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Analysis of RNA modifications in peripheral white blood cells from breast cancer patients by mass spectrometry

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ABSTRACT

RNA modifications play vital regulatory roles in biological systems. Dysregulated RNA modifications themselves or their regulators are associated with various diseases, including cancers and immune related diseases. However, to the best of our knowledge, RNA modifications in peripheral white blood cells (immune cells) have not been systematically investigated before. Here we utilized hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) for the quantification of 19 chemical modifications in total RNA and 17 chemical modifications in small RNA in peripheral white blood cells from breast cancer patients and healthy controls. We found out 13 RNA modifications were up-regulated in total RNA samples of breast cancer patients. For small RNA samples, only N^6-methyladenosine (m^6A) was down-regulated in breast cancer patients ($P < 0.0001$). Receiver operating characteristic (ROC) curves analysis showed that N^4-acetylcytidine (ac^4C) in total RNA had an area under curve (AUC) value of 0.833, and m^6A in small RNA had an AUC value of 0.994. Our results further illustrated that RNA modifications may play vital roles in immune cell biology of breast cancer, and may act as novel biomarkers for the diagnosis of breast cancer.

Beyond the classical central dogma of molecular biology, DNA modifications, RNA modifications and protein post-transcriptional modifications play vital roles in biological systems [1]. More than 170 RNA modifications have been reported [2,3], and novel RNA modifications are discovered almost every year [4–6]. Especially, small RNAs (<200 nt, mainly tRNAs) are considered heavily modified [7–9], and these modifications are related to the diagnosis and treatment of human diseases [10]. Increasing evidence illustrates that the levels of RNA modifications themselves or related proteins (writers, readers and erasers) are dysregulated in various diseases, especially cancers [11–16].

Blood can be separated into different fractions, for example, serum or plasma, platelets or circulating tumor cells (CTCs), in order to enrich for tumor biomarkers [17]. Mass spectrometry is a powerful tool for the identification and quantification of RNA modifications [18,19]. Numerous studies have been conducted on free modified nucleosides in the serum or plasma from patients with different cancer types [20]. RNA modifications in blood have also been shown to be related to various physiological or pathological processes, for example, aging [21], adolescent alcohol exposure [22], smoking and air pollution [23], colorectal cancer [24,25] and pulmonary hypertension [26]. RNA modifications in CTCs of lung cancer patients have also been investigated [27].

However, to the best of our knowledge, RNA modifications in peripheral white blood cells of breast cancer patients have not been systematically investigated before. Considering white blood cells are parts of the immune system, and RNA modifications are involved in immune cell biology and cancer immunity [28–30], it is important and desirable to investigate RNA modifications in peripheral white blood cells.

In this work, we aimed to study RNA modifications in total RNA and small RNA in peripheral white blood cells from breast cancer patients. As shown in Fig. 1, total RNA was extracted using Trizol reagent, and small RNA was extracted using a commercially avail-

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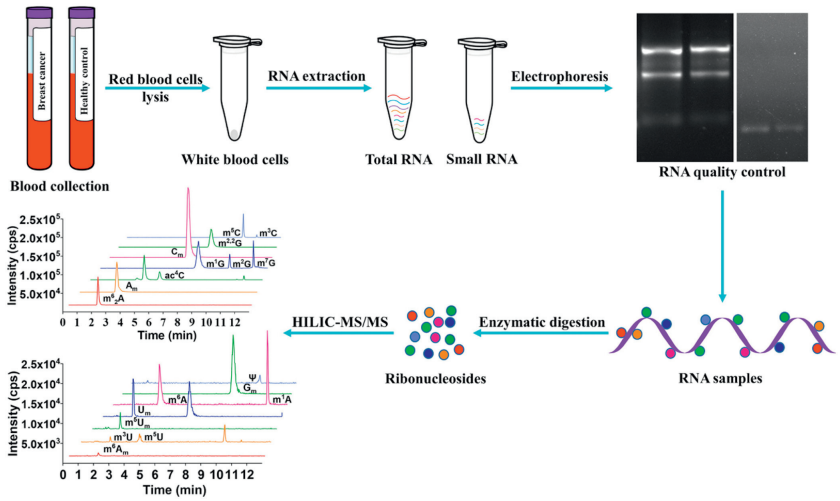

