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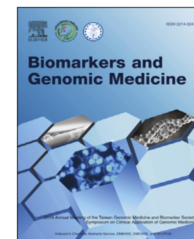

SHORT COMMUNICATION

# Autophagy regulation in heme-induced neutrophil activation is associated with microRNA expression on transfusion-related acute lung injury

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**Abstract** Transfusion-related acute lung injury (TRALI) is the leading cause of death after transfusion therapy. The pathogenesis of TRALI is associated with neutrophil activation in the lungs, causing endothelial damage and capillary leakage, and thus neutrophil extravasation. Heme-related molecules derived from the hemolysis of red blood cell components have been recognized as a stimulator, inducing neutrophil activation at TRALI. To investigate post-transcriptional changes of the neutrophil at TRALI, we performed heme-related molecules induced reactive oxygen species production in the neutrophil as a model. Neutrophils were isolated from heparinized peripheral blood and stimulated with heme-related molecules. After stimulation, reactive oxygen species production, degranulation, phagocytosis activity, and miRNA expression profile of neutrophil were analyzed by luminol assay, flow cytometry, and real-time polymerase chain reaction. The expression of miRNA targeting NADPH oxidase and autophagy in the neutrophil activation of TRALI was explored. The expression profile of miRNAs will be a useful predictor of disease severity and for the grading of patients for transfusion.
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## Introduction

Transfusion-related acute lung injury (TRALI) is associated with vascular endothelial cell injury following neutrophil activation. Recently, it has been considered that heme-related molecules from erythrocyte-derived substances contained in blood preparations induce the activation of neutrophils, which are involved in TRALI.[1–3] Heme-related molecules can induce neutrophil activation, resulting in reactive oxygen species (ROS) production that is generated by inducible NADPH oxidase[1]. During ROS production, autophagic machinery is considered to be a survival mechanism as it counteracts the oxidative burst-dependent cell damage caused by a neutrophil.[4] Autophagy is a homeostatic mechanism involved in the clearance of damaged organelles or proteins, providing essential nutrient supply through the recycling of cytosolic macromolecules and organelles.[5,6] The enhancing of autophagic activity has been reported to protect sepsis-induced acute lung injury.[7,8] During TRALI, excessive neutrophils and the accumulation of neutrophil extracellular traps (NETs) contribute to its pathogenesis. The autophagic activity of neutrophils is required for the release of NETs, which might contribute to TRALI.[9] A number of miRNAs have been recognized as regulators of the gene expression that controls ROS generation and the autophagy of neutrophils.[6] In this report, we try to identify the miRNA biomarker of TRALI via the autophagic pathway in heme-induced neutrophil.

## Materials and methods

Heparinized peripheral blood was collected from healthy donors for neutrophil isolation using a density gradient method. The study protocol was developed in accordance with the Declaration of Helsinki and the procedures were approved by Tzu Chi Hospital, Haulien, Taiwan. The neutrophils were stimulated with phorbol myristate acetate and heme-related molecules (heme, hemi, protoporphyrin IX, and ferric citrate; Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 37°C. To study the effect of autophagy and ROS production, neutrophils were pretreated for 15 minutes at 37°C with 3-methyladenine (3-MA, 5mM), diphenylene iodonium (DPI, 10μm), butylated hydroxyanisole (BHA, 100μm), and 4-aminobenzoic acid hydrazide (ABAH, 100μm). All of these chemicals were purchased from Sigma-Aldrich. Neutrophil degranulation was assessed by myeloperoxidase release. ROS were measured by monitoring luminol-amplified chemiluminescence. Luminol fluorescence (50μM) in the presence of 1.2 U/mL horseradish peroxidase was added to the neutrophils and the mixture was incubated for 30 minutes at 37°C. The chemiluminescence signal was measured at various time intervals after heme-related molecule stimulation in a Luminometer (Luminoskan Ascent Microplate Luminometer, Thermo Scientific, Waltham, MA, USA) over a period of 30 minutes. The mean fluorescent intensity values of ROS production and phagocytosis activity in the neutrophils were assessed by flow cytometry-based assay using CM-$H_2$DCFDA 2μm (Invitrogen, Carlsbad, CA, USA) and fluorescein isothiocyanate-dextran 2μm (Invitrogen). For real-time quantitative polymerase chain reaction (PCR), total RNA was prepared using the mirVana miRNA isolation kit (Life Technologies, Carlsbad, CA, USA). The Taqman microRNA reverse transcription kit (Applied Biosystems, Warrington, UK) and Taqman microRNA assays (Applied Biosystems) were used according to the manufacturer's instructions and plates were read using an ABI Prism 7700 Real-Time PCR system (Applied Biosystems). RNU44 was used as an endogenous control and all samples were run in triplicate.

