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RAPID COMMUNICATION

Systematic analysis of clinical relevance and molecular characterization of m^{6}A in COVID-19 patients

N^6-methyladenosine (m^{6}A) is the most prevalent post-transcriptional RNA modification in mRNA and long non-coding RNAs of eukaryotes, and its biological functions are mediated by m^{6}A writers, erasers and readers.[1] A nuclear methyltransferase complex consisting of METTL3, METTL14, WTAP, VIRMA, ZC3H13, RBM15 (or RBM15B), YWHAG, TRA2A and CAPRIN1 catalyzes the m^{6}A modifications, acting as m^{6}A writers.[1] m^{6}A demethylase ALKBH5 as well as m^{6}A demethylase FTO mediate the demethylation of m^{6}As, acting as the m^{6}A erasers.[1] A variety of proteins including YTH domain-containing proteins can bind m^{6}A marks as the m^{6}A readers. The role of mRNA m^{6}A methylation in COVID-19 patients is of great concern[2,3] due to reports that m^{6}A may provide potential new strategies for the development of vaccine and antiviral drug. For instance, Jun'e et al reported that m^{6}A regulators regulate m^{6}A during SARS-CoV-2 infection in Huh7 cells.[3] Other studies also show that m^{6}A regulators METTL3 and RBM15 are able to regulate host cell innate immune responses during SARS-CoV-2 infection in Caco-2 cells and HuT 78 cells, respectively.[2] Hannah et al found that METTL3 affects SARS-CoV-2 replication in A549 cells, and they found that targeting the m^{6}A RNA modification pathway can block SARS-CoV-2 replication.[2] Although many studies have focused on the function and molecular mechanism of m^{6}A in cell lines infected with SARS-CoV-2, the clinical relevance and basic molecular characterization of m^{6}A *in vivo* have been neglected, which deserves further exploration.

One recent study by Katherine et al measured RNA-seq data in a population with current largest sample size (126) and complete clinical data, therefore we used their data for our analysis of clinical relevance with m^{6}A in COVID-19 patients (https://www.ncbi.nlm.nih.gov/sra/, SRP279280).

To explore molecular characterization of m^{6}A in real COVID-19 patients, we obtained peripheral blood mononuclear cells (PBMCs) from two COVID-19 patients and two control subjects for vaccination over the same period, and performed the m^{6}A sequencing of the RNAs isolated from PBMCs. These two COVID-19 patients received intensive care unit (ICU) care, and PBMCs from two COVID-19 samples were collected before recovery. Two vaccine recipients received two doses of BBIBP-CorV developed by the Beijing Institute of Biological Products (Beijing, China), manufactured as previously described.[4] The raw sequencing reads of m^{6}A-seq have been deposited in Genome Sequence Archive (GSA) for Human under the accession code PRJNA753626.

RNA-seq reads were mapped to rRNA sequences to remove potential rRNA reads and then mapped to the human genome (hg38) with GENCODE gene annotation (v32) following the guideline of ENCODE RNA-seq pipeline (https://github.com/ENCODE-DCC/long-rna-seq-pipeline).[5] The mapping results were visualized using the Integrative Genomics Viewer (IGV) tool. We used StringTie (v1.3.4d) to calculate the TPMs (Transcripts Per Million) of each sample.[5] Gene Ontology analysis was performed using DAVID.[5] Differential gene expression analyses were performed based on the input data using DESeq2.[5]

m^{6}A peaks were identified according to the methods as described previously.[1] In brief, we made sliding windows of 100 bp with 50 bp overlap on the exon regions and calculated the RPKM (Reads Per Kilobase Million) of each window. The sliding windows with winscore (enrichment score) $>$ 2 were identified as m^{6}A peaks. The m^{6}A ratio of each m^{6}A peak was calculated as the RPKM (without adding 1) of IP library divided by the RPKM (without adding 1) of input library. m^{6}A ratios based on the denominators

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(peak RPKM of input) < 5 were treated as NAs (not available) in the downstream analyses.

