Original Research *Feature article*

Systematic analysis of molecular characterization and clinical relevance of m6A regulators in digestive system pan-cancers

Tiankuo Kou[1,*], Ruizhi Chai[1,*], Lan Jin[1], Zhigang Bai[1], Yun Yang[1], Yongtian Zhao[2], Dongfang Wu[2], Zhongtao Zhang[1] and Yingchi Yang[1]

[1]Department of General Surgery, Beijing Friendship Hospital, Capital Medical University, Beijing Key Laboratory of Cancer Invasion and Metastasis Research & National Clinical Research Center for Digestive Diseases, Beijing 100032, China; [2]YuceBio Technology Co., Ltd., Shenzhen, Guangdong 518000, China

Corresponding authors: Zhongtao Zhang. Email: zhangzht@ccmu.edu.cn; Yingchi Yang. Email: yangyingchi@ccum.edu.cn

*These authors contributed equally to this work.

Impact statement

The current research discovered that N6-methyladenosine (m6A) regulators have extensive genetic changes and highly consistent expression regulation. The m6A expression is significantly correlated with the activity of cancer pathways. At the same time, we also identified m6A regulators significantly related to the common cancer pathways of digestive system tumors and specific cancer pathways of digestive tracts and digestive glands. The current findings show that the similarities and difference of the action mechanism m6A regulators in the digestive tract and digestive gland tumor progression could guide potential drug development.

Abstract

Digestive system tumors, which mainly include esophagus, stomach, colorectum, liver, pancreas, bile duct, and some other tumors, often have a poor prognosis. N6-methyladenosine (m6A) has critical functions in development and tumorigenesis and may help improve the molecular mechanisms of digestive system tumors. However, current understanding of the reconstitution of m6A in digestive system tumors is far from comprehensive. Herein, this study systematically analyzed multi-layered genomic characteristics and clinical relevance of m6A regulators in 1906 patients involving seven digestive system tumor types. We discovered that m6A regulators showed extensive genetic changes and highly consistent expression regulation. The m6A expression was closely related to the activity of cancer pathways. At the same time, we also identified m6A regulators significantly related to the common cancer pathways of digestive system tumors and specific cancer pathways of digestive tract and digestive glands. These cancer pathways may explain the prognostic differences of patients with digestive tract tumors. In addition, m6A regulators demonstrated strong potential in prognostic stratification and drug development, especially in multiple research cohorts on pancreatic cancer, pointing to a strong prognostic stratification capability of m6A regulators. Finally, a m6A scoring model significantly related to highly active ubiquitin-mediated proteolysis, mismatch repair, cell cycle, ebasal transcription factors was constructed and had a strong prognostic stratification ability in digestive gland tumors. The score showed a significant negative correlation with the tumor immune microenvironment. This study demonstrated that the similarities and difference of the action mechanism m6A regulators in the digestive tract and digestive gland tumor progression could guide potential drug development.

Keywords: Bioinformatics, copy number variation, N6-methyladenosine, The Cancer Genome Atlas, digestive system pan-cancers

Experimental Biology and Medicine **2021; 246: 2007–2018. DOI: 10.1177/15353702211019681**

Introduction

As the most common form of mRNA modification,[1] N6-methyladenosine (m6A) is widely present in some viruses and almost all eukaryotes.[2] The presence of m6A on the transcripts helps realize a variety of basic cellular functions, as m6A could regulate exon inclusion, translation, 3′-end processing, nuclear export, alternative splicing, miRNA processing, degradation, and circRNA translation.[3–5] In addition, m6A modification also actively participates in many important physiological processes such as obesity, immune regulation, circadian cycle, stem cell differentiation pluripotency, DNA damage response, embryogenesis,

yeast meiosis, and carcinogenesis.[2,6–10] The abundance and effect of m6A on RNA depends on the dynamic interaction between methyltransferases (Writers), demethylases (Readers), and binding proteins (Erasers).[11] Thus, discovery of these different m6A regulators of RNA methylation greatly increases our understanding of the role of m6A regulation of gene expressions.[12,13] Recent studies demonstrated that m6A mediated by adjusted disturbance shows dysregulate cell death and cell effort and promotes a variety of human disease progression, for example, m6A regulators facilitate the malignant transformation of glioma and gastric cancer.[14,15] RNA N6-methyladenosine demethylase fat mass and obesity associated (FTO) promotes the progression of breast tumor through suppressing BNIP3.[16] Study also showed that m6A demethylase FTO enhances tumorigenesis of hepatocellular carcinoma through mediating PKM2 demethylation.[17] To elucidate RNA-based therapeutic targets, the genetic changes and expression perturbations in cancer cell heterogeneity should be more comprehensively explored.

Digestive tract tumors such as tumors of the stomach, rectum, esophagus, colon, liver, bile duct, and pancreas, which are the most frequent malignancy in the world, cause more than 2.5 million death cases in the world annually.[18,19] Although medical treatments have been greatly improved over the past half century, overall five-year survival rates of cancer patients remain dismal, especially for those with digestive gland tumors. An early diagnosis will greatly promote the clinical treatment outcome of gastrointestinal tumors, but most tumors are diagnosed at a late stage, making advanced digestive tumors one of the most challenging cancers in the world.[20,21] Thus, to explore the molecular mechanism of prognosis of patients with digestive system tumors is highly necessary. In particular, m6A is a new field that may improve the current understanding of the molecular mechanisms of digestive tumors.

This study was designed to systematically examine clinical relevance of m6A regulators and molecular changes in tumors of the digestive system. By analyzing the data materials collected from by The Cancer Genome Atlas (TCGA), the study evaluated the genetic changes and clinical prognostic significance of m6A regulators in tumor tissues of the digestive system. We also identified extensive genetic changes and high co-expression rich in digestive system tumors. Moreover, after analyzing the perturbation of m6A regulators expression and cancer pathway activity, this study proved the clinical prognostic significance of m6A regulators; moreover, m6A regulators were confirmed to be potential prognostic stratification indicators. This analysis well demonstrated the important role of m6A regulators in the development of digestive system tumors, and their similarities and differences in the progression of digestive tract and digestive gland tumors, laying the foundation for the development of RNA methylation-based therapeutic strategies.

Materials and methods

Data acquisition and pre-processing

TCGA GDC API (https://gdc.cancer.gov/developers/gdc-application-programming-interface-api) was used to download TCGA (https://portal.gdc.cancer.gov/) for acquiring the latest clinical follow-up data materials of digestive system tumors (esophageal cancer, rectal cancer, gastric cancer, bile duct cancer, colon cancer, pancreatic cancer, and liver cancer) in 24 October 2019. Copy number variation (CNV), methylation and RNA-seq data (including count and Fragments Per Kilobase of transcript per Million Fragments [FPKM] quantitative expression profile data), and SNP data were also collected. Sample statistics of each digestive system tumor are shown in Table 1.

Collection of m6A regulators

We manually retrieved m6A-related literature from PubMed (https://pubmed.ncbi.nlm.nih.gov/) and collected m6A regulators from previous literature; specifically, 20 genes comes from Yang *et al*.[11] and Pinello *et al*.[12] including 2 Erasers, 11 Readers, and 7 Writers (online Table S1). All of these genes were collected from genecards (https://www.genecards.org/) and integrated into the Entrez ID and Gene Nomenclature Committee (HGNC) symbol.

The role of m6A regulator in cell growth

The crispr-cas9 gene scale screen was collected from cell lines from three types of gastrointestinal cancer from Behan *et al*.[22] The proportion of cell lines observed by each regulator as essential genes was calculated to analyze the functions of m6A regulator in cell growth during gastrointestinal cancers.

Table 1. Tumor of digestive system in TCGA pancancer project.

Cancers	Abbr	Mutation	RNA_Seq_normal	RNA_Seq_cancer	CNV	Methylation_normal	Methylation_normal
Rectum adenocarcinoma	READ	137	10	166	164	7	98
Colon adenocarcinoma	COAD	399	41	456	451	38	297
Stomach adenocarcinoma	STAD	437	32	375	441	2	396
Liver hepatocellular carcinoma	LIHC	364	50	374	375	50	377
Pancreatic adenocarcinoma	PAAD	178	4	177	184	10	184
Esophageal carcinoma	ESCA	184	11	161	184	16	185
Cholangiocarcinoma	CHOL	51	9	36	35	9	36
In total		1750	157	1745	1834	132	1573

CNV: copy number variation.

