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Review Article

Platelet Transcriptome-Based Approaches in the Fight against Dengue and Other Diseases

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ABSTRACT

Human platelets are anucleate cells that lack in deoxyribonucleic acid (DNA), thus hampering genomic study on them. However, the presence of their own messenger ribonucleic acid (mRNA) transcript allows functional study via the transcriptome approach. Transcriptome not only allows profiling of platelet but also aids in studying gene regulation in virus infections and other diseases that have an impact on platelets. Some viruses are known to affect the platelet either by causing a reduction or destruction. Dengue virus is one of the most postulated virus having such effect and frequently linked to platelet reduction. The transcriptome approach has a pivotal role in providing a deeper insight to link certain diseases and their effect on platelets. This review critically discusses role of platelet in dengue and other viral diseases of public health relevance, with a specific focus on the methods currently used in platelet transcriptome profiling.

Keywords: Anucleate cells, arbovirus, dengue, platelet transcriptome profiling, viral diseases

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INTRODUCTION

Platelets are a component of blood that generally contributes to haemostasis, a process to stop bleeding by clumping and clotting blood vessel injuries. Platelets are anucleate cells derived from megakaryocytes in the bone marrow. Once endoplasmatic

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maturation takes place, megakaryocytes develop pro-platelets, which bud off numerous platelets into the bloodstream. This mechanism is accentuated by cytokines such as interleukin 3 (IL-3), interleukin 6 (IL-6), granulocyte macrophage colony-stimulating factor (GM-CSF), stromal cell-derived factor-1 (SDF-1) and vitally by thrombopoietin (TPO) (Kuter, 2014). Platelet is uniquely found in mammals, whereas in other animals such as amphibians and avian, it circulates as intact mononuclear cells. The regulation of megakaryocyte and platelet production is done by TPO, a hormone produced in the kidneys and liver. In a healthy adult, approximately 10^{11} platelets are continuously produced and cleared daily to maintain 150–400 × 10^9/L of blood level (Balduni & Noris, 2014; Grozovsky, Giannini, Falet, & Hoffmeister, 2015).

To examine in depth the function of platelets, researchers have attempted to investigate the products of the genome, which are proteins. Proteomics provides details on protein quantity and diversity, but on the other hand, it may not depict a cell's entire story. Moreover, translational modification of proteins poses a technical challenge in characterising proteome (Gregorich & Ge, 2014). In order to overcome this, measuring the intermediate step between genes and proteins, called transcripts of messenger ribonucleic acid (transcriptome), alternatively bridges the gap between the genomics and proteomics.

Transcriptome profiling provides information on up-regulation or down-regulation of genes and quantity of gene expression in a cell. This information will be useful to understand the mechanism of certain diseases caused by thrombocytopenic condition involving platelets. For instance, transcriptional profiling of platelets reveals WD-40 repeat domain 1 (WDR1) vanquishes platelet activity and is associated with cardiovascular disease (Montenont et al., 2016). Other applications of platelet transcriptome profiling include identifying altered platelet function due to mutations (Noetzli et al., 2015), monitoring response of anti-platelet therapy in hyperthrombic patients (Mao et al., 2014) and potential biomarkers of disease (Best et al., 2015).

In addition to these applications, platelet transcriptome profiling has the potential to provide a breakthrough in viral infections that affect platelets. It is known that thrombocytopenia is the hallmark of dengue infection, however only in recent years, researchers have been able to prove the replication of dengue virus in platelet (Sutherland et al., 2016; Simon, Sutherland, & Pryzdial, 2015). Platelet transcriptome profiling, yet to be used in dengue research could be another stepping stone in understanding mechanism of dengue infection, similarly in other viral infections too.