In the analysis of the RNA-seq data and clinical data in 100 COVID-19 patients and 26 non-COVID-19 patients, we observed the expression levels of most m^{6}A regulators increased in COVID-19 patients (Fig. 1A, B). ALKBH5 and FTO are specific m^{6}A demethylase,[1] which tend to regulate site-specific m^{6}A rather than global m^{6}A,[1] it was the reason for METTL3 and FTO both increased in COVID group. In order to further investigate the clinical relevance of m^{6}A in COVID-19, we conducted a principal component analysis (PCA) on expression levels of m^{6}A regulators, and constructed m^{6}A signatures as described previously[5] (Fig. 1C). Two significant components were identified to explain 81% of the m^{6}A regulator variation. The first principal component (PC) mainly separates COVID-19 patients with non-COVID-19 patients in m^{6}A regulators (Fig. 1C). Then, first principal component was selected to use as m^{6}A signature scores to predict patient's COVID-19 clinical characteristics as described previously.[5] As shown in Figure 1D, m^{6}A signature scores vary in different age groups. In addition, there were significant trends for increasing m^{6}A signature scores with decreasing value of Charlson score, indicating that m^{6}A modification may play an important role in clinical status of COVID-19 patients. Moreover, patients in the intensive care unit (ICU) have lower m^{6}A signature scores, which is consistent with the result of ICU in Figure 1A. Receiver operating characteristic (ROC) curves were constructed to compare the classification accuracy of the m^{6}A signature scores and transcriptional signatures for distinguishing between COVID-19 and non-COVID-19 patients, ICU and non-ICU patients with COVID-19 (Fig. 1E). Among these, m^{6}A signature showed the higher AUC value (0.78, 0.66) than scores and transcriptional signatures (0.72, 0.63) for distinguishing between COVID-19 and non-COVID-19 patients, ICU and non-ICU patients with COVID-19. Even transcriptional signatures can identify the risk of COVID-19 progression. Due to that m^{6}A regulates mRNA stability and translation, so m^{6}A gave better predictions than the transcriptional signatures.

In general, m^{6}A regulators regulate gene expression through regulating the m^{6}A levels of key genes in host cells. To further investigate molecular characterization of control subjects and COVID-19 patients' m^{6}A *in vivo*, we analyzed the PBMC m^{6}A-RNA-sequence (m^{6}A-seq) data from two COVID-19 patients and two control subjects. We found that the m^{6}A peaks in control subjects were highly enriched near stop codons (Fig. S1A). GGACU motif was enriched in m^{6}A sites in COVID-19 patients. However, more [GA][GA]C[UAC] motifs were enriched in m^{6}A sites in control subjects (Fig. S1B). We obtained about 10,000 m^{6}A peaks for control subjects and COVID-19 patients, and there are 13,823 different m^{6}A peaks between control subjects and COVID-19 patients (Table S1, and Fig. S1C). As shown in Figure S1D, most m^{6}A targeted gene are differently expressed genes, suggesting that the changes of m^{6}A affect the expression levels of host transcripts in COVID-19 patients. The Gene ontology (GO) enrichment analysis on differently expressed genes regulated by m^{6}A indicates that the top regulated functions include viral genome replication, regulation of Ras protein signal transduction, and GTPase mediated signal transduction (Fig. S1E), which are consistent with the results of one previous study.[2] In that study, the mechanism of m^{6}A affecting SARS-CoV-2 replication is verified by wet experiments *in vitro*.[2] Furthermore, the pathway enrichment analysis show that immune functions were disturbed (Fig. S1F).

Taken together, our study reported that m^{6}A is associated with multiple clinical state of COVID-19 patients, supporting the strategy that m^{6}A could be act as a therapeutic target for COVID-19 patients. In fact, one previous study has shown that a highly specific METTL3 inhibitor is able to inhibit the replication of SARS-CoV-2.[2] Moreover, m^{6}A-seq technique is the most widely used method to identify the methylation level of m^{6}A on a large scale and with high throughput at the transcriptional level. However, m^{6}A-seq is not applied on patients with COVID-19 *in vivo* before. We firstly used m^{6}A-seq data to analyze m^{6}A characteristics of COVID-19 patients, not only revealing the molecular characteristics of m^{6}A *in vivo* rather than *in vitro*, but also providing valuable resources for future research on m^{6}A in COVID-19. In addition, the different m^{6}A characteristics between COVID-19 patients and vaccinated control subjects indicated m^{6}A may be involved in mechanism of breaking-vaccination infection, which is also an interesting research topic in the future.

Author contributions

SQA, LY and HL designed this study. SQA, YQL and YL performed data collections. SQA, WFY wrote the manuscript. WD, JJ, JL and FY revised the manuscript. WXL, HL and JL directed and supervised the project. All authors read and approved the final manuscript.

Conflict of interests

Authors declare no conflict of interests.