Correlation analysis of m6A regulator and Kyoto encyclopedia of genes and genomes pathway

Gene set variation analysis (GSVA)[23] was conducted with the R software using Molecular Signatures Database (MSigDB)[24] C2 Canonical pathways gene set collection, which contained 1320 gene sets. Single-sample gene set enrichment analysis (ssGSEA) on each pathway was performed for obtaining enrichment score of the sample in the pathway. Finally, the correlation between the enrichment score and m6A regulators was calculated. The threshold was correlation greater than 0.6 and false discovery rate <0.05.

Building cancer knowledge base

Based on Hanahan and Weinberg's reviews on the hallmarks of cancer,[25,26] the knowledge base of cancer-related genes was summarized and contained the genes in the following 25 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms: (1) MAPK signaling pathway, (2) pathways in cancer, (3) PPAR signaling pathway, (4) mTOR signaling pathway, (5) Jak-STAT signaling pathway, (6) cytokine-cytokine receptor interaction, (7) cell cycle, (8) ErbB signaling pathway, (9) telomere maintenance, (10) apoptosis, (11) TGF-β signaling pathway, (12) VEGF signaling pathway, (13) Wnt signaling pathway, (14) adherens junction, (15) ECM-receptor interaction, (16) mismatch repair, (17) p53 signaling pathway, (18) inflammatory response, (19) focal adhesion, (20) nucleotide excision repair, (21) base excision repair, (22) B cell receptor signaling pathway, (23) natural killer cell-mediated cytotoxicity (24) glycolysis/gluconeogenesis, and (25) T cell receptor signaling pathway. Genes for these pathways were downloaded from the MSigDB[27] as a cancer pathway gene knowledge base.

Interaction of m6A regulator

Pearson correlation coefficient (PCC) in m6A regulators was calculated based on gene expression for all cancer types. Corrplot software package in the R program (https://github.com/taiyun/corrplot)[28] was used to visualize the PCC in the m6A regulators. In addition, protein–protein interactions between m6A regulators were determined based on the hippie database.[29] Among them, the genes in the cancer pathway gene knowledge base were combined to study their interaction with m6A regulatory genes. Only the interaction between m6A regulators and cancer pathway genes was visualized by Cytoscape.[30]

Clinical significance of m6A regulators

Here, we examined whether patient survival was related to the expression of m6A regulators. The relationship between expression changes and patient prognosis was analyzed with Cox proportional-hazards model analysis according to the level of m6A regulators. Receiver operating characteristic (ROC) analysis was performed using the R software timeROC. All the patients were assigned into two groups by Youden's index. Difference in survival rates of the two groups was analyzed with log rank by the package survival in the R program. A P value of <0.05 was defined as significant.

The m6A score

A two-phase strategy was implemented to develop m6A_score. Firstly, the prognostic significance of m6A regulators in different digestive system tumors was analyzed to select prognosis-related genes in more than three digestive system tumors as the feature gene set. We used ssGSEA to define the samples. The enrichment score in the feature gene set was used as the m6A_score of the sample, and the ssGSEA analysis was performed in the R package GSVA.[23]

Data and code availability

Analyses were performed in R 3.6.0, all the code and data could be found in GitHub (https://github.com/biocn/SigestiveSystem). The work flow chart is shown in Figure 1.

Results

m6A regulators showed extensive genetic changes in different digestive system tumors

At present, the m6A regulators mainly include Writers, Readers, and Erasers. After extensive literature review, a catalogue of 20 genes including 2 Erasers, 7 Writers, and 11 Readers that mainly act as regulators of RNA methylation was screened (Figure 2(a)). Data of cell mutations were first integrated to determine the incidence of m6A regulator in seven types of digestive system tumors. The overall mean mutation frequency of m6A regulator was low, ranging from 0 to 11% (Figure 2(b)). Cancers that had a higher global mutation burden (such as COAD, STAD) also demonstrated higher mutation frequencies, with ZC3H13 and KIAA1429 showing relatively higher mutation frequencies. Investigation of the frequency of CNV changes in m6A regulators found that CNV changes were common except PAAD, and that IGF2BP1/2/3, YTHDF1/3, and KIAA1429 showed extensive CNV amplification. In contrast, the CNV of ALKBH5, METTL14 were generally absent (Figure 2(c)). Interestingly, except for PAAD, the samples with copy number mutations or amplifications were significantly different when compared with the normal samples (online Figure S1). The change of CNV may be one of the important mechanisms of interference expression of m6A regulator. Furthermore, the methylation differences in the promoter region of m6A regulators were analyzed. Among them, the m6A regulators differentially methylated in promoters were not exactly the same in different tumors, and generally more than 50% of the promoter methylation of m6A regulators were different (Figure 2(d)), indicating that DNA methylation functioned critically in expression regulation of m6A regulators. Here, the results demonstrated a highly heterogeneous genetic and expression change in m6A regulators in digestive system tumor types, meaning that m6A regulators play significant roles in different digestive system tumors.

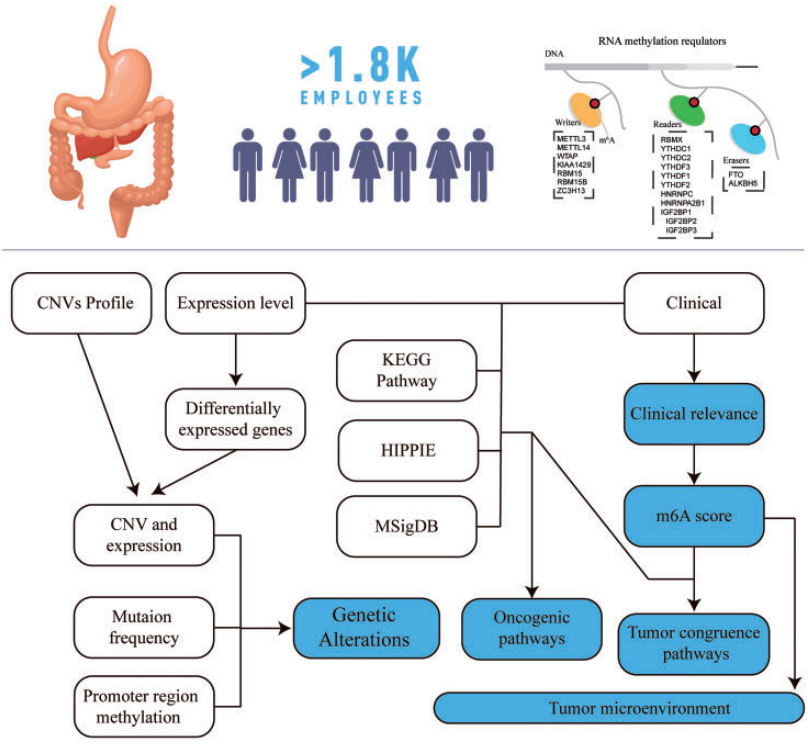

Figure 1. The workflow of the systematic method. (A color version of this figure is available in the online journal.)

High co-expression of m6A regulators in tumors of the digestive system

Gene expression data were integrated to analyze the simultaneous occurrence and expression correlation of gene changes of 20 m6a regulators in seven digestive systems tumors. It could be found that the genes in a same functional category demonstrated significant co-genetic changes and highly correlated expression patterns, and high correlations among Readers, Writers, and Erasers (Figure 3(a)). There was a high correlation between genes in the same protein complex, for example, METTL3 and WTAP of the spliceosome complex. Furthermore, after analyzing the differences between cancers and adjacent cancers in various digestive system tumors, it could be found that except PAAD, the expression of most m6A regulators in 6 tumors was different (Figure 3(b)). These differential m6A regulators were almost uniformly and significantly overexpressed in tumor samples (online Figure S2(a) and (b)), suggesting that m6A regulators may have highly consistent expression regulation in these tumors. Similarly, the proportion of cell lines observed as essential genes for each regulatory factor was calculated (Figure 3(c)), and the data demonstrated that m6A regulators were likely to be the key genes in different cell lines, meaning that m6A regulators play a key role in cell growth of m6A regulators.

