



RAPID COMMUNICATION

Systematic analysis of clinical relevance and molecular characterization of m⁶A in COVID-19 patients



N⁶-methyladenosine (m⁶A) is the most prevalent post-transcriptional RNA modification in mRNA and long non-coding RNAs of eukaryotes, and its biological functions are mediated by m⁶A 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 m⁶A modifications, acting as m⁶A writers.¹ m⁶A demethylase ALKBH5 as well as m⁶A demethylase FTO mediate the demethylation of m⁶As, acting as the m⁶A erasers.¹ A variety of proteins including YTH domain-containing proteins can bind m⁶A marks as the m⁶A readers. The role of mRNA m⁶A methylation in COVID-19 patients is of great concern^{2,3} due to reports that m⁶A may provide potential new strategies for the development of vaccine and antiviral drug. For instance, Jun'e et al reported that m⁶A regulators regulate m⁶A during SARS-CoV-2 infection in Huh7 cells.³ Other studies also show that m⁶A 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 m⁶A RNA modification pathway can block SARS-CoV-2 replication.² Although many studies have focused on the function and molecular mechanism of m⁶A in cell lines infected with SARS-CoV-2, the clinical relevance and basic molecular characterization of m⁶A *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 m⁶A in COVID-19 patients (<https://www.ncbi.nlm.nih.gov/sra/>, SRP279280).

To explore molecular characterization of m⁶A 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 m⁶A 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 m⁶A-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

m⁶A 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 m⁶A peaks. The m⁶A ratio of each m⁶A peak was calculated as the RPKM (without adding 1) of IP library divided by the RPKM (without adding 1) of input library. m⁶A ratios based on the denominators

Peer review under responsibility of Chongqing Medical University.

<https://doi.org/10.1016/j.gendis.2021.12.005>

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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 m⁶A regulators increased in COVID-19 patients (Fig. 1A, B). ALKBH5 and FTO are specific m⁶A demethylase,¹ which tend to regulate site-specific m⁶A rather than global m⁶A,¹ it was the reason for METTL3 and FTO both increased in COVID group. In order to further investigate the clinical relevance of m⁶A in COVID-19, we conducted a principal component analysis (PCA) on expression levels of m⁶A regulators, and constructed m⁶A signatures as described previously⁵ (Fig. 1C). Two significant components were identified to explain 81% of the m⁶A regulator variation. The first principal component (PC) mainly separates COVID-19 patients with non-COVID-19 patients in m⁶A regulators (Fig. 1C). Then, first principal component was selected to use as m⁶A signature scores to predict patient's COVID-19 clinical characteristics as described previously.⁵ As shown in Figure 1D, m⁶A signature scores vary in different age groups. In addition, there were significant trends for increasing m⁶A signature scores with decreasing value of Charlson score, indicating that m⁶A modification may play an important role in clinical status of COVID-19 patients. Moreover, patients in the intensive care unit (ICU) have lower m⁶A 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 m⁶A 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, m⁶A 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 m⁶A regulates mRNA stability and translation, so m⁶A gave better predictions than the transcriptional signatures.

In general, m⁶A regulators regulate gene expression through regulating the m⁶A levels of key genes in host cells. To further investigate molecular characterization of control subjects and COVID-19 patients' m⁶A *in vivo*, we analyzed the PBMC m⁶A-RNA-sequence (m⁶A-seq) data from two COVID-19 patients and two control subjects. We found that the m⁶A peaks in control subjects were highly enriched near stop codons (Fig. S1A). GGACU motif was enriched in m⁶A sites in COVID-19 patients. However, more [GA][GA]C[UAC] motifs were enriched in m⁶A sites in control subjects (Fig. S1B). We obtained about 10,000 m⁶A peaks for control subjects and COVID-19 patients, and there are 13,823 different m⁶A peaks between control subjects and COVID-19 patients (Table S1, and Fig. S1C).

As shown in Figure S1D, most m⁶A targeted gene are differently expressed genes, suggesting that the changes of m⁶A affect the expression levels of host transcripts in COVID-19 patients. The Gene ontology (GO) enrichment analysis on differently expressed genes regulated by m⁶A 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 m⁶A 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 m⁶A is associated with multiple clinical state of COVID-19 patients, supporting the strategy that m⁶A 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, m⁶A-seq technique is the most widely used method to identify the methylation level of m⁶A on a large scale and with high throughput at the transcriptional level. However, m⁶A-seq is not applied on patients with COVID-19 *in vivo* before. We firstly used m⁶A-seq data to analyze m⁶A characteristics of COVID-19 patients, not only revealing the molecular characteristics of m⁶A *in vivo* rather than *in vitro*, but also providing valuable resources for future research on m⁶A in COVID-19. In addition, the different m⁶A characteristics between COVID-19 patients and vaccinated control subjects indicated m⁶A 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.

Funding

This work was supported by China Postdoctoral Science Foundation (No. 2020M683623XB); National Natural Science Foundation of China (No. 82160389); Guangxi Medical University Training Program for Distinguished Young Scholars (to Junjun Jiang); Guangxi Science Fund for Distinguished Young Scholars (No. 2018GXNSFFA281001).