## Results

To better understand the potential regulation of neutrophil activation at TRALI, neutrophils were stimulated with heme-related molecules derived from the hemolysis of red blood cell components. ROS production was observed in heme-induced neutrophils (Fig. 1). NADPH oxidase (NOX) activation or autophagy could be associated with heme-induced neutrophil ROS production. To investigate the roles of NOX and autophagy in heme-induced neutrophils, we added a Class III phosphoinositide 3-kinase (PI3K) inhibitor, (3-MA, which suppresses the autophagic/lysosomal pathway), an inhibitor of NOX and Complex I of the mitochondrial electron transport chain (DPI), a mitochondrial ROS scavenger (BHA) and a myeloperoxidase inhibitor (ABAH) during neutrophil stimulation. As shown in Fig. 2, 3-MA and DPI significantly reduced the ROS production of heme-induced neutrophils, whereas BHA and ABAH did not. Our data indicate that autophagy and NOX are important mediators of heme-induced neutrophil ROS production. To uncover the roles of miRNA in regulating the autophagy of heme-induced neutrophils, we focused on miRNA genes that are reported to regulate autophagic genes (Table 1). As shown in Fig. 3A, miRNA-30a, -17, and -20 were downregulated in heme-induced neutrophils. Moreover, markers related to neutrophil functions (miRNA-223, -29b, and -25) were upregulated by protoporphyrin IX-induced neutrophils

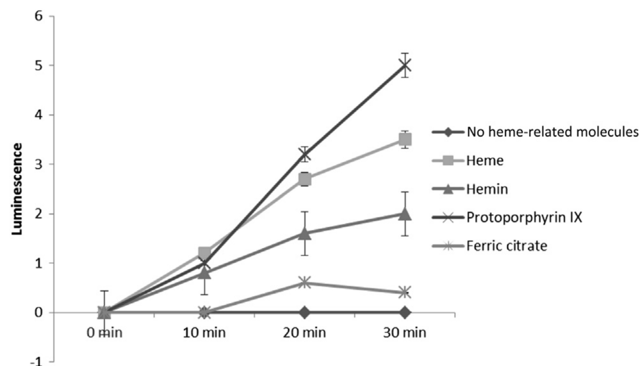


**Figure 1** Extracellular reactive oxygen species (ROS) production of neutrophils incubated with heme-related molecules as measured by the luminol-amplified chemiluminescence assay. Freshly isolated human neutrophils were incubated with heme-related molecules (40μM heme, 40μM hemin, 0.8μM protoporphyrin and 40μM ferric citrate) for 30 minutes at 37°C. Subsequently, luminol was added, and ROS production was induced with 20nM 4-MA. The ROS production was detected by measuring chemiluminescence for a period of 30 minutes at 37°C. Data shown give the mean ± SD from two independent experiments.

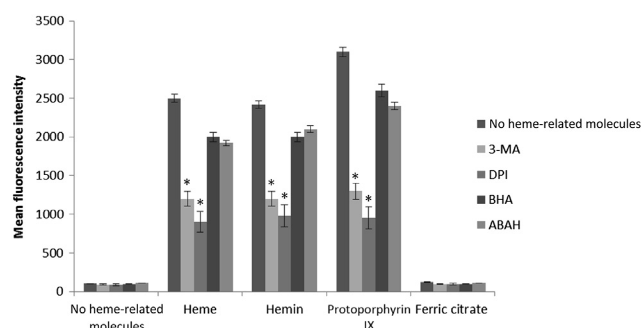


**Figure 2** Intracellular reactive oxygen species production of heme-induced neutrophils after exposure to various inhibitors. Freshly isolated human neutrophils were pre-incubated with or without inhibitors (5mM 3-MA, 10μM DPI, 100μM BHA and 100μM ABAH) for 15 minutes at 37°C. Cells were loaded with CM-H2DCFDA (2μM), and the samples were stimulated with heme-related molecules. The intracellular production of reactive oxygen species was assessed using flow cytometry. Data shown give the mean ± SD from two independent experiments. 3-MA = 3-methyladenine; ABAH = 4-aminobenzoic acid hydrazide; BHA = butylated hydroxyanisole; DPI = diphenylene iodonium. *$p < 0.05$ as compared to the 4-MA-stimulated sample without inhibitor (median).

(Fig. 3A). Heme-induced miRNA downregulation was significantly inhibited by the Class III PI3K inhibitor 3-MA but not by other inhibitors (Fig. 3B). Previous studies have demonstrated that Class III PI3Ks, AMP-activated protein kinase and mammalian target of rapamycin are essential kinases required for autophagy[10]; therefore, this result indicated the critical role of PI3K signaling in the regulation of autophagic miRNAs transcription in neutrophil during heme-related molecule induction. Other autophagic signaling pathways (AMP-activated protein kinase or mammalian target of rapamycin) involved in neutrophil function at TRALI require further investigation.

## Discussion

Heme is derived from free hemoglobin of red blood cells that are stored for long periods of time.[11] *In vivo*, heme is normally scavenged by hemopexin. After that, heme is delivered to the liver and degraded by heme oxygenase. When hemolysis collapses, free heme accumulates in the circulation. Heme-related molecules derived from hemolysis have been reported to induce the production of ROS from neutrophils. ROS generation in neutrophils results in increased autophagic activity. Recently, NETs induced by heme-related molecules have been shown to be associated with the onset of TRALI.[12] Activated neutrophils undergo NETosis and release nuclear DNA in long chromatin filaments generating web-like structures decorated with granular proteins.[13] Whether or not miRNA regulation is required for NETosis requires further investigation.