M6A regulators widely participated in the regulating carcinogenic pathways in digestive system tumors

Genes do not function isolation, and evidence suggests an interaction between Readers, Writers, and Erasers in cancers.[31,32] Therefore, we also examined the interactions of these m6A regulators and the genes in 25 Cancer Knowledge Bases in which all m6A regulators have at least two or more gene interactions. The current results found significant identical interaction gene clusters of the genes in the same functional class, and that Reader and Writers interact particularly frequently; moreover, at least 75% of m7a regulators participate in the regulation of multiple cancer pathways (Figure 4(a)). Furthermore, the molecular mechanism of m6A regulators in tumors was studied, and the relationship between the activity of the KEGG pathway and the expression of m6A regulators was analyzed. It was found that a total of 51 pathways were noticeably related to the expression of m6A regulators in 7 digestive system tumors. These pathways were divided into three categories (C1: $n=29$, C2: $n=6$, C3: $n=16$) (Figure 4(b)). Specifically, the C1 pathway showed a high correlation in CHOL, LIHC, and PAAD. These pathways were mainly Wnt signaling pathway, basal cell carcinoma, bladder cancer, focal adhesion, adherens junction, glioma, renal cell carcinoma, prostate cancer, and ERBB signaling pathway. C2 had a high correlation in ESCA, COAD, STAD, and these pathways were mainly base excision repair, pyrimidine metabolism, nucleotide excision repair, RNA degradation. Pathways in class C3 had a highly consistent high correlation in seven tumors (Figure 4(c)), and these pathways were mainly p53 signaling pathway, cell cycle, mismatch repair, homologous recombination, DNA replication, spliceosome, non-homologous end joining, chronic myeloid leukemia, colorectal cancer, pancreatic cancer, small cell lung cancer, and pathways in cancers. Interestingly, the above pathways are significantly important pathways in tumorigenesis, invasion and metastasis, indicating that m6A regulators are widely present in the regulation

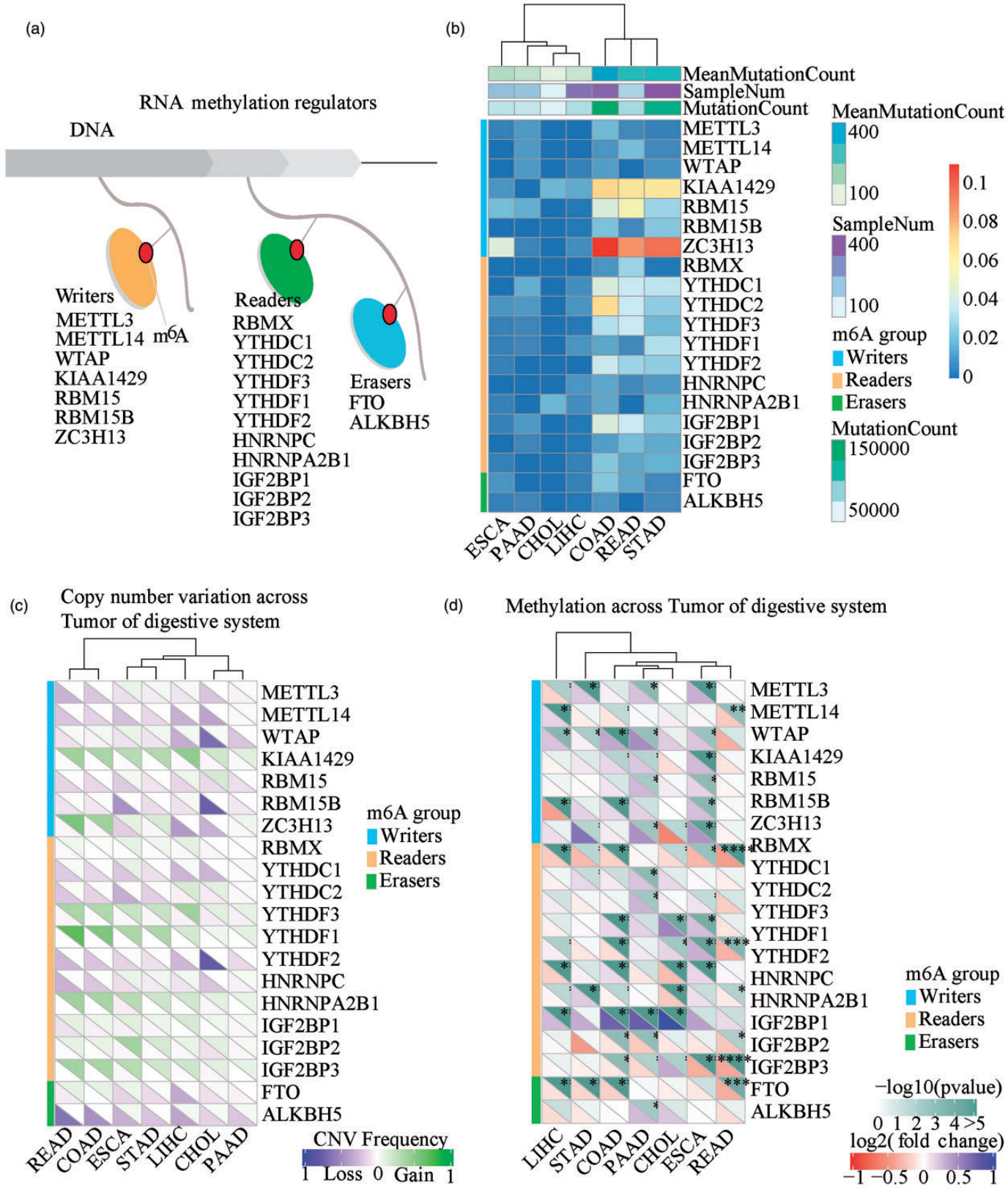

Figure 2. Tumor of digestive system genetic and expression alterations of m6A regulators. (a) Diagram of m6A regulators. (b) The mutation frequency of m6A regulators in seven tumors of digestive system. (c) The CNV alteration frequency of m6A regulators in different cancer types. The bottom part of each grid shows the deletion frequency, and the upper part shows the amplification frequency. When it exceeds 10%, the value is displayed in the graph. (d) The gene promoter methylation differences of m6A regulators in seven tumor of digestive system types. The bottom part of each grid shows the fold changes, and the upper part shows the *P* value, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$. (A color version of this figure is available in the online journal.)