HUMAN PLATELET TRANSCRIPTOME

Human platelets are known to be anucleate, lacking in DNA and produced as cytoplasmic buds from megakaryocytes, they retain megakaryocyte-derived messenger ribonucleic acid (mRNA) (Bahou & Gnatenko, 2004). There is a paucity of studies on platelet transcriptome; however, steady observations support the fact that platelet mRNA is biologically and patho-physiologically significant. Earlier studies show platelet transcriptome directly correlates with proteomic data indicating that transcriptional analysis can provide insight on platelet function and disorders. Adversely, one study reports a weak association of proteome data with human platelet transcriptome obtained from the healthy male individual (Londin et al., 2014). Having

said that, to date, it has been revealed that approximately 69% of secreted platelet-associated proteins are detectable at the mRNA level (McRedmond et al., 2004).

Additionally, 2000 mRNA transcripts are found to be present in unstimulated platelets, thus providing a better chance for evaluation of the transcriptome (Gnatenko et al., 2003). It is also believed that platelet transcriptome is fixed, with minimal changes indicating that significant changes in transcripts may need days compared to hours for nucleated cells. An interesting data to note, 58% of the mRNAs expressed by human platelets are found in mouse platelets while 83% vice versa (Rowley et al., 2011). In addition, transcriptome profiling reveal platelet has 50%-90% less ribosomal RNA, high levels mRNA and small RNAs and contains various isoforms of mRNA (Bray et al., 2010).

Prior investigation on platelet mRNA show integrins and glycoproteins are significantly higher compared with other components (Bugert & Kluter, 2006). The over-presentation of integrins may reflect their utmost importance in biological functions of the platelet. Transcriptome data have allowed novel discovery of transporters for gamma-aminobutyric acid (GABA), glutamate and dopamine molecules in platelets (Frankhauser et al., 2006; Rainesalo, Keranen, Saransaari, & Honkaniemi, 2005).

It is useful to characterise platelet transcriptome to identify the genetic aspect of disease and disease traits. Capturing primary megakaryocytes mRNA profile is not feasible when it involves many subjects. Thus, profiling platelet RNA is an alternative as it is easy to be procured compared with to megakaryocytes. There are several methods to study the platelet transcriptome of which some may become obsolete due to advancement in molecular methods (Figure 1).

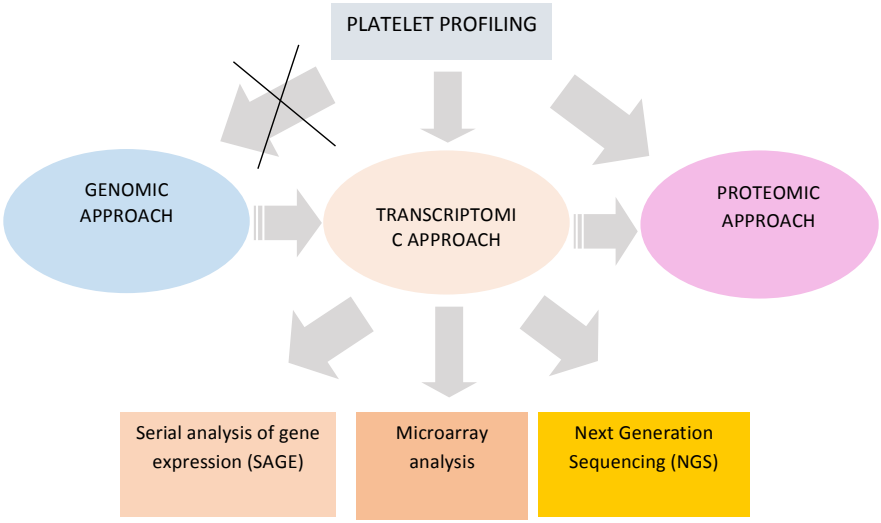

Figure 1. Methods currently available for platelet transcriptome profiling

Platelet profiling can be performed via the transcriptomic and proteomic approaches. On the other hand, genomic approach is not possible as the platelets do not have nuclear DNA. Transcriptomic profiling enables characterisation of platelet at the stage of mRNA transcription, a step ahead of protein translation (proteomic approach). Three identified methods of platelet transcriptome profiling are serial analysis of gene expression (SAGE), microarray analysis and next generation sequencing (NGS). Microarray analysis and NGS are advanced methods which are currently preferred by researchers as tools for platelet profiling.

