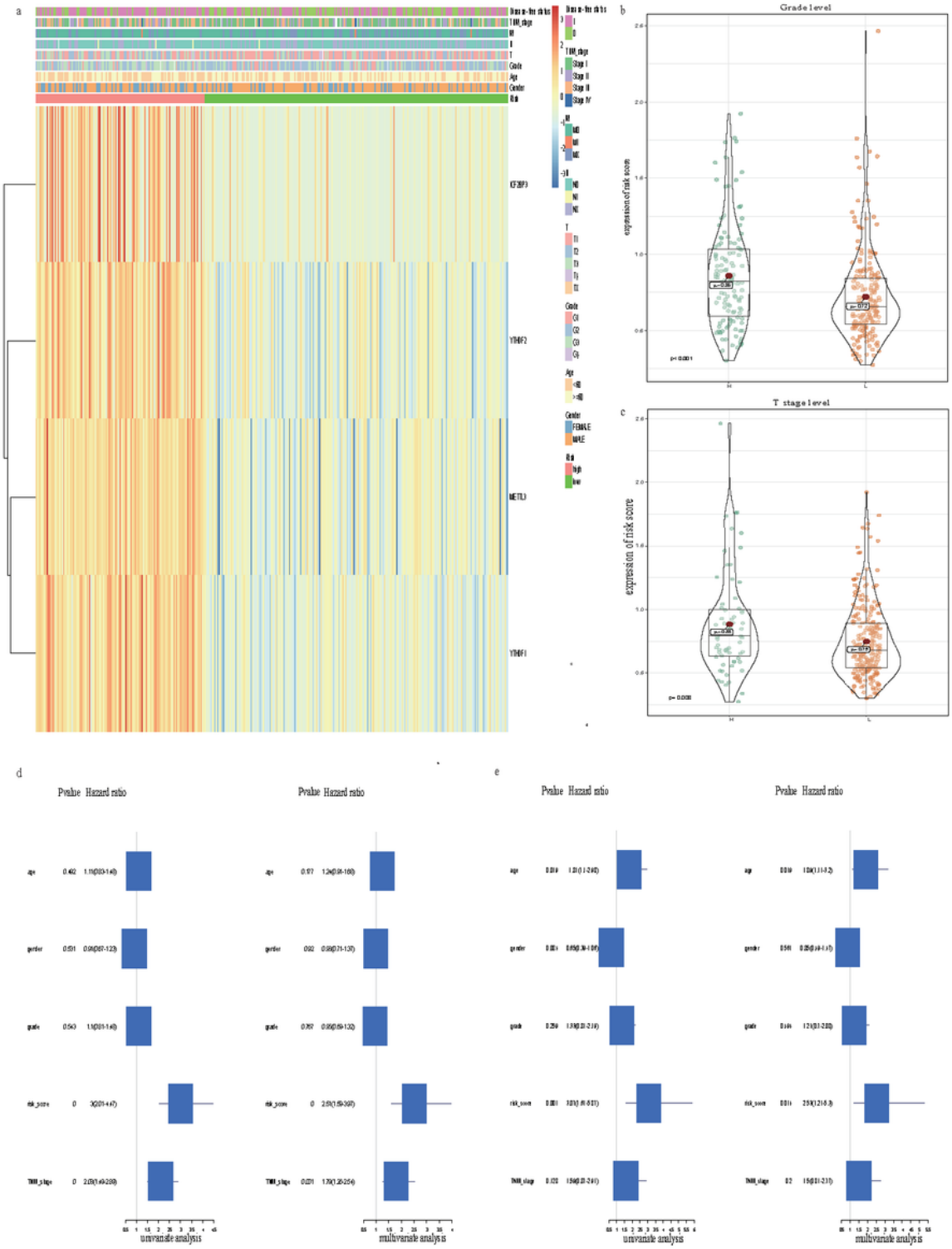

Figure 12

Characteristics of risk-signature subtypes. (a) The heatmap shows the clinical characteristics of risk-high group and risk-low group. (b-c) Significant differences were found for the pathological grade and T-stage between risk-high and risk-low groups. (d-e) Univariate and multivariate regression analysis of the relation between the riskscore and clinicopathological features regarding OS/DFS.