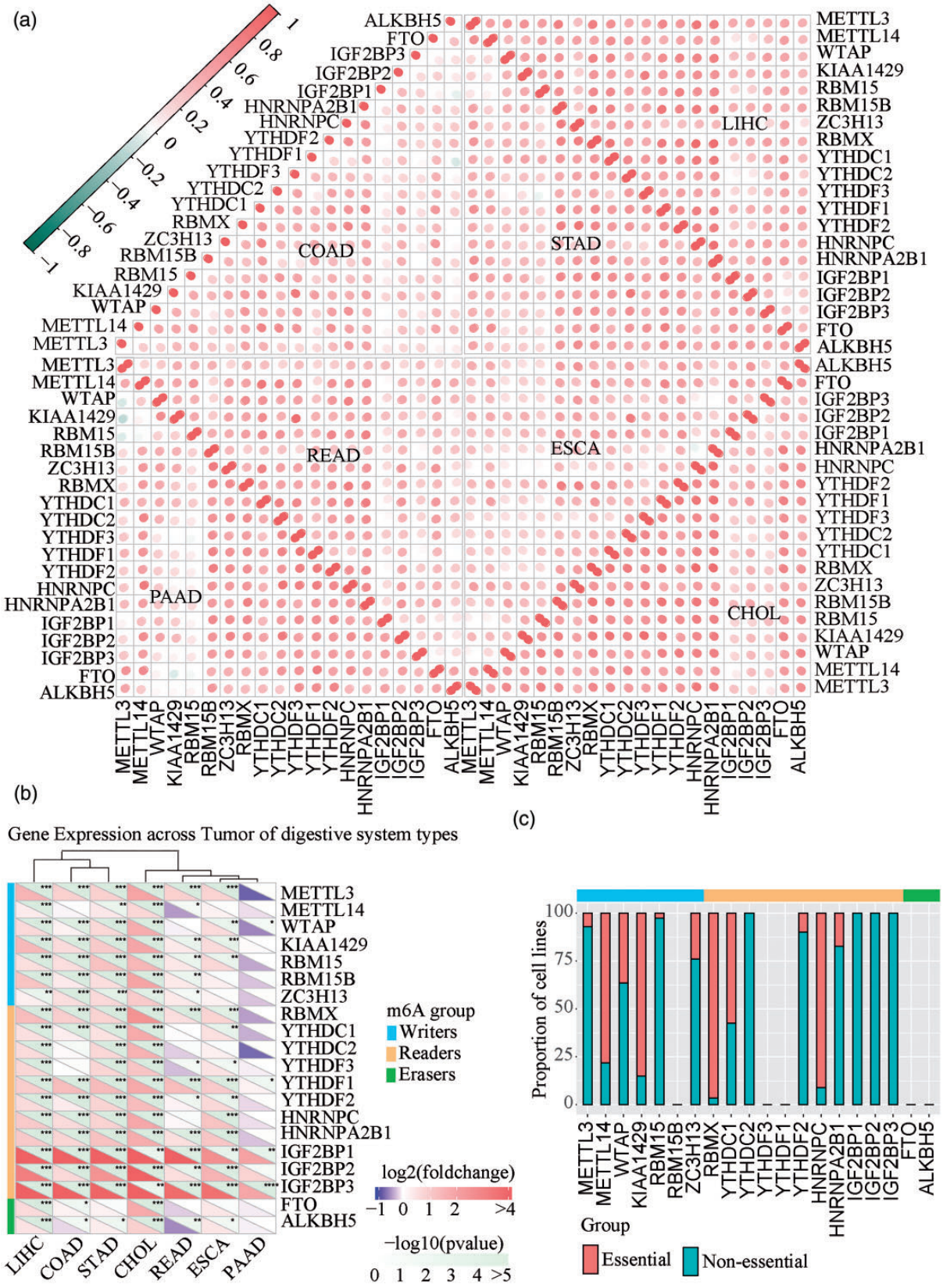

Figure 3. (a) The co-expression relationship of the m6A regulator in seven tumors of digestive system. Correlation is calculated using Pearson correlation coefficient. The larger the red dot in the figure, the more relevant it is. *indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$. (b) The expression differences of m6A in seven tumors of digestive system. The bottom part of each grid shows the fold changes, and the upper part shows the P value. * indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$, **** indicates $P < 0.0001$. t-test was used for statistical tests. (c) Function of m6A regulators in cell growth. The proportion of cell lines that each regulator was identified as essential gene. (A color version of this figure is available in the online journal.)

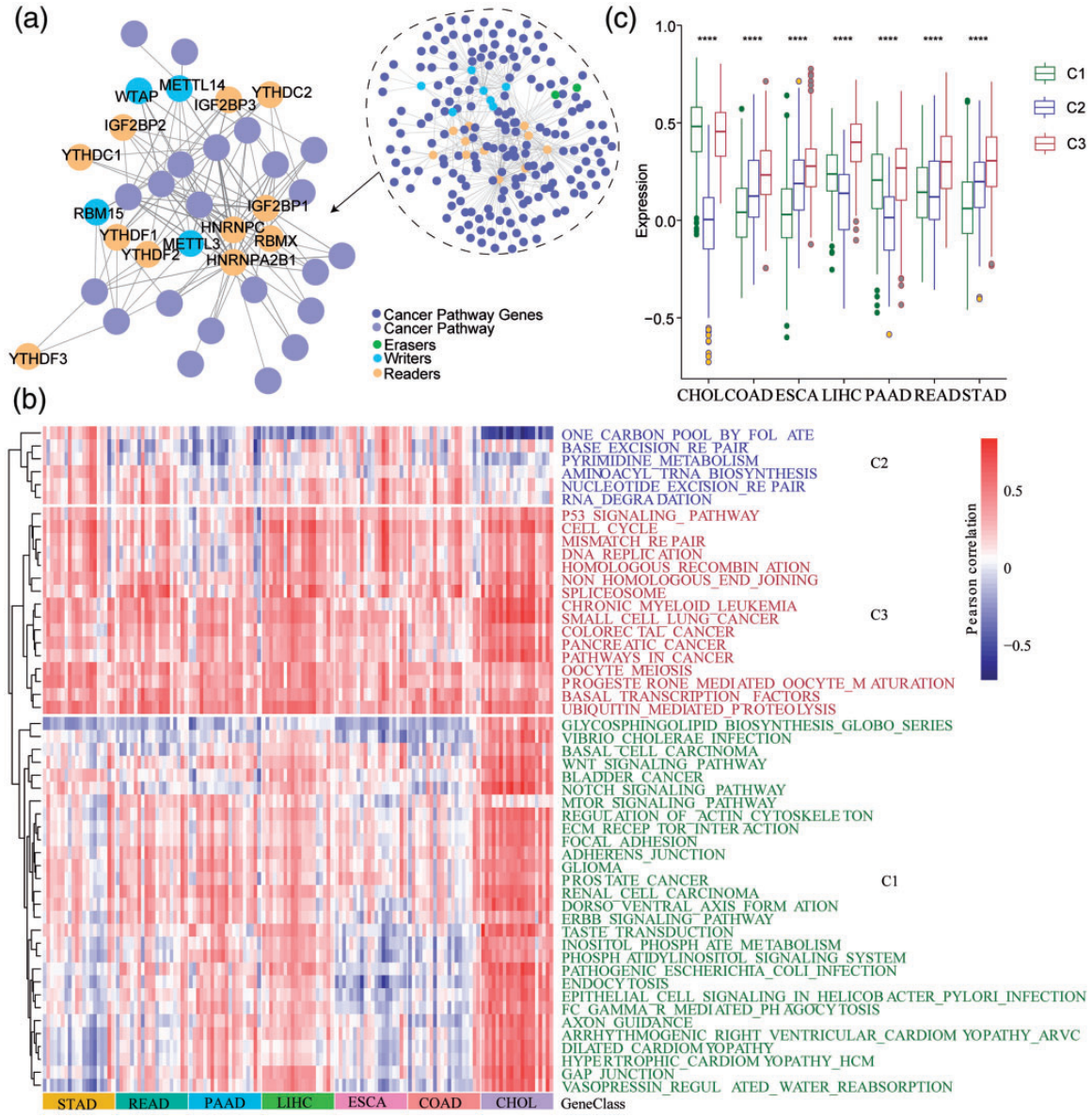

Figure 4. (a) The expression differences of m6A in seven tumors of digestive system. The bottom part of each grid shows the fold changes, and the upper part shows the *P* value, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. *t*-test was used for statistical tests. (b) The hierarchical clustering of the KEGG pathway, highly correlated with the expression of m6A regulators, in seven tumors of digestive system. Euclidean distance was used to calculate the distance between paths. (c) The distribution of correlation coefficients between the three types of pathways was different in seven tumors. (A color version of this figure is available in the online journal.)

of oncogenic pathways in digestive system tumors, and that changes in cancer pathways vary in different tumors.

The clinical significance of m6A regulator in digestive tumors

The general genetic and expression changes of m6A regulatory factor in tumors of the digestive system could provide important insights into the development of translational medicine. The relationship between prognostic survival of patients and m6A regulatory factor expression in the seven digestive system tumors was examined, and it was found that the m6A regulatory genes were all related to the prognosis of patients with at least one type of cancer. A majority of m6A regulators, such as igf2bp1/2/3, METTL3, showed oncogenic characteristics, and these genes with a high expression were correlated with poor survival. Moreover, we also found that some m6A regulators, such as YTHDC2 and RBM15, demonstrated tumor suppressor characteristics. A high expression of YTHDC2 was significantly associated with a better survival of READ, and high-expressed RBM15 was closely related to a progression-free rate of CHOL (Figure 5(a)). At the same time, it was also observed that the prognosis of PAAD and LIHC tumors in the seven digestive system tumors is more closely related to m6A regulator expression. It is known that patients with PAAD and LIHC tumors often develop a worse prognosis, and both the two cancers are digestive gland tumors, indicating different prognostic patterns of the expression of m6A regulators in the digestive tracts and glands, and may play an important role in digestive gland cancers with a worse prognosis. ssGSEA was used to establish m6A_score to determine the