Fig. 1. Workflow for the analysis of RNA modifications in peripheral white blood cells from breast cancer patients. The procedure involves collection of blood samples, red blood cells lysis for the isolation of white blood cells, total RNA and small RNA (<200 nt) extraction, agarose gel electrophoresis for RNA quality control, enzymatic digestion into ribonucleosides and HILIC-MS/MS quantification. A_m, 2′-*O*-methyladenosine; m^1A, N^1-methyladenosine; m^6A, N^6-methyladenosine; m^6A_m, N^6,2′-*O*-dimethyladenosine; $m^6{}_2A$, N^6,N^6-dimethyladenosine; G_m, 2′-*O*-methylguanosine; m^1G, N^1-methylguanosine; m^2G, N^2-methylguanosine; m^7G, N^7-methylguanosine; $m^{2,2}G$, N^2,N^2-dimethylguanosine; C_m, 2′-*O*-methylcytidine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; ac^4C, N^4-acetylcytidine; U_m, 2′-*O*-methyluridine; m^3U, 3-methyluridine; m^5U, 5-methyluridine; m^5U_m, 5,2′-*O*-dimethyluridine; ψ, pseudouridine.

able kit. The purities of RNA samples were verified by agarose gel electrophoresis. Total RNA and small RNA samples were enzymatically digested into ribonucleosides. Then, hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) was used for the quantification of these RNA modifications.

Firstly, we used Trizol reagent for extraction of total RNA from whole blood cells. However, we found out RNA was degraded and DNA was mainly obtained from blood samples whether frozen at −80 °C or freshly collected (Fig. S1 in Supporting information). This might be attributed to the presence of abundant RNases in red blood cells [31]. With the help of red blood lysis, we could remove red blood cells before RNA extraction. And high purity RNA samples were obtained based on non-denaturing agarose gel electrophoresis (Fig. S2 in Supporting information). Small RNA samples were isolated by a commercially available kit, the extraction process was repeated once in order to guarantee their purity, and the purities of small RNA samples were also evaluated by non-denaturing agarose gel electrophoresis (Fig. S2). Detailed protocols for the isolation of total RNA and small RNA were shown in Supporting information.

For enzymatic digestion, about 1 μg of total RNA or 500 ng of small RNA samples were digested into ribonucleosides with the help of nuclease P1, phosphodiesterase 2 and antarctic phosphatase, and detailed protocols were shown in the Supporting information [32]. HILIC-MS/MS was used for the detection and quantification of these RNA modifications based on Acquity UPLC system and QTRAP 4000 mass spectrometer. Optimized mobile phase elution gradient and MRM parameters were shown in materials and methods section and Table S1 (Supporting information) [33–35].

The chemical structures and detailed information of RNA modifications and their isotope-labeled internal standards tested in this study were shown in Fig. S3, Fig. S4 and Table S2 (Supporting information), respectively. Compared with retention time of their isotope-labeled standards (for A_m, m^1A, m^6A, $m^6{}_2A$, G_m, m^1G, m^7G, $m^{2,2}G$, C_m, m^5C, ac^4C, U_m, m^3U, m^5U and ψ) or standards (for m^6A_m, m^2G, m^3C and m^5U_m), we could detect 19 RNA modifications in total RNA samples (A_m, m^1A, m^6A, m^6A_m, $m^6{}_2A$, G_m, m^1G, m^2G, m^7G, $m^{2,2}G$, C_m, m^3C, m^5C, ac^4C, U_m, m^3U, m^5U, m^5U_m and ψ), and 18 RNA modifications in small RNA samples (except m^6A_m, compared with modifications in total RNA) (Fig. 2). In order to exclude the contamination from enzymes, enzyme blank experiments were carried out and the results indicated these RNA modifications were not detected in the enzyme blank samples (Fig. S5 in Supporting information).