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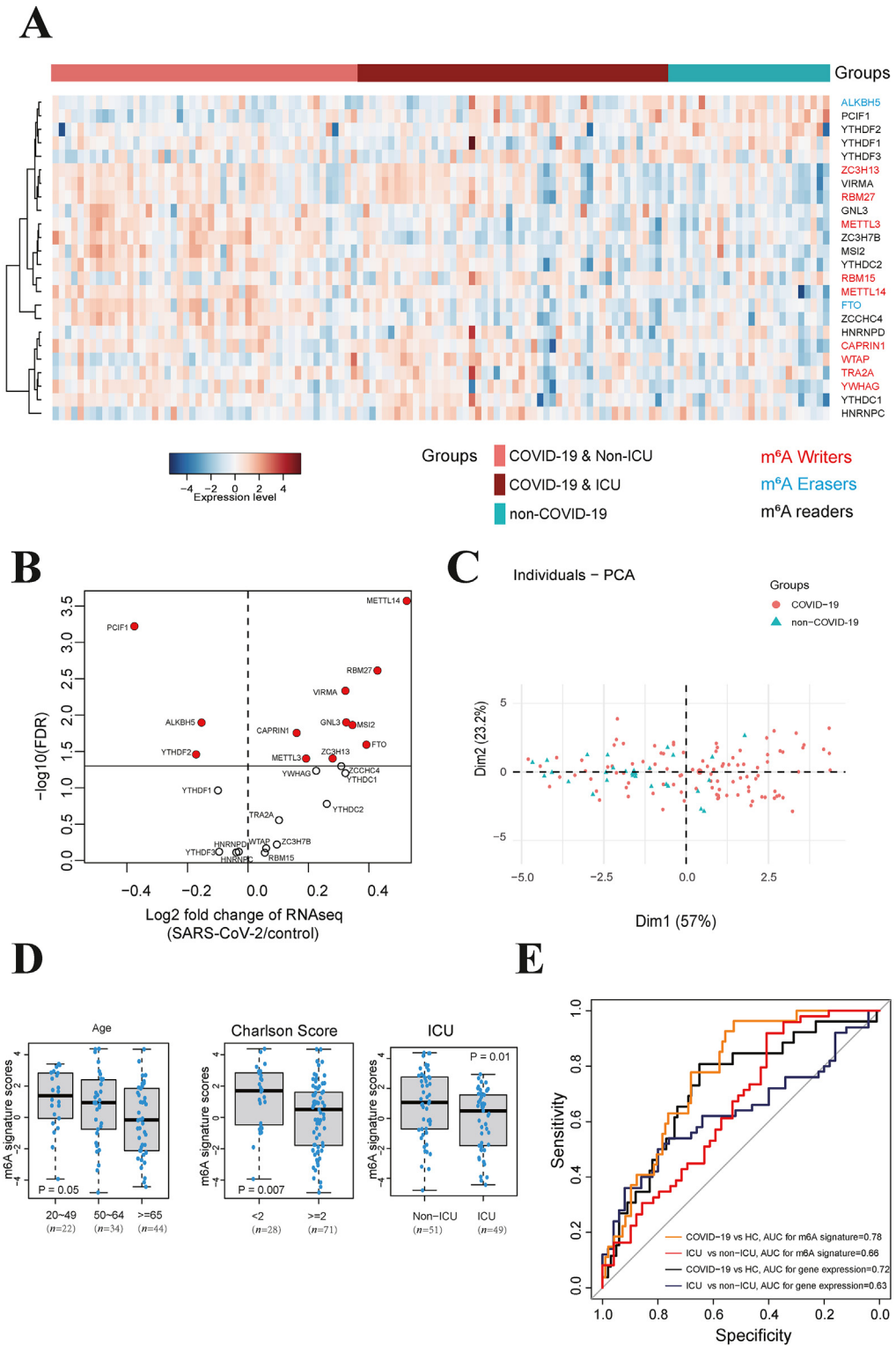

Figure 1 The expression level of most m^{6}A regulators is changed in COVID-19 patients. **(A)** Heatmaps representing the Z scores of gene expression of the m^{6}A regulators in COVID-19 patients PBMC (including ICU and non-ICU group). **(B)** Volcano plot of m^{6}A regulators' expression level in COVID-19 patients. **(C)** Principal component analysis (PCA) of expression level of m^{6}A regulators **(D)** Patients are classified according to different clinical features including different age, different Charlson score, different ICU status. Differences of m^{6}A signature scores between groups was analyzed. The two-tailed Wilcoxon test was used to assess the significance of differences between two subtypes. **(E)** Area under the receiver operating characteristic curve (AUROCC) of m^{6}A signature as well as differently gene expression in discrimination among COVID-19 patients and non-COVID-19, ICU patient and non-ICU patients with COVID-19.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.12.005.

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