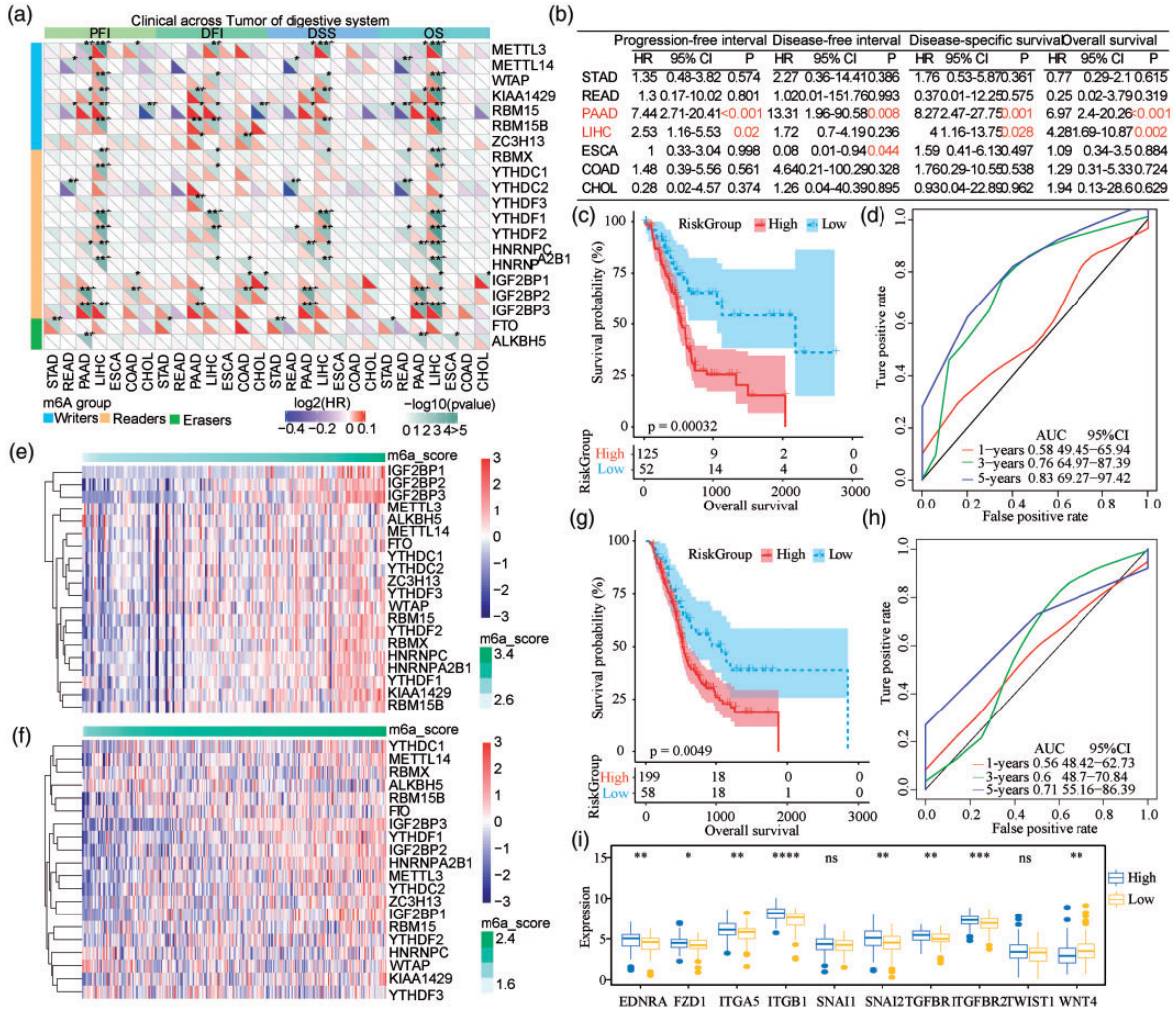

Figure 5. (a) Relationship between the expression of the m6A regulator and prognosis in seven digestive system tumor types. The bottom part of each grid shows the $\log_2$ (HR), and the upper part shows the *P* value. *indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$**** indicates $P < 0.0001$. (b) The prognostic relationship between m6A_score and seven tumors of the digestive system. (c) The prognostic difference between high m6A_score and low m6A_score samples in PAAD. Youden's index as cutoff=−0.6099466. (d) ROC curve of total survival between m6A_score and PAAD. (e) Heat map of the expression relationship between m6A_score and m6A regulator. (f) Heat map of expression relationship between m6A_score and m6A regulator in ICGC queue. (g) Prognostic differences between the high m6A_score group and the low m6A_score group in the ICGC cohort with the same cutoff. (h) ROC curve of total survival between m6A_score and PAAD in ICGC queue. (i) Differences in the expression of EMT-related genes between the high m6A_score group and the low m6A_score group. (A color version of this figure is available in the online journal.)

expression score of m6A regulators in the samples. The m6A_score of LIHC and PAAD in the seven digestive system tumors was significantly related to the patients' tumor progression and overall survival (Figure 5(b)). Patients with PAAD showed significant prognostic differences in terms of their disease progression, disease-free survival and overall survival. Therefore, we focused on the prognostic relationship between m6A_score and PAAD. As expected, high m6A_score in PAAD correlated with a poor prognosis (Figure 5(c)), and the area under the curve (AUC) of m6A_score five-year survival rate in PAAD reached 0.83 (Figure 5(d)). High m6A_score samples had a higher expression of m6A regulators (Figure 5(e)). The same phenomenon was seen in the ICGC pancreatic cancer cohort (Figure 5(f)). Similarly, a high m6A_score in the ICGC cohort correlated with poor prognosis (Figure 5(g)), and five-year survival AUC reached 0.71 (Figure 5(h)). We selected several gene features from recent studies, such as, Li *et al.*'s three-gene feature,[33] Yang *et al.*'s six-gene feature,[34] and Yan *et al.*'s four-gene feature.[35] Comparing these three gene characteristics with the AUC and C-index of m6AScore, they all have the best predictive performance for the five-year survival rate. The risk models of Li, Yang, and Yan have lower AUC than m6AScore (online Figure S3(a) to (c)). In addition, compared to other models, m6AScore has the highest C-index (online Figure S3(d)).

Epithelial-mesenchymal transition (EMT) gene expression characteristics are indicative of metastasis and degree of malignancy of human tumors.[36,37] It is interesting to observe in the current study that the expression of EMT-related genes was noticeably higher in the samples of high-risk groups than in samples of low-risk groups (Figure 5(i)). These results showed that m6A_score could serve as a prognostic marker of pancreatic cancer, and that m6A regulators had different potentials in development of new treatment strategies and prognostic classification of cancers.