To realize accurate quantitative analysis, calibration curves of canonical ribonucleosides (adenosine (A), guanosine (G), cytidine (C) and uridine (U)) and these RNA modifications were built by serial dilution of standards and addition of isotope-labeled internal standards (detailed methods in Supporting information). For those without isotope-labeled standards, $[D_3]A_m$, $[D_6]m^{2,2}G$, $[^{13}CD_3]m^5C$ and $[D_3]U_m$ were used as internal standards for the quantification of m^6A_m, m^2G, m^3C and m^5U_m, respectively. Calibration curves had excellent linearities, with R^2 values larger than 0.998 (Table S3 in Supporting information). Limits of detection (LODs) and limits of quantification (LOQs) were in the range of 0.02–40 nmol/L and 0.05–80 nmol/L (Table S3), based on signal-to-noise ratio (S/N) of 3 and 10, respectively. In order to validate the developed method, accuracy and precision were tested. The intra-day accuracy values ranged from 81.0% to 112.9%, and the intra-day precision, as reflected by relative standard deviation (RSD), was within 8.6%. The inter-day accuracy values ranged from 80.7% to 112.6%, and the inter-day precision was within 11.8% (Table S4 in Supporting information).

Blood samples were obtained from the Second Affiliated Hospital, Zhejiang University School of Medicine (SAHZU). An approval was granted by the Medical Ethics Committee of SAHZU and subjects signed an informed consent form. We obtained total RNA samples from the white blood cells of 42 breast cancer patients and 43 healthy controls, and small RNA samples from 17 breast cancer patients and 20 healthy controls. Blood samples from breast cancer patients were obtained before surgery and without radiation or chemotherapy. Details of these samples were shown in Tables S5 and S6 (Supporting information). By using the established and validated method, we quantified RNA modifications in these samples.

As for total RNA in peripheral white blood cells, most of the quantified RNA modifications were up-regulated in breast cancer patients compared with healthy controls (Fig. 3). The statistical analysis revealed that among these RNA modifications, m^6A_m,