THE LINK BETWEEN PLATELET AND DENGUE INFECTION

Dengue is a mosquito-borne viral disease of public health importance (Benelli & Mehlhorn, 2016). Platelet reduction, also known as thrombocytopenia is a hallmark of dengue virus infection. However, the actual mechanism on how dengue virus affects platelet during infection is not well understood. Besides, there are variations in research findings that make it difficult to come to a consensus conclusion on the link between platelet and dengue virus.

Recent studies demonstrated that dengue virus binds to lectin receptor and triggers platelet activation and apoptosis, which generates inflammatory responses in target monocytes (Hottz et al., 2014; Sun et al., 2007). Contradictory to this, a study observed the presence of dengue RNA in a highly augmented CD61 (+) cell population from infected rhesus monkey during the acute phase (Noisakran et al., 2012). This result may suggest that dengue virus utilises CD61 receptor for binding to the platelet. Another research reported the detection of dengue virus in platelets isolated from dengue patients (Noisakran et al., 2009). This raised the possibility of dengue virus replicating in platelet cells. In a most recent study in 2015, researchers provide the first evidence that dengue virus attacks platelets and utilise their translational machinery to replicate and produce infectious virus (Simon, Sutherland, & Pryzdial, 2015).

A prospective observational study in India found significant correlation between platelet and duration of hospitalisation of dengue patients. A decline in platelet level increases complication rate and prolonged hospitalisation, thus platelet count can be utilised as a predictive parameter (Jayanthi & Thulasi, 2016). In another study, correlation of bleeding manifestation and platelet count is assessed in patients with severe dengue. The study, however, finds poor correlation between thrombocytopenia and bleeding manifestations (Sreenivasa, Manjunatha, & Nivil, 2016). This perhaps indicates that abnormal platelet aggregation or disseminated intravascular coagulation may contribute to bleeding in severe dengue cases rather than number of platelets.

New evidence on correlation between activation and depletion of platelets in dengue patients with thrombocytopenia has surfaced. The findings a study show a substantial decrease in platelet count occur on day four of fever in dengue patients. Furthermore, high copy numbers of dengue virus genome in platelet pellet directly correlated with the platelet activation markers during day four, six and eight of fever are observed. Based on the observation that platelet activation is an important determinant of thrombocytopenia in dengue infections, the study suggests a controlled strategy of platelet inactivation may save them from rapid decline during dengue infections (Ojha et al., 2017).

PLATELETS AND OTHER VIRAL INFECTIONS

Platelet activation is not only limited to responding to injury but also indicates host response and virus survival. Thrombocytopenia, a condition due to either decreased in platelet synthesis or increased in platelet destruction, is common following certain viral infections (Table 1). Viruses' ability to diminish the levels of circulating platelets is a multifactorial event and differs based on the mechanism. For instance, simian immunodeficiency virus (SIV) alters TPO production in the liver by up-regulating tumour growth factor beta (TGF β) (Pate et al., 2014) whereas hepatitis C virus (HCV) induces bone marrow suppression and hypersplenism (Fouad, 2013). The spleen size is inversely correlated to platelet count in patients with chronic HCV infection (Medeiros, Domingues, Luna, & Lopes, 2014; van der Meer et al., 2016).

Platelet destruction, on the other hand, occurs via direct interaction of platelets and virus. Some viruses have the tendency to bind to platelet by utilising receptors, which enhance the interaction of platelets and neutrophils. Subsequently, platelets are phagocytised, thus, reducing the number of circulating platelets. Besides that, a reduction in platelet count sometimes is caused by self-destruction due to the presence of anti-platelet autoantibody. This leads to a condition called idiopathic thrombocytopenic purpura (ITP) that is commonly caused in HCV, cytomegalovirus (CMV), Human Immunodeficiency virus (HIV), Epstein Barr virus (EBV), Varicella Zoster virus (VZ) and herpes viruses (Assinger, 2014; Assinger et al., 2014; Goeijenbier et al., 2012). Studies have shown antibodies produced by B-lymphocytes against these viruses interrupt the surface integrins on the platelet such as the GPIIb/IIIa or GPIb-IX-V and diminish survival of platelets (Hamidpour, Behrendt, Griffiths, Partridge, & Lindsey, 2006; Najaoui et al., 2012).