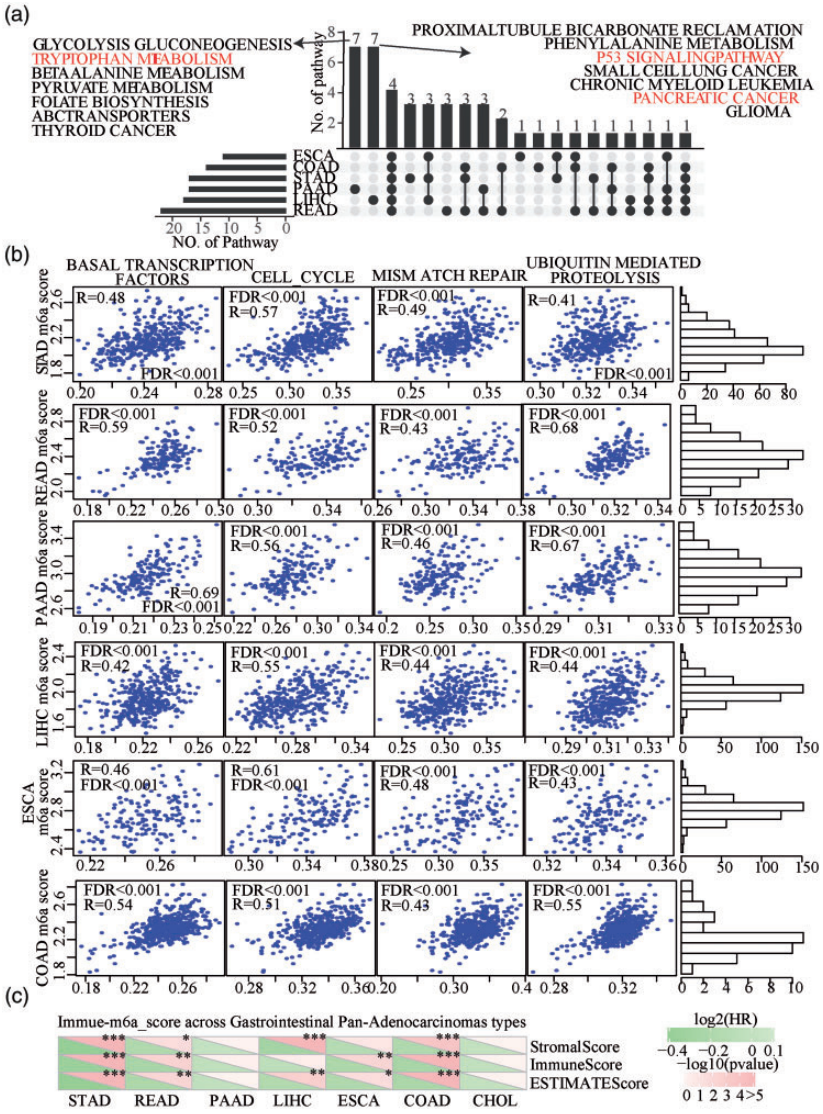

Figure 6. The potential regulatory mechanism of m6A regulator in digestive tumors. (a) The intersection distribution of pathways highly associated with m6A_score in each tumor. The left side is the number of highly correlated pathways of each tumor. The upper part is the distribution of the number of intersection between each tumor. The lower part is the intersection type, and the circle line represents the intersection of corresponding tumors. (b) The correlation between m6A_score and UBIQUITIN MEDIATED protein-mediated, MISMATCH REPAIR, CELL CYCL and EBASAL TRANSCRIPTIONF ACTORS in seven digestive system tumors. The right side is the distribution of m6A_score in the corresponding tumor. The horizontal axis is the enrichment score of the pathway, and the vertical axis is m6A_score. (c) Correlation between m6A_score and immune score. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$**** $P < 0.0001$. (A color version of this figure is available in the online journal.)

The potential regulatory mechanism of m6A regulators in digestive tumors

For examining the molecular mechanism of m6A regulatory factor expression in the seven digestive system tumors, the correlation between m6A_score and various pathways was determined. Here, a total of 45 significantly correlated pathways were found in six tumors except CHOL (online Table S2), and 51% of these pathways were significantly correlated in at least two tumors. Specific pathways were also found to exist in different tumors, including 7 (41.2%) specific pathways in PAAD and LIHC (Figure 6(a)); moreover, these pathways may be the key to the specific regulation of m6A regulators in specific tumors, such as pancreatic cancer in PAAD and tryptophan metabolism in LIHC. In addition, four pathways were significantly correlated in the six tumors (Figure 6(b)). High m6A_score was also related to highly active ubiquitin mediated proteolysis, mismatch repair, cell cycle, and ebasal transcription factors, which are all important pathways in tumor proliferation and metastasis. Abnormal cell cycle is one of the markers of tumorigenesis, and abnormal ebasal transcription factors can lead to the transcription and overexpression of proto-oncogenes, suggesting that m6A regulator may promote the overexpression of proto-oncogenes through ebasal transcription factors, thereby activating cell cycle and leading to tumor cell proliferation. Repair deficiency can greatly improve the effectiveness of cancer immunotherapy under certain conditions,[38,39] which suggests that the m6A regulator may be related to the immune microenvironment. Furthermore, to determine the correlation between immune infiltration and m6A_score, we found that in COAD, ESCA, READ and STAD, high m6A_score

was associated with low immune infiltration, while in LIHC, it was negatively correlated with matrix components, and there was no significant correlation in CHOL and PAAD (Figure 6(c)). We used the online database Timmer[40] to evaluate the scores of the 6 types of immune cells in pancreatic cancer patients, and compared the differences in the scores of the 6 types of immune cells in the low- and high-risk groups. The data revealed that high-risk patients were significantly higher than those in low-risk patients (online Figure S3(e)), which suggests that patients in different risk groups have different immune microenvironment characteristics. Furthermore, we obtained the PD-L1 immunotherapy cohort data set from the previous study (10.1038/nature25501), calculated the m6AScore of each patient using the same method, and compared the difference in m6AScore of patients with different immune responses. Interestingly, patients in the immunotherapy progression group have significantly higher m6AScore (online Figure S3(f)), which suggests that high m6AScore may be a potential marker for immunotherapy. These data revealed that in gastrointestinal tumors there was a specific association between m6A_score and immunity, which could lead to different clinical outcomes in gastrointestinal and digestive gland tumors.

Discussion

In most cancer types, genomic instability, which is considered as a hallmark of malignancy, will lead to DNA copy number changes.[25,41] These CNVs are important determinants of gene expression in cancer.[42] DNA methylation is another important regulator of gene transcription in addition to copy number abnormalities and a widely explored epigenetic modification.[43] Abnormal hypomethylation induces genomic instability and oncogene overexpression, while hypermethylation of tumor suppressor gene promoter regions may disrupt DNA repair, cell apoptosis, and cell cycle regulation, thereby causing malignant cell transformation.[44] Recent studies showed that epigenomics, genomics, and transcriptomics all have critical functions in the progression of tumors.[45,46] A comprehensive analysis on multi-layered genomic characteristics of cancer can help better classify molecular subtypes, explore precise mechanisms and tumor heterogeneity, and reveal candidate therapeutic targets and biomarkers. Multilayer genome features of m6A regulators analysis found that CNV change of m6A regulators is widely present in the digestive system tumors and also anomalies such as a vast majority of abnormal promoter methylation may lead to the change gene expression in tumor. For example, the samples with copy number mutation or amplification are significantly different from the normal samples. More interestingly, a majority of m6A regulators were almost identical in digestive system tumors, and in the tumor samples, the level of m6A was noticeably higher. In addition, we also observed that m6A regulators play a critical role in cell growth and may be necessary genes across cell lines, and that they had a high co-expression relationship in the seven digestive system tumors. These results demonstrated that m6A regulators function critically in the digestive system. Tumors may have highly consistent regulation of expression, and there may be a highly heterogeneous genetic and expression change in specific digestive system tumor types.

The m6A regulators have critical functions in the carcinogenic pathway.[47] In our research, it was found that these m6A regulators widely participated in the regulation of cancer pathways, and that genes belonging to the same functional class showed significant identical interactions with cancer pathway gene aggregation; moreover, the interaction between Reader and Writers was particularly frequent. In addition, we also found that there was a set of highly consistent pathways related to m6A regulator expression among digestive tract tumors, such as p53 signaling pathway, mismatch repair, cell cycle, pathways in cancer, and DNA replication, all of which are related to tumorigenesis. In addition, there were two groups of pathways related to m6A regulator expression and were highly consistent in the digestive tracts and gland tumors. Specifically, pathways in the digestive tract were base excision repair, pyrimidine metabolism, nucleotide excision repair, and RNA degradation. Specific highly related pathways in the digestive glands were basal cell carcinoma, Wnt signaling pathway, bladder cancer, focal adhesion, adherens junction, and ERBB signaling pathway. Although these pathways play an important role in tumor progression, m6A regulator relationship shows expression differences in different tumor types; therefore, m6A regulators could function similarly in different types of cancer control, at the same time, differences still exist in regulation and control, which may be the cause of different tumor types of clinical results.