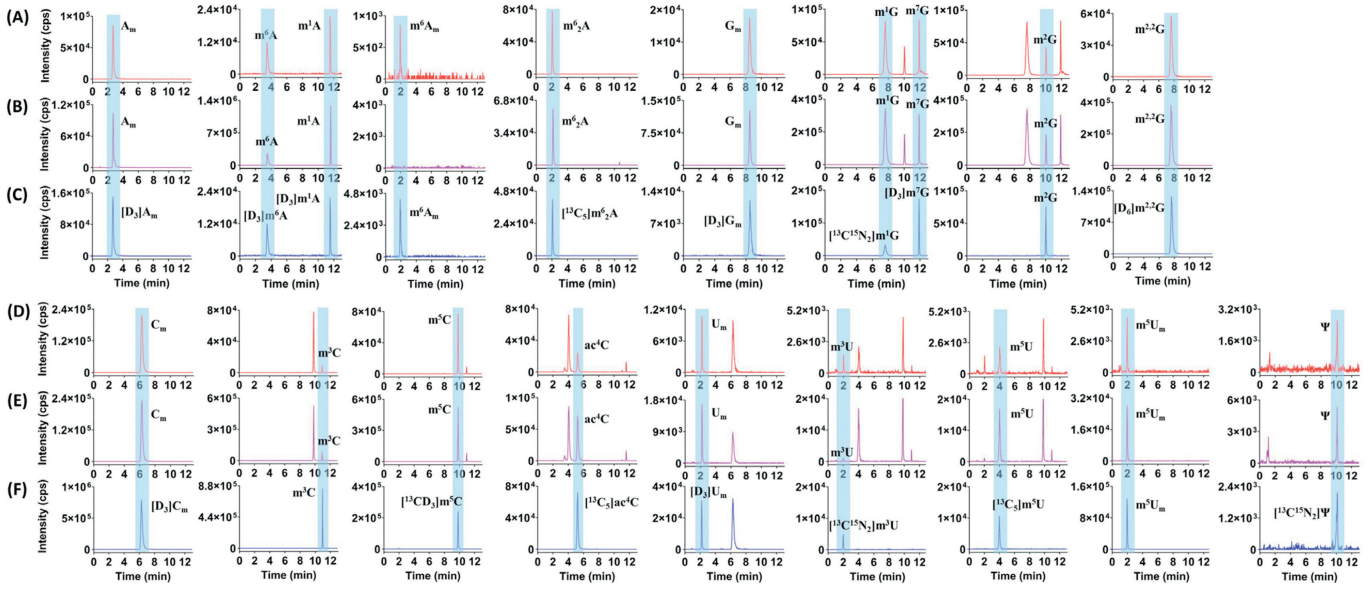

Fig. 2. Extraction-ion chromatograms of RNA modifications of white blood cells in total RNA and small RNA (<200 nt) samples from breast cancer patients. RNA modifications in total RNA (A, D), small RNA (B, E) and isotope-labeled internal standards or standards (C, F).

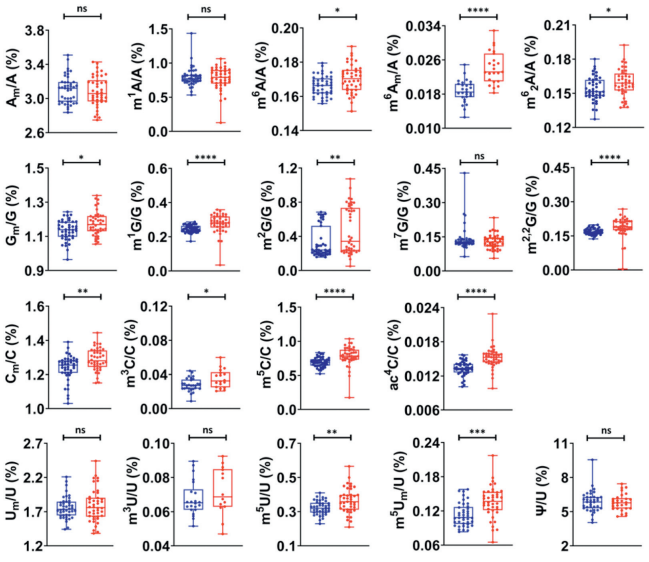

Fig. 3. Total RNA modification alterations in white blood cells of breast cancer patients (red) and normal controls (blue). m^6A_m, m^3C, m^3U, m^5U, m^5U_m and Ψ could not be quantified in all samples. *P* values were based on the Mann-Whitney test. ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