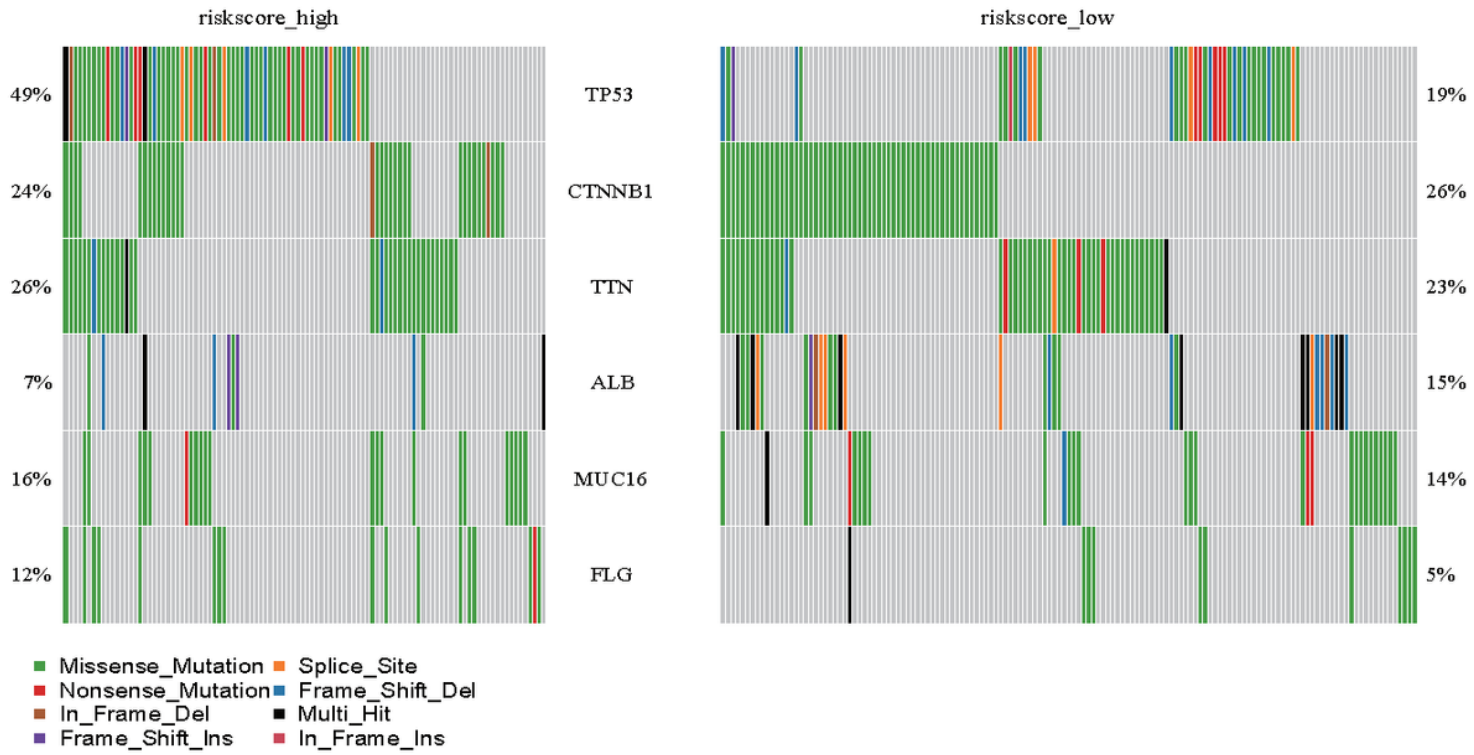

Figure 14

Genetic alteration between the riskscore subtypes. Oncoprint shows highly variant mutated genes correlated with riskscore subtypes.

Supplementary Files

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