HOW PLATELET TRANSCRIPTOME CAN BE USED TO STUDY MECHANISMS OF DENGUE INFECTION IN PLATELET

To establish an infection, almost all viruses exhibit the same modus operandi, which includes entry to the host's cells, replication via hosts' internal machinery and release of new virions (Li & Nagy, 2011). However, some viral replication mechanisms are less understood due to lack of laboratory evidences. For instance, replication of dengue virus in platelets remains unclear until recent study shows that dengue virus uses platelets to replicate (Simon, Sutherland, & Pryzdial, 2015). Thus, the key to unveiling further about dengue replication lies within the platelet, and platelet transcriptome can be utilised to study the mechanism of infection by dengue virus. To date, platelet transcriptome profiling has yet to be used as a tool to study the mechanism of dengue infection. One proposed method for future research includes *in vitro* study comparing the platelet transcriptome before and after dengue virus infection to identify genes, which are involved in virus transcription. Apart from that, it would be interesting to analyse platelet transcriptome profile at different phases of dengue infection or based on the severity of the disease.

Table 1
Mechanisms of action of selected viral infections on platelet

Virus	Mechanism of action on platelet	Study
Simian Immunodeficiency Virus (SIV)	SIV alters Hepatic thrombopoietin (TPO) production by up-regulating tumour growth factor.	Pate et al., 2014
Hepatitis C virus (HCV)	HCV interacts with platelets through collagen receptor GPVI. It stimulates bone marrow suppression and hypersplenism. The size of the spleen is inversely related to platelet count.	van der Meer et al., 2016
Cytomegalovirus (CMV)	CMV is known to replicate in megakaryocyte. CMV binds to platelet via the TLR2 receptor, initiating platelet activation and degranulation. This results in enhanced platelet interaction with neutrophils, phagocytosis, and drop in platelet count.	Assinger et al., 2014
Human immunodeficiency virus (HIV)	Multiple mechanisms: (1) Reduce survival of megakaryocytes and precursors; (2) HIV surface glycoprotein gp120 binds to lection receptor and leads to increased megakaryocyte apoptosis; (3) reduce TPO receptor (c-Mpl) expression.	Assinger, 2014
Hantavirus	Binds to αvβ3 or αIIbβ3 integrins expressed on platelets and endothelial cells, contributing to viral dissemination, platelet activation, and induction of endothelial cell functions.	Zapata, Cox, & Salvato, 2014
Epstein Barr Virus (EBV) Dengue virus	Interact with platelets by complementing receptor 2 (CR2) and induces platelet activation. Bind to lectin receptor and enhance platelet activation and apoptosis, which generates inflammatory responses in target monocytes. Anti-non-structural protein-1 (NS-1) triggers complement-mediated lysis of platelets which further accelerates thrombocytopenia. Dengue virus has been detected inside the platelet, raising the possibility of direct replication in platelet.	Ahmad & Menezes, 1997 Hottz et al., 2014 Sun et al., 2007 Noisakran et al., 2009
Rotavirus	Rotavirus binds to platelet using collagen receptor GPIa/IIa thus triggering platelet activation and destruction.	Assinger, 2014

METHODS OF PLATELET TRANSCRIPTOME PROFILING

Platelet Transcriptome Profiling Approach by SAGE

The serial analysis of gene expression (SAGE) is a quantitative method for measuring gene expression; it was first, developed and published in 1995 (Velculescu, Zhang, Vogelstein, & Kinzler, 1995). Though lower in throughput than microarray analysis, SAGE provides a more detailed transcriptome profiling due to its quantitative property while microarray has been just a qualitative method. The difference between SAGE and microarray has been widely

studied (Gnatenko et al., 2003). The study identified 89% of platelet mRNA transcripts are of mitochondrial origin, while 11% corresponds to unique genes which are not even detected by microarray. Therefore, SAGE is a good technique that may permit full characterisation of the mRNA transcripts including non-mitochondrial origin. Nevertheless, with the development of high-throughput sequencing technologies and more advanced microarray techniques, it appears possible that SAGE may be surpassed in future studies as detailed in Table 2.