Abnormal expression in m6A regulators is observed in many cancer types. For example, WTAP expression is upregulated in acute myeloid leukemia (AML) cell lines and samples. In addition, WTAP knockout inhibits the survival and proliferation of AML cells, suggesting that WTAP could be a potential oncogene in AML.[48] Similarly, in bladder cancer tissue samples, the downregulation of METTL3 inhibits the migration, invasion, and proliferation of cancer cells *in vitro* and tumor growth *in vivo*.[49] This suggests that the m6A regulators has great potential in serving as a prognostic marker. In our analysis, we also observed that m6A regulators were correlated with the prognosis of patients with at least one type of cancer. Most of the m6A regulators showed oncogenic characteristics, while a few had tumor suppressor characteristics, and there were different prognostic patterns in the digestive tract and digestive glands. This study established a cross-cancer m6A_score model and found that m6A_score was significantly correlated with prognosis in LIHC and PAAD, especially with significant prognostic differences in total survival, progression and disease-free survival in PAAD. In addition, the five-year survival rate of AUC in PAAD reached 0.83, and the AUC in the validation cohort reached 0.71, suggesting that m6A_score was likely to be a prognostic marker for pancreatic cancer. m6A_score in the seven digestive system tumors was highly positively correlated with highly active ubiquitin mediated proteolysis, mismatch repair, cell cycle, and EBASAL transcription factors. Cell cycle abnormalities are signs of tumorigenesis. Abnormal EBASAL transcription factors can cause transcription and overexpression of proto-oncogenes, which suggests that

m6A regulators may promote overexpression of proto-oncogenes through EBASAL transcription factors to activate cell cycle and result in tumor cell proliferation. Under certain conditions, mismatch repair deficiency can greatly improve the efficacy of cancer immunotherapy.[38,39] Furthermore, the relationship between m6A_score and immune infiltration showed that m6A_score was significantly negatively related to immune infiltration in gastrointestinal tumors, but such phenomenon was not found in gastrointestinal tumors. We suspected that this may be related to the presence of more microorganisms in the digestive tract. Gut microbes are decisive to the efficacy of immunotherapy.[50] PD1 expression is found in regulatory T cells of patients with Helicobacter pylori infection.[51] This suggests that the level of m6A regulators may be closely related to the immune microenvironment and microecology, and such a relationship could result in different clinical outcomes of digestive tract and digestive gland tumors.

Conclusions

In summary, we systematically studied the clinical relevance of m6A regulators and molecular changes in digestive system tumors, identified extensive genetic changes and high co-expression in rich digestive system tumors, and also evaluated the perturbation of m6A regulators expression and the cancer pathway. This study proved the clinical prognostic significance of m6A regulators. Here, m6A regulators were confirmed as potential indicators of prognostic stratification, and we established the m6A_score model across tumors, highlighting a critical role of m6A_score in the development of digestive system tumors. Similarities and differences in the progression of digestive tract and gland tumors were also identified. Those results may contribute to the understanding of the development of therapeutic strategies based on RNA methylation.

AUTHORS' CONTRIBUTIONS

TKK and YCY designed the study. LJ, ZGB, and YY contributed to the literature search. YTZ and DFW analyzed and interpreted data. ZTZ and RZC wrote the initial draft of the manuscript. YCY reviewed and edited the manuscript. All authors read and approved the manuscript. TKK and RZC contributed equally to this work.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by National Key Technologies R&D Program (No. 2015BAI13B09), National Key Technologies R&D Program of China (No. 2017YFC0110904), and Clinical Center for Colorectal Cancer, Capital Medical University (No. 1192070313).

ORCID iD

Yingchi Yang https://orcid.org/0000-0002-9126-3281

Supplemental material

Supplementary material for this article is available online.

REFERENCES

1. Desrosiers R, Friderici K, Rottman F. Identification of methylated nucleosides in messenger RNA from novikoff hepatoma cells. *Proc Natl Acad Sci U S A* 1974;**71**:3971–5
2. Wei W, Ji X, Guo X, Ji S. Regulatory role of N(6)-methyladenosine (m(6)A) methylation in RNA processing and human diseases. *J Cell Biochem* 2017;**118**:2534–43
3. Roignant JY, Soller M. M6A in mRNA: an ancient mechanism for fine-tuning gene expression. *Trends Genet* 2017;**33**:380–90
4. Shafik A, Schumann U, Evers M, Sibbritt T, Preiss T. The emerging epitranscriptomics of long noncoding RNAs. *Biochim Biophys Acta* 2016;**1859**:59–70
5. Yang Y, Fan X, Mao M, Song X, Wu P, Zhang Y, Jin Y, Yang Y, Chen LL, Wang Y, Wong CC, Xiao X, Wang Z. Extensive translation of circular RNAs driven by N(6)-methyladenosine. *Cell Res* 2017;**27**:626–41
6. Wang S, Sun C, Li J, Zhang E, Ma Z, Xu W, Li H, Qiu M, Xu Y, Xia W, Xu L, Yin R. Roles of RNA methylation by means of N(6)-methyladenosine (m(6)A) in human cancers. *Cancer Lett* 2017;**408**:112–20
7. Geula S, Moshitch-Moshkovitz S, Dominissini D, Mansour AA, Kol N, Salmon-Divon M, Hershkovitz V, Peer E, Mor N, Manor YS, Ben-Haim MS, Eyal E, Yunger S, Pinto Y, Jaitin DA, Viukov S, Rais Y, Krupalnik V, Chomsky E, Zerbib M, Maza I, Rechavi Y, Massarwa R, Hanna S, Amit I, Levanon EY, Amariglio N, Stern-Ginossar N, Novershtern N, Rechavi G, Hanna JH. Stem cells. m6A mRNA methylation facilitates resolution of naive pluripotency toward differentiation. *Science* 2015;**347**:1002–6
8. Fustin JM, Doi M, Yamaguchi Y, Hida H, Nishimura S, Yoshida M, Isagawa T, Morioka MS, Kakeya H, Manabe I, Okamura H. RNA-methylation-dependent RNA processing controls the speed of the circadian clock. *Cell* 2013;**155**:793–806
9. Mendel M, Chen KM, Homolka D, Gos P, Pandey RR, McCarthy AA, Pillai RS. Methylation of structured RNA by the m(6)a writer METTL16 is essential for mouse embryonic development. *Mol Cell* 2018;**71**:986–1000
10. Xiang Y, Laurent B, Hsu CH, Nachtergaele S, Lu Z, Sheng W, Xu C, Chen H, Ouyang J, Wang S, Ling D, Hsu PH, Zou L, Jambhekar A, He C, Shi Y. RNA m(6)a methylation regulates the ultraviolet-induced DNA damage response. *Nature* 2017;**543**:573–6
11. Yang Y, Hsu PJ, Chen YS, Yang YG. Dynamic transcriptomic m(6)a decoration: writers, erasers, readers and functions in RNA metabolism. *Cell Res* 2018;**28**:616–24
12. Pinello N, Sun S, Wong JJ. Aberrant expression of enzymes regulating m(6)a mRNA methylation: implication in cancer. *Cancer Biol Med* 2018;**15**:323–34
13. Roundtree IA, Evans ME, Pan T, He C. Dynamic RNA modifications in gene expression regulation. *Cell* 2017;**169**:1187–200
14. Su Y, Huang J, Hu J. M. A RNA methylation regulators contribute to malignant progression and have clinical prognostic impact in gastric cancer. *Front Oncol* 2019;**9**:1038
15. Chai RC, Wu F, Wang QX, Zhang S, Zhang KN, Liu YQ, Zhao Z, Jiang T, Wang YZ, Kang CS. m(6)a RNA methylation regulators contribute to malignant progression and have clinical prognostic impact in gliomas. *Aging (Albany NY)* 2019;**11**:1204–25
16. Niu Y, Lin Z, Wan A, Chen H, Liang H, Sun L, Wang Y, Li X, Xiong XF, Wei B, Wu X, Wan G. RNA N6-methyladenosine demethylase FTO promotes breast tumor progression through inhibiting BNIP3. *Mol Cancer* 2019;**18**:46
17. Li J, Zhu L, Shi Y, Liu J, Lin L, Chen X. m6A demethylase FTO promotes hepatocellular carcinoma tumorigenesis via mediating PKM2 demethylation. *Am J Transl Res* 2019;**11**:6084–92