TRALI is a potentially fatal adverse transfusion reaction; however, the pathogenesis of TRALI is not completely understood. The essential finding in studies of TRALI is that lung endothelial cell damage is caused by neutrophil overactivation. The clinical diagnosis of a TRALI reaction remains a challenge. The current laboratory investigations of TRALI are based on TRALI-associated symptoms and neutrophil and human leukocyte antigen antibody screening of the associated donors.[14] Understanding the role of miRNAs in TRALI will provide clinically useful

**Table 1** Autophagy-related miRNAs.

| MicroRNA | Possible conserved targets/ neutrophil function | Mature miRNA sequence |
|---|---|---|
| miR-30a | BECN1 | hsa-miR-30a-3p<br>CUUUCAGUCGGAUGUUUGCAGC<br>hsa-miR-30b-5p<br>UGUAAACAUCCUACACUCAGCU |
| miR-17, -20, -93, and -106 | SQSTM1(p62) | hsa-miR-17-3p<br>ACUGCAGUGAAGGCACUUGU<br>hsa-miR-20a-5p<br>UAAAGUGCUUAUAGUGCAGGUAG |
| miR-204 | MAP1LC3 | hsa-miR-204-5p<br>UUCCCUUUGUCAUCCUAUGCCU |
| miR-196 | IRGM | hsa-miR-196a-5p<br>UAGGUAGUUUCAUGUUGUUGGG<br>hsa-miR-196b-5p<br>UAGGUAGUUUCCUGUUGUUGGG |
| miR-135a | NIX | hsa-miR-135a-5p<br>UAUGGCUUUUUAUUCCUAUGUGA |
| miR-223 | Neutrophil trafficking | hsa-miR-223-3p<br>UGUCAGUUUGUCAAAUACCCCA |
| miR-29B | Neutrophil differentiation | hsa-miR-29b-3p<br>UAGCACCAUUUGAAAUCAGUGUU |
| miR-25 | NOX4 expression | hsa-miR-25-3p<br>CAUUGCACUUGUCUCGGUCUGA |

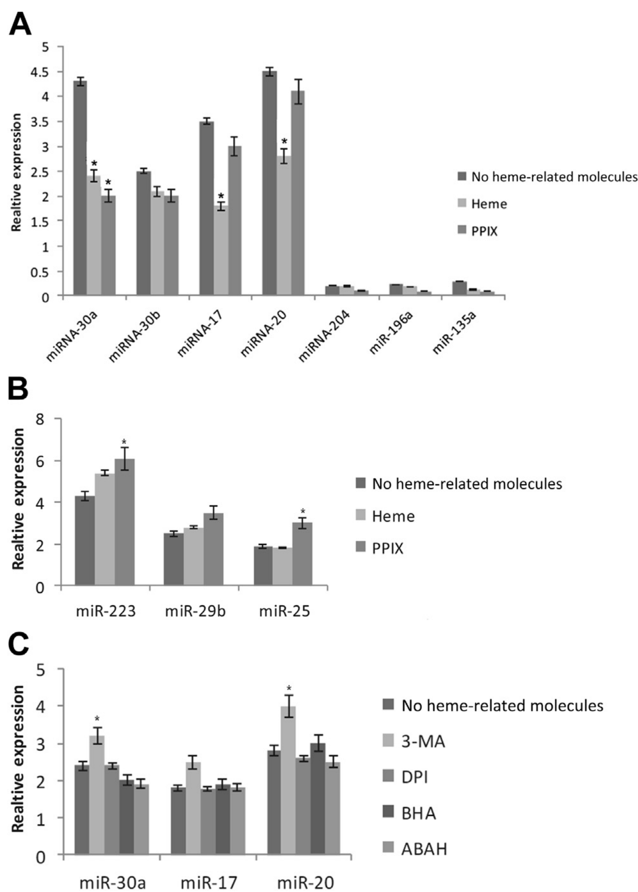


**Figure 3** Quantification of miRNA in heme-induced neutrophils. (A, B) The relative expression of miRNAs in heme-related molecule-induced neutrophils was determined by real-time polymerase chain reaction (Student *t* test). (C) Freshly isolated human neutrophils were treated with heme-related molecules in the presence of inhibitors (5mM 3-MA, 10μM DPI, 100μM BHA, and 100μM ABAH). Mature miRNA expression at 12 hours after treatment was quantitated by real-time polymerase chain reaction. The fold change of relative miRNAs expression (miRNA/RNU44) was normalized to that observed in control cells. 3-MA = 3-methyladenine; ABAH = 4-aminobenzoic acid hydrazide; BHA = butylated hydroxyanisole; DPI = diphenylene iodonium; PPIX = protoporphyrin IX. *$p < 0.05$.

information that will hopefully lead to the discovery of the predictor for disease severity and grading for monitoring the TRALI responses of patients.

## Conflicts of interest

All authors declare no conflicts of interest.

## Acknowledgments

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