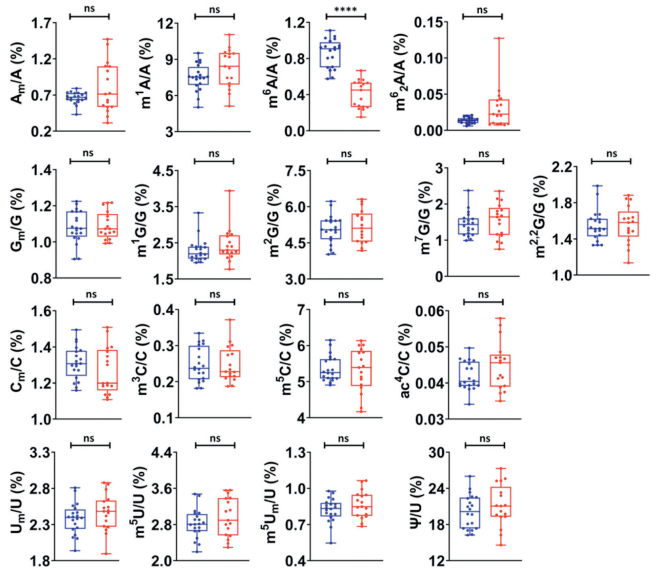

Fig. 4. Small RNA modification alterations in white blood cells of breast cancer patients (red) and normal controls (blue). m^6A_m couldnot be detected, and m^3U could be detected but couldnot be quantified in small RNA samples. *P* values were based on the Mann-Whitney test. ns, $P > 0.05$; ****, $P < 0.0001$.

m^1G, $m^{2,2}G$, m^5C, and ac^4C had *P* values less than 0.0001; m^5U_m had *P* values less than 0.001; m^2G, C_m and m^5U had *P* values less than 0.01; m^6A, $m^6{}_2A$, G_m and m^3C had *P* values less than 0.05.

As for small RNA in peripheral white blood cells, only m^6A was down-regulated in small RNA samples from breast cancer patients ($P < 0.0001$), and other modifications in small RNA had no significant difference between breast cancer patients and healthy controls (Fig. 4).

In addition, we found small RNA samples had relatively higher levels of m^1A, m^6A, m^3C, m^5C, ac^4C, m^1G, m^2G, m^7G, $m^{2,2}G$, m^5U, m^5U_m and Ψ, lower levels of A_m and $m^6{}_2A$, and undetectable level of m^6A_m, compared with total RNA (Figs. 3 and 4 and Table S7 in Supporting information). These results were consistent with previously reported relative levels of these modifications in small RNA or tRNA, compared with their levels in total RNA [36–39], which further confirmed the purity of the small RNA samples we extracted.

We further performed receiver operating characteristic (ROC) curves analysis. It is interesting that ac^4C in total RNA had an area under curve (AUC) value of 0.833, and m^6A in small RNA had an AUC value of 0.994 (Fig. 5). AUC values of other significantly changed modifications in total RNA were shown in Fig. S6 (Supporting information). The combination of a panel of significantly changed modifications in total RNA ($m^6A + m^6{}_2A + G_m + m^1G + m^2G + m^{2,2}G + C_m + m^5C + ac^4C$) had an AUC value of 0.892 (Fig. 5). m^6A_m, m^5U_m, m^3C and m^5U were excluded from total RNA diagnostic panel because they couldnot be quantified in all total RNA samples (Fig. 3).

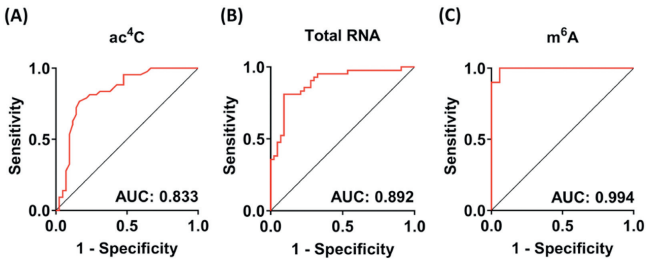

Fig. 5. ROC curves of ac^4C in total RNA (A), the combination of a panel of total RNA modifications ($m^6A + m^6{}_2A + G_m + m^1G + m^2G + m^{2,2}G + C_m + m^5C + ac^4C$) (B), and m^6A in small RNA samples (C) for the discrimination of breast cancer patients and healthy controls.

We clarified 42 breast cancer patients into 27 early stage breast cancer (grades 0 and I) and 15 locally advanced breast cancer (groups II and III) [40], and compared total RNA modification levels in the two groups. Only ac^4C had significant differences between the two groups ($P < 0.01$), based on the Mann-Whitney test. Locally advanced breast cancer patients had lower levels of ac^4C, compared with early stage breast cancer. Based on One-way ANOVA analysis, ac^4C may act as a biomarker for early stage breast cancer (Fig. S7 in Supporting information).