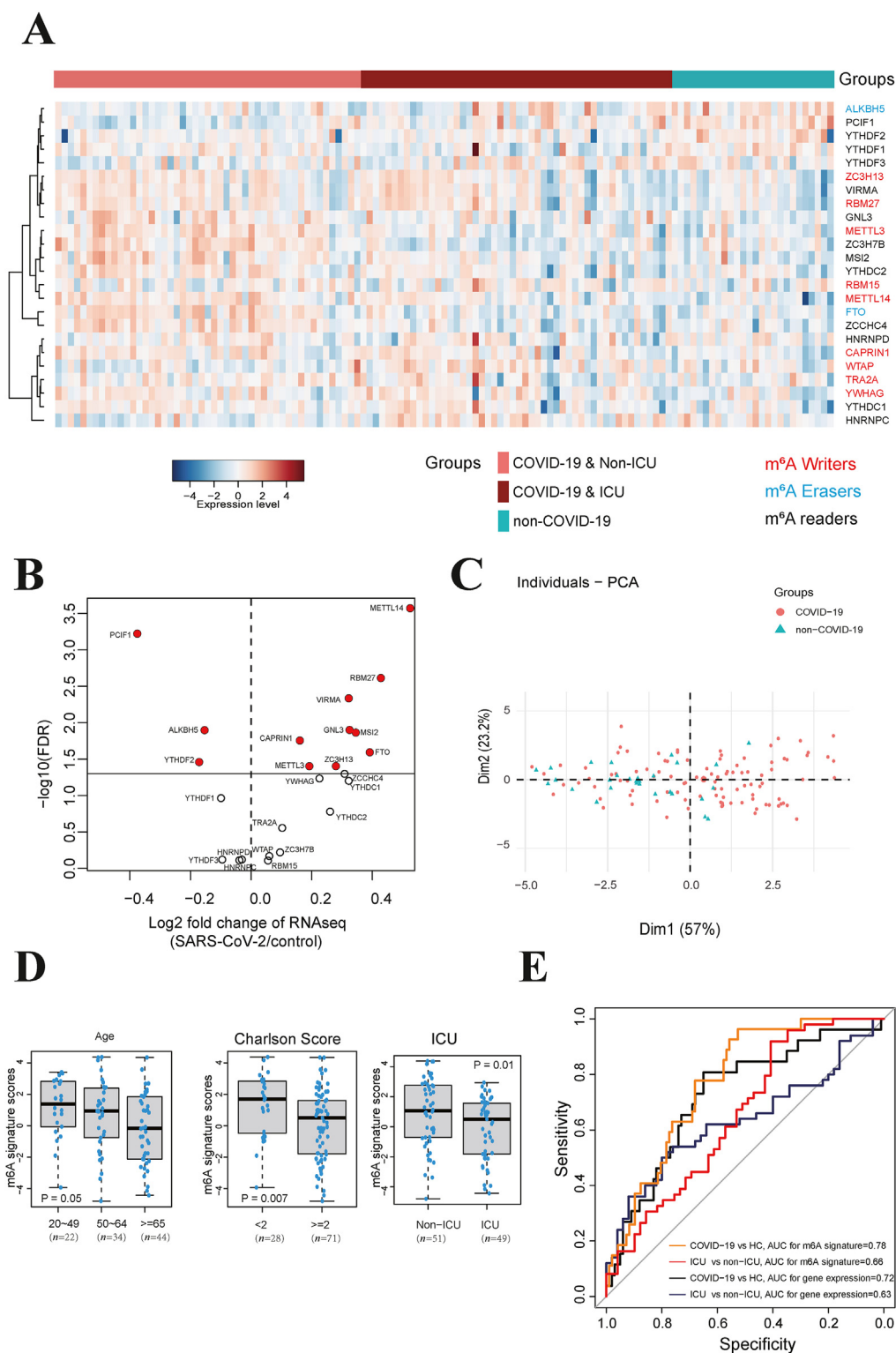


Figure 1 The expression level of most m⁶A regulators is changed in COVID-19 patients. **(A)** Heatmaps representing the Z scores of gene expression of the m⁶A regulators in COVID-19 patients PBMC (including ICU and non-ICU group). **(B)** Volcano plot of m⁶A regulators' expression level in COVID-19 patients. **(C)** Principal component analysis (PCA) of expression level of m⁶A regulators **(D)** Patients are classified according to different clinical features including different age, different Charlson score, different ICU status. Differences of m⁶A 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 (AUROC) of m⁶A 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.

Acknowledgements

We thank Hailong Wang, Peijiang Pan, Yao Lin and Jingyi Li for the suggestions in preparing the manuscript.

Appendix A. Supplementary data

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

References

1. An S, Huang W, Huang X, et al. Integrative network analysis identifies cell-specific trans regulators of m6A. *Nucleic Acids Res.* 2020;48(4):1715–1729.
2. Burgess HM, Depledge DP, Thompson L, et al. Targeting the m(6) A RNA modification pathway blocks SARS-CoV-2 and HCoV-OC43 replication. *Genes Dev.* 2021;35(13–14):1005–1019.
3. Liu J, Xu YP, Li K, et al. The m(6)A methylome of SARS-CoV-2 in host cells. *Cell Res.* 2021;31(4):404–414.
4. Xia S, Zhang Y, Wang Y, et al. Safety and immunogenicity of an inactivated SARS-CoV-2 vaccine, BBIBP-CorV: a randomised, double-blind, placebo-controlled, phase 1/2 trial. *Lancet Infect Dis.* 2021;21(1):39–51.
5. An S, Li Y, Lin Y, et al. Genome-wide profiling reveals alternative polyadenylation of innate immune-related mRNA in patients with COVID-19. *Front Immunol.* 2021;12:756288.

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14 September 2021

Available online 5 January 2022

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