Platelet Transcriptome Profiling by NGS

Next-generation sequencing (NGS) of platelets has enabled exceptional quantification and profiling of platelet transcriptome and discovered the diversity of mRNAs. The NGS has wide applications in this aspect, such as comparing platelet transcriptome between virus-infected and non-infected cells. One study uses NGS approach to evaluate various pathogen reduction systems (PR) for platelet storage (Osman, Hitzler, Ameur, & Provost, 2015). PR systems function to extend the shelf life of stored blood components, such as platelets and inhibit infections transmitted via transfusion. It is found that use of certain PR systems greatly reduces mRNA level in PR-treated platelet concentrates compared with non-treated platelet. This finding will be useful in alerting the blood bank authorities in implementing appropriate PR system for blood component storage (Osman, Hitzler, & Provost, 2016).

The NGS is also used to compare platelet transcriptome between mice and human (Rowley et al., 2011). Mouse platelets are commonly used as surrogates to study in vivo human platelet function. Still, uncertainty often arises regarding the functional differences and similarities between mouse and human platelets. This is the first study that utilises NGS to analyse human and mice platelet. The study discovered 95% conserved platelet transcriptome between human and mice. Conservation of platelet mRNA between two species enables mice model to be used to study haemostasis.

Though next-generation sequencing enables large scale platelet transcriptome profiling, its application for clinical studies is confined. NGS is foreseen to replace some of the old techniques used in the clinical setting. For instance, it may be used in assessing aspirin resistance in heart disease patients. To date, the influence of aspirin is commonly evaluated using light transmission aggregometry (LTA) and thrombelastography platelet mapping assay (TEG) (Liu et al., 2013). NGS approach to determine the platelet mRNA profile in association with aspirin resistance will provide more data in predicting genetic markers of aspirin responsiveness. The NGS generates a large transcript data per sample, and therefore, it is challenging to perform analysis with such data. The RNA sequencing in a much larger population will need to be performed so that a normal platelet transcript can be established (Freedman, 2011).

Microarray Analysis in Characterisation of Platelet Transcriptome

Apart from NGS, microarray analysis is another method commonly used for platelet transcriptome profiling. This method is beneficial in observing up-regulation and down-regulation of genes without the need for quantification as applied by NGS. One study had

attempted to compare platelet transcriptome from three unrelated donors by using leukocyte depleted platelet subjected to hybridisation to the Affymetrix HG-U95Av2 GeneChip, consisting of 12,600 probe sets. It found exceptional concordance, reproducibility and discrete profiles of human platelets among the three donors (Bahou & Gnatenko, 2004). In a most recent study, microarray analysis is performed to compare neonate and adult human platelet transcriptomes using pure platelet RNA obtained from adult and cord blood. Interestingly, 201 genes are found to be differentially expressed between these two groups of which neonatal platelets had higher amounts of mRNA that are associated with protein synthesis and lower levels of genes involved in calcium metabolism and cell signalling (Caparrós-Pérez et al., 2017).

Microarray approach seems to be more feasible for clinical application. Coronary heart disease is a condition in which platelet aggregation occurs and genetically modulated (Floyd, Ellis, & Ferro, 2014). Hereditary platelet glycoprotein polymorphisms are a known risk factor for coronary heart disease. The gene encoding glycoprotein GPIIIa has displayed a polymorphism (PlA2 allele) that is linked to vascular disease. Several case report studies identified the presence of this polymorphism in patients who died of myocardial infarction, stroke and related heart diseases (O'Donnell et al., 2001). It would be interesting to utilise microarray for comparison profiling of this platelet-related gene in patients who possess heterozygous or homozygous condition of the allele. In addition, platelet transcriptome analysis by microarray is useful in chronic kidney disease patients (CKD). An alteration in the uremic platelet mRNA is observed in CKD patients (Ple et al., 2012). However, dialysis appeared to have corrected this defect, which is consistent with the favourable effect of dialysis.