18. Torre LA, Siegel RL, Ward EM, Jemal A. Global cancer incidence and mortality rates and trends–an update. *Cancer Epidemiol Biomarkers Prev* 2016;**25**:16–27
19. Arnold M, Soerjomataram I, Ferlay J, Forman D. Global incidence of oesophageal cancer by histological subtype in 2012. *Gut* 2015;**64**:381–7
20. Sehdev V, Katsha A, Ecsedy J, Zaika A, Belkhiri A, El-Rifai W. The combination of alisertib, an investigational Aurora kinase a inhibitor, and docetaxel promotes cell death and reduces tumor growth in pre-clinical cell models of upper gastrointestinal adenocarcinomas. *Cancer* 2013;**119**:904–14
21. Winn JN, Sathyamurthy A, Kneib JL, Ibdah JA, Tahan V. Synchronous gastrointestinal carcinoid tumor and colon adenocarcinoma: case reports and literature review. *Am J Case Rep* 2017;**18**:626–30
22. Behan FM, Iorio F, Picco G, Goncalves E, Beaver CM, Migliardi G, Santos R, Rao Y, Sassi F, Pinnelli M, Ansari R, Harper S, Jackson DA, McRae R, Pooley R, Wilkinson P, van der Meer D, Dow D, Buser-Doepner C, Bertotti A, Trusolino L, Stronach EA, Saez-Rodriguez J, Yusa K, Garnett MJ. Prioritization of cancer therapeutic targets using CRISPR-Cas9 screens. *Nature* 2019;**568**:511–6
23. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinform* 2013;**14**:7
24. Liberzon A, Subramanian A, Pinchback R, Thorvaldsdottir H, Tamayo P, Mesirov JP. Molecular signatures database (MSigDB) 3.0. *Bioinformatics* 2011;**27**:1739–40
25. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:646–74
26. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100**:57–70
27. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, Tamayo P. The molecular signatures database (MSigDB) hallmark gene set collection. *Cell Syst* 2015;**1**:417–25
28. Geraerts I, Van Poppel H, Devoogdt N, Joniau S, Van Cleynenbreugel B, De Groef A, Van Kampen M. Influence of preoperative and postoperative pelvic floor muscle training (PFMT) compared with postoperative PFMT on urinary incontinence after radical prostatectomy: a randomized controlled trial. *Eur Urol* 2013;**64**:766–72
29. Schaefer MH, Lopes TJ, Mah N, Shoemaker JE, Matsuoka Y, Fontaine JF, Louis-Jeune C, Eisfeld AJ, Neumann G, Perez-Iratxeta C, Kawaoka Y, Kitano H, Andrade-Navarro MA. Adding protein context to the human protein-protein interaction network to reveal meaningful interactions. *PLoS Comput Biol* 2013;**9**:e1002860
30. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;**13**:2498–504
31. Panneerdoss S, Eedunuri VK, Yadav P, Timilsina S, Rajamanickam S, Viswanadhapalli S, Abdelfattah N, Onyeagucha BC, Cui X, Lai Z, Mohammad TA, Gupta YK, Huang TH, Huang Y, Chen Y, Rao MK. Cross-talk among writers, readers, and erasers of m(6)a regulates cancer growth and progression. *Sci Adv* 2018;**4**:eaar8263
32. Barabasi AL, Gulbahce N, Loscalzo J. Network medicine: a network-based approach to human disease. *Nat Rev Genet* 2011;**12**:56–68
33. Li A, Hou S, Chen J, Jiang Y. Development and validation of a novel Glycolysis-Related risk signature for predicting survival in pancreatic adenocarcinoma. *Clinica Chimica Acta* 2021;**518**:156–61
34. Yang J, Shi W, Zhu S, Yang C. Construction of a 6-gene prognostic signature to assess prognosis of patients with pancreatic cancer. *Medicine* 2020;**99**:e22092
35. Yan X, Wan H, Hao X, Lan T, Li W, Xu L, Yuan K, Wu H. Importance of gene expression signatures in pancreatic cancer prognosis and the establishment of a prediction model. *Cancer Manag Res* 2019;**11**:273–83
36. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008;**40**:499–507
37. Polyak K, Weinberg RA. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 2009;**9**:265–73
38. Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, Skora AD, Luber BS, Azad NS, Laheru D, Biedrzycki B, Donehower RC, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Duffy SM, Goldberg RM, de la Chapelle A, Koshiji M, Bhaijee F, Huebner T, Hruban RH, Wood LD, Cuka N, Pardoll DM, Papadopoulos N, Kinzler KW, Zhou S, Cornish TC, Taube JM, Anders RA, Eshleman JR, Vogelstein B, Diaz LA Jr. PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med* 2015;**372**:2509–20
39. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, Lu S, Kemberling H, Wilt C, Luber BS, Wong F, Azad NS, Rucki AA, Laheru D, Donehower R, Zaheer A, Fisher GA, Crocenzi TS, Lee JJ, Greten TF, Duffy AG, Ciombor KK, Eyring AD, Lam BH, Joe A, Kang SP, Holdhoff M, Danilova L, Cope L, Meyer C, Zhou S, Goldberg RM, Armstrong DK, Bever KM, Fader AN, Taube J, Housseau F, Spetzler D, Xiao N, Pardoll DM, Papadopoulos N, Kinzler KW, Eshleman JR, Vogelstein B, Anders RA, Diaz LA Jr. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. *Science* 2017;**357**:409–13
40. Li T, Fu J, Zeng Z, Cohen D, Li J, Chen Q, Li B, Liu XS. TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res* 2020;**48**: W509–14
41. Stratton MR, Campbell PJ, Futreal PA. The cancer genome. *Nature* 2009;**458**:719–24
42. Stranger BE, Forrest MS, Dunning M, Ingle CE, Beazley C, Thorne N, Redon R, Bird CP, de Grassi A, Lee C, Tyler-Smith C, Carter N, Scherer SW, Tavare S, Deloukas P, Hurles ME, Dermitzakis ET. Relative impact of nucleotide and copy number variation on gene expression phenotypes. *Science* 2007;**315**:848–53
43. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 2009;**462**:315–22
44. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, Cui H, Gabo K, Rongione M, Webster M, Ji H, Potash J, Sabunciyan S, Feinberg AP. The human Colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet* 2009;**41**:178–86
45. Lin DC, Wang MR, Koeffler HP. Genomic and epigenomic aberrations in esophageal squamous cell carcinoma and implications for patients. *Gastroenterology* 2018;**154**:374–89
46. Hoshimoto S, Takeuchi H, Ono S, Sim MS, Huynh JL, Huang SK, Marzese DM, Kitagawa Y, Hoon DS. Genome-wide hypomethylation and specific tumor-related gene hypermethylation are associated with esophageal squamous cell carcinoma outcome. *J Thorac Oncol* 2015;**10**:509–17
47. Li Y, Xiao J, Bai J, Tian Y, Qu Y, Chen X, Wang Q, Li X, Zhang Y, Xu J. Molecular characterization and clinical relevance of m(6)a regulators across 33 cancer types. *Mol Cancer* 2019;**18**:137
48. Bansal H, Yihua Q, Iyer SP, Ganapathy S, Proia DA, Penalva LO, Uren PJ, Suresh U, Carew JS, Karnad AB, Weitman S, Tomlinson GE, Rao MK, Kornblau SM, Bansal S. WTAP is a novel oncogenic protein in acute myeloid leukemia. *Leukemia* 2014;**28**:1171–4
49. Cheng M, Sheng L, Gao Q, Xiong Q, Zhang H, Wu M, Liang Y, Zhu F, Zhang Y, Zhang X, Yuan Q, Li Y. The m(6)a methyltransferase METTL3 promotes bladder cancer progression via AFF4/NF-kappaB/MYC signaling network. *Oncogene* 2019;**38**:3667–80
50. Tanoue T, Morita S, Plichta DR, Skelly AN, Suda W, Sugiura Y, Narushima S, Vlamakis H, Motoo I, Sugita K, Shiota A, Takeshita K, Yasuma-Mitobe K, Riethmacher D, Kaisho T, Norman JM, Mucida D, Suematsu M, Yaguchi T, Bucci V, Inoue T, Kawakami Y, Olle B, Roberts B, Hattori M, Xavier RJ, Atarashi K, Honda K. A defined commensal consortium elicits CD8 T cells and anti-cancer immunity. *Nature* 2019;**565**:600–5
51. Wu YY, Chen JH, Kao JT, Liu KC, Lai CH, Wang YM, Hsieh CT, Tzen JT, Hsu PN. Expression of CD25(high) regulatory T cells and PD-1 in gastric infiltrating CD4(+) T lymphocytes in patients with Helicobacter pylori infection. *Clin Vaccine Immunol* 2011;**18**:1198–201

(Received March 12, 2021, Accepted May 1, 2021)