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# RNA sequencing enables systematic identification of platelet transcriptomic alterations in NSCLC patients

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ABSTRACT

Platelets are implicated as key players in the metastatic dissemination of tumor cells. Previous evidence demonstrated platelets retained cytoplasmic RNAs with physiologically activity, splicing pre-mRNA to mRNA and translating into functional proteins in response to external stimulation. Recently, platelets gene profile of healthy or diseased individuals were characterized with the help of RNA sequencing (RNA-Seq) in some studies, leading to new insights into the mechanisms underlying disease pathogenesis. In this study, we performed RNA-seq in platelets from 7 healthy individuals and 15 non-small cell lung cancer (NSCLC) patients. Our data revealed a subset of near universal differently expressed gene (DEG) profiles in platelets of metastatic NSCLC compared to healthy individuals, including 626 up-regulated RNAs (mRNAs and ncRNAs) and 1497 down-regulated genes. The significant over-expressed genes showed enrichment in focal adhesion, platelets activation, gap junction and adherens junction pathways. The DEGs also included previously reported tumor-related genes such as *PDGFR*, *VEGF*, *EGF*, etc., verifying the consistence and significance of platelet RNA-Seq in oncology study. We also validated several up-regulated DEGs involved in tumor cell–induced platelet aggregation (TCIPA) and tumorigenesis. Additionally, transcriptomic comparison analyses of NSCLC subgroups were conducted. Between non-metastatic and metastatic NSCLC patients, 526 platelet DEGs were identified with the most altered expression. The outcomes from subgroup analysis between lung adenocarcinoma and lung squamous cell carcinoma demonstrated the diagnostic potential of platelet RNA-Seq on distinguishing tumor histological types.

## 1. Introduction

Accumulating data suggested platelets could promote tumor growth, angiogenesis, metastasis and tumor immune escape by releasing growth factors and directly interacting with circulating tumor cells (CTCs) [1]. On the other hand, tumor cells alter the characteristics of blood platelets. For example, the predictive significance of platelets numerical count, and platelet lymphocyte ratio (PLR) in cancer diagnosis have been well-acknowledged [2]. Besides numerical changes, tumors can induce platelets functional abnormalities to promote thrombosis, namely tumor cell–induced platelet aggregation (TCIPA) [3]. During TCIPA, platelets are “educated” by tumor cells, which in turn enhances tumor progression. Therefore, the cross-talk between platelets and tumor cells appears reciprocal on that the tumors activating platelets by changing its genomic and proteomic profiles, while platelets modulate tumor development and metastasis.

Platelets lack genomic DNA but possess a relative diverse RNA repository including mRNAs, microRNAs, and non-coding RNAs inherited from megakaryocytes or microvesicles. Platelets are capable to splice pre-mRNA to mRNA and modulate certain RNA modifications [4,5]. Moreover, considering the existence of ribosomes, platelets also retain the capacity of protein translation [6]. Several published studies have revealed the protein profile was consistent with transcriptome analyses in platelets [7,8], indicating platelets transcriptome would be useful for

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exploring platelets functions and pro-metastasis ability. For example, protein encoding genes related to chemokine signaling, cell adhesion, aggregation and cellular interaction were identified by analyzing platelet RNA sequencing (RNA-seq) results [9]. It has been increasingly appreciated that alterations in platelets transcriptome provides a potential platform for cancer diagnosis [10].

Currently, lung cancer is the leading cause of cancer death [11]. Non-small cell lung cancer (NSCLC) accounts for about 80–85% of lung cancer, of which lung adenocarcinoma (LAC) and lung squamous cell carcinoma (LSC) are the most common pathological subtypes. It has been reported that the platelet count in patients with distant metastasized NSCLC was significantly higher than that of localized patients without metastasis [12]. Intriguingly, CTCs from lung cancer can be coated by activated platelet to shield from the natural killer (NK) cell attacks, subsequently promoting the hematogenous metastasis of lung cancer cells [13]. In addition, the existence of EML4-ALK mRNA rearrangements in platelets from NSCLC patients has been detected, which served as an invaluable tool to perform cancer diagnosis by blood-based “liquid biopsy” [14]. These evidences imply the participation of platelets in the progression of NSCLC, however, the global transcriptome landscape in NSCLC patients’ platelets, especially in those with distant metastasis remains unclear.

Here, we performed next-generation RNA-seq on isolated peripheral blood platelets to explore the relationship between platelet gene expression and NSCLC metastasis. The platelets transcriptome between healthy donors (HDs) showed little difference, while universal altered expression of mRNA as well as noncoding RNAs was identified between HDs and metastasis cases, indicating platelet function was significantly adapted during tumor metastasis. Meanwhile, we investigated the differentially expressed genes in platelets between metastasis-negative and metastasis patients. Similarly, the subgroup comparison between LAC and LSC was analyzed, which also showed significant platelet transcriptomic differences. By elucidating the platelet transcriptome changes in NSCLC patients, our data reveal insights into responsive or causal platelet factors possibly associated with NSCLC events, providing novel directions in diagnostic and therapeutic strategies for NSCLC.