The microarray platelet profile is also helpful in providing insight into transcriptional signaling pathways in platelets in sickle cell anaemia disease. A study utilising microarray identified the absence of expression of CD45 and CD5 in sickle cell anaemic patients. These are lymphocytes and T cells markers. On the other hand, significant up-regulation of mRNAs encoding arginase II and ornithine decarboxylase antizyme in patients with sickle cell disease is observed in comparison with control subjects. It is known that arginine metabolic enzymes are involved in homeostasis (Raghavachari et al., 2007).

Microarray approach may have its limitations. There is no standardised method for microarray data analysis that, can contribute to inter-laboratory variations. Microarray assays also require a minimum of 5 mg of total cellular RNA as starting material, thus to prepare pure leukocyte depleted platelet RNA, a large volume of blood may be needed (Gnatenko et al., 2003). In addition, the microarray is no doubt a very costly method, which may be a prohibitive factor in a large-scale study of platelet transcriptome. This factor should be taken into stringent consideration when customising the microarray probe chips in regard to which gene should be spotted (Eicher et al., 2016). Prioritising the importance of genes to be spotted and prior knowledge is required to prevent wastage of data and prohibitive cost.

Table 2
Current knowledge on platelet profiling

Method	Key findings	Up-regulated/down-regulated	Reference
SAGE	Detected 89% mitochondrial transcripts and 11% of non-mitochondrial transcripts.	-	Gnatenko et al., 2003
Microarray	Detected mainly unknown function genes, genes involve in metabolism and receptors.	Majority of the up-regulated genes are unknown functional genes.	Gnatenko et al., 2003
Microarray	Detected abundant glycoproteins and integrins	Glycoprotein/integrins and receptors are majorly up-regulated.	Bugert & Kluter, 2006
Microarray	Human platelet transcriptome correlated well with proteome profile.	-	McRedmond et al., 2004
Microarray	Evaluation of platelet profile from 3 healthy donors revealed concordance, reproducibility and distinct profiles of human platelets.	-	Bahou & Gnatenko, 2004
Microarray	An alteration of the uremic platelet mRNA is observed in CKD patients. Dialysis appeared to have corrected this defect, which is consistent with the favourable effect of dialysis.	23 and 17 mRNAs are differentially expressed in platelets from dialysis and uremic patients respectively Defective genes such as PCTP, RAB1A, ZFAND5, and USP15 are corrected by dialysis.	Ple et al., 2012
Microarray	Demonstrated down-regulation of transcriptional signaling pathways in platelets in sickle cell anaemia patients.	CD45 and CD5 are down-regulated; arginase II and ornithine decarboxylase antizyme ae up-regulated.	Raghavachari et al., 2007
NGS	Abnormal transcript reads detected from an individual with autosomal recessive gray platelet syndrome.	Mutation in NBEAL2 gene responsible for causing gray platelet syndrome.	Kahr et al., 2011
NGS	Evaluated various pathogen reduction systems (PR) for platelet storage and finds that Intercept system alters platelet quality.	-	Osman et al., 2015
NGS	95% conserved platelet transcriptome between human and mice enables mice model to be used to study haemostasis.	-	Rowley et al., 2011

CONCLUSION

In sum, the study has highlighted that platelets are well suited for transcriptomic studies, as they lack nuclear DNA and their genome consists of megakaryocytes derived mRNA transcripts. There are many available transcriptomic approaches and analysis methods. While microarray is one of the widely-employed methods, next-generation sequencing is gradually becoming a favoured tool for transcriptome analysis. However, the limitations of modern transcript profiling include lack of reproducibility, difficulty in data processing and complexity of data

produced. Despite that, these methods are still reliable in clinical applications. In future, platelet transcriptome studies investigating a more diverse set of healthy and diseased samples will add value to the existing knowledge on platelet thrombotic and non-thrombotic functions.

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