These discoveries facilitated us to think about RNA modifications in immune cells and cancer biology. Although the roles of RNA modifications in breast cancer immunity are not well understood, various modifications such as m^1A, m^6A, m^5C, ac^4C, m^7G and Ψ have been reported to play diverse roles in different aspects of immune cell biology [28]. As the most extensively investigated RNA modification [13,41], m^6A has been shown to play vital roles in tumor immune microenvironment (TIME) and cancer progression [41,42]. For example, loss of function of METTL3 (a writer protein for the formation of m^6A) has been found to suppress colorectal cancer cell growth through inhibition of the accumulation of myeloid-derived suppressor cells [43]. Besides, m^6A regulators play important roles in antitumor immune response [44]. In addition, ac^4C has been reported existing in tRNA, rRNA and mRNA [45,46], and recent discoveries have found out ac^4C could facilitate cervical cancer progression and immunosuppression [47]. Synthetic mRNA with ac^4C modification has been shown to be less inflammatory to immune cells than cytidine [48]. m^5C has also been shown to play important roles in TIME, and related regulators may be valuable as biomarkers for the diagnosis and prognosis of cancers [49]. m^6A_m is part of cap structure in eukaryotic mRNA, and it is important in the immunorecognition of self and non-self [50]. m^5U_m in tRNA has been reported to decrease immune response [51]. Furthermore, m^1A, m^5C, ac^4C, m^7G and Ψ are found to be related to immune cell infiltration in cancers [41]. RNA with modifications like m^5C, m^6A, m^5U, 2-thiouridine (s^2U) and Ψ can help to get rid of organismic immune response [52]. Other significantly changed modifications in this study, such as $m^6{}_2A$, G_m, m^1G, m^2G, $m^{2,2}G$, C_m and m^3C, may be less investigated in immune cell biology and cancer immunity, and future work need to be done to further understand their roles in these aspects.

As for future work, we should use a larger sample size to validate the ability of RNA modifications in peripheral white blood cells as biomarkers for detection of breast cancer. Besides, RNA modifications in white blood cells may be used for the differentiation of different subtypes or stages of breast cancer patients. Furthermore, the investigation of RNA modifications in different subtypes of immune cells (*e.g.*, $CD4^+$ T and $CD8^+$ T lymphocytes, B lymphocytes, dendritic cells, monocytes or macrophages) is desirable and may benefit clinical practice [28,53]. In future studies, it would be also valuable to investigate RNA modifications in other types of blood RNA, such as poly(A)-tailed RNA (mainly mRNA), diverse rRNAs (5S, 5.8S, 18S and 28S rRNA) and small RNA (10–50 nt).

In conclusion, we extracted high quality total RNA and small RNA samples (confirmed by gel electrophoresis) in peripheral white blood cells from breast cancer patients and healthy controls. By using our established and validated HILIC-MS/MS method, we quantified 19 RNA modifications in total RNA samples and 17 RNA modifications in small RNA samples. 13 RNA modifications were found to be up-regulated in total RNA samples from breast cancer patients, especially for ac^4C, with a $P < 0.0001$ and an AUC value of 0.833. As for small RNA, only m^6A has significant difference between breast cancer patients and healthy controls, with a $P < 0.0001$ and an AUC value of 0.994. This work may contribute to not only the discovery of novel biomarkers based on RNA modifications in peripheral white blood cells, but also the deep understanding of RNA modifications in immune cell biology and cancer immunity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Keqiang Shi: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Xiujuan Hong:** Methodology, Investigation, Formal analysis, Data curation. **Dongyan Xu:** Resources. **Tao Pan:** Resources. **Huiwen Wang:** Writing – review & editing. **Hongru Feng:** Writing – review & editing. **Cheng Guo:** Writing – review & editing, Supervision, Funding acquisition. **Yuanjiang Pan:** Writing – review & editing, Supervision, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2024.110079.

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