## 2. Materials and methods

### 2.1. Patients and sample collection

This study was approved by the Ethics Committee of Jinling Hospital, and written informed consent was obtained from all participants. Blood samples of NSCLC patients were collected pre-operatively from June 2016 to December 2016, and all the final diagnoses were based on pathology examinations. None of the patients received any pre-operative chemotherapy or radiotherapy at the time of phlebotomize. In addition, patients with previous history of anti-platelet medications within 10 days or hematological disease such as thrombosis and bleeding, were excluded from our cohort. Localized NSCLC patients (M0-NSCLC) were defined as those without detectable distant metastasis from the primary tumor [10]. After excluded those cases, we selected 15 NSCLC patients as the exploration cohort in the current study for RNA-Seq. Besides NSCLC patients, we also collected platelet samples from 7 healthy individuals during blood donation. During data validation, we enrolled another 5 healthy individuals and 15 NSCLC patients as the validation cohort. A quantity of 6 mL peripheral blood was drawn from patients or healthy donors into EDTA anti-coagulant vacutainers containing 15% acid citrate dextrose.

### 2.2. Platelet isolation, purification, and RNA extraction

Platelets were isolated from blood samples within 4 h after collection to minimize RNA degradation. The workflow for platelet purification was shown in Fig. 1A. Briefly, karyocytes were firstly removed by centrifugation at 120 *g* for 20 min at room temperature [15]. To avoid leukocytes and erythrocytes contamination, only the upper 2/3 of the platelet-rich plasma was used for further collecting platelets. Platelets were then pelleted at 700 *g* for 10 min and gently resuspended with 80 μL beads buffer (0.8% NaCl, 0.02% KCl, 0.144% $Na_2HPO_4$, 0.024% $KH_2PO_4$, 0.5% bovine serum albumin, and 2 mM ethylenediaminetetraacetic acid, Miltenyi Biotec), incubating with 20 μL human monoclonal anti-CD45 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min at room temperature. Magnetic separation columns (MS Separation Columns, Miltenyi Biotec) were loaded with platelets suspension to delete CD45+ cells by negative selection. The purified platelets were further collected by centrifugation at 1500*g* for 10 min at room temperature, after which the platelet pellets were saved in 50 μL RNAlater buffer and stored at −80 °C.

The platelets purity was assessed by both morphological [10] and flow cytometry experiments [16], with the criteria of less than one leukocyte per one million platelets. Total platelet RNA was extracted using the mirVana PARIS kit (Ambion) from purified platelets according to the suppliers’ instructions. RNA integrity was assessed by an Agilent Bioanalyzer, and RNA samples with integrity numbers > 7.0 were qualified for further next-generation sequencing [17].

### 2.3. Quantitative reverse transcription–PCR

Total RNA extraction, reversed transcription and quantitative reverse transcription–PCR were performed as our previously described [18]. The measurements were assessed with ΔCT normalized by GAPDH. The primers used were listed as following: GAPDH (forward: ACAACTTTGGTATCGTGGAAGG, reverse: GCCATCACGCCACAGTTTC); ITGA1 (forward: GTGCTTATTGGTTCTCCGTTAGT, reverse: CACAAGC CAGAAATCCTCCAT); VEGFC (forward: GGCTGGCAACATAACAGA GAA, reverse: CCCCACATCTATACACACCTCC); MYL9 (forward: TCTT CGCAATGTTTGACCAGT, reverse: GTTGAAAGCCTCCTTAAACTCCT); FLNA (forward: CGAGGTCGAGGTTGTGATCC, reverse: GCAGGCACTC GGGTTACAG); TLN1 (forward: AGCTGAACCTCCTGTATGTGC, reverse: CAATTCTTGTGTGCCTGGAAGAT).

### 2.4. Platelets RNA-sequencing

Platelets total RNA was reversely transcribed to cDNA and amplified by Discover-scTM WTA kit V2 (Vazyme, N711) according to the manufacturer’s instructions. The amplification of cDNA was quantified using QuBit (Life Technologies) and quality controlled by Agilent 2100 Bioanalyzer with High Sensitivity DNA Chip. Sample preparation after Biorupter shearing by sonication for sequencing was performed using the TruePrep TM DNA Library Prep Kit V2 for Illumina® (Vazyme, TD503). Size-selection of 350 bp was loaded on Illumina X ten platform.

### 2.5. Sequence reads analysis

Raw sequencing data was converted to raw reads using CASAVA base calling. Quality control of the raw reads was performed by calculating Qphred score (Qphred = −10log10(e)) with Fast QC. Clean reads were obtained by removing low quality reads (50% of the reads were bases of Qphred ≤ 10), sequencing adapters, and reads containing unrecognized bases with a percentage of higher than five. Clean reads alignment to reference genome sequencing using TopHat2, and FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) was computed with cufflinks-2.2.1.

### 2.6. Differential expression of gene

Genes with less than ten reads counts in all platelets samples were excluded from calculation of differentially expressed transcripts and ncRNAs. Differentially expressed genes (DEGs) were measured with the help of Cuffdiff analysis. ANOVA testing for difference was performed to obtain corresponding *p* value. The false discovery rate (FDR),