Preliminary study of urinary biomarker, miR-21, for kidney injury detection

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Abstract: Herbicide, namely glyphosate, has long been reported to cause adverse effects in human especially the induction of kidney injury and disturbance of kidney functions. Several biomarkers were demonstrated to be used for early kidney injury detection. However, some biomarkers have a limitation and low sensitivity to detect early phase of kidney injury. Therefore, we aimed to establish two urinary kidney injury biomarkers, urinary kidney injury molecule-1 (KIM-1) and microRNA-21 (miR-21), in glyphosate-exposed farmers. Two spot urine samples consisting of pre-work urine sample (before glyphosate application) and post-work urine sample (after glyphosate application 24 hours) were obtained. Urinary KIM-1 concentrations and miR-21 were analyzed. The average level of urinary KIM-1 was 1.34 μg/g Cr in pre-work urine and 1.26 μg/g Cr in post-work urine. The average fold of miR-21 expression was 1.40 and 1.21-fold in the pre- and post-work urine sample. There were no significant differences of the two biomarkers between the pre-work and post-work urine sample. However, the ΔmiR-21 expression moderately correlated with the volume of glyphosate used. Although two biomarkers were not significant different between pre- and post-work urine sample, the miR-21 expression may relate with the dose of glyphosate application in farmers. The number of subjects in this is limited, therefore a population size should be increased in future study.

Keywords: glyphosate, urinary biomarker, KIM-1, miR-21, occupational exposure, kidney injury

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1. Introduction

Glyphosate is globally used in agricultural activities to control weeds in cropped and non-cropped fields. The glyphosate and glyphosate-based herbicides toxicity has long been reported to exert harmless in human and animal [1]. Therefore, the potential health risks from glyphosate and glyphosate-based herbicides exposure have long been concerned. The farmers are faced with increasing risks related to expose glyphosate during working in farm through inhalation, skin absorption, and oral ingestion. After that, glyphosate and its metabolites were excreted through kidney [1]. According to the study of Gao et al., 2019, the renal proximal tubule was identified as a main target of glyphosate and glyphosate-based herbicides [2]. Also, several studies reported that the history exposure of glyphosate associated with changes in kidney injury and functions [3-7].

Many biomarkers such as creatinine, blood urea nitrogen, and urine markers of kidney injury (epithelial cells, tubular casts, urinary concentrating ability, etc.) were generally used to evaluate kidney injury and function [8]. However, some biomarkers such

as serum creatinine (sCr) and urine output could be detected in late kidney injury and showed low sensitivity and specificity [9]. In addition, urinary kidney injury molecule-1 (KIM-1), urinary beta2-microglobulin (B2M), urinary total protein, urinary albumin, urinary clusterin, urinary cystatin c (Cys C), and urinary trefoil factor 3 (TFF-3) have been reported to show high sensitivity to detect early acute kidney injury (AKI) [9-11]. In the case of KIM-1, it was highly localized in proximal tubule cells and can be detected in urine [12]. This biomarker has been reported to use for early AKI diagnosis and xenobiotic-induced kidney injury monitoring [13, 14]. The meta-analysis demonstrated that urinary KIM-1 level was specific to diagnose AKI with specificity at 86.0% and sensitivity at 74.0% [15]. In addition, the KIM-1 level was represented to predict kidney function deterioration since this level was significantly correlated with a decrease in estimated glomerular filtration rate (eGFR) [16].

With the recent developments in high-throughput technologies, the pace of advances in the functional genomics and proteomics that are applicable to establish novel biomarker for several diseases especially kidney disease has noticeably accelerated [17]. The microRNAs (miRNAs) have been recently emerged as promising biomarker candidates for several diseases such as cancer, Alzheimer's, and kidney diseases [18-20]. The miRNAs are endogenous short RNA molecules and play important role to regulate mRNA expression by mRNA destabilization and translational repression [21]. The great advantage of miRNAs to serve potentially excellent biomarker was the stability in biological samples such as blood, serum, urine, and other sources [22]. The miR-21 function has been proposed to be involved in cellular apoptosis, inflammation, and fibrosis signaling pathways in AKI [18, 23, 24]. In the model of drug-induced nephrotoxicity, the upregulation of miR-21 was found in damaged kidney in order to decrease lipid peroxidation and reactive oxygen species as well as deregulate metabolic processes [25]. Previous study illustrated that the significant alteration of miR-21 was observed in both acute as well as chronic animal models of kidney injury [26]. Moreover, the regulation of TGF-β signaling which directly acted to control reactive oxygen species (ROS) production was reported to implicate with miR-21in tubulointerstitial fibrosis associating with kidney dysfunction [27]. Hence, it was likely that the function of miR-21 in kidney disease was involved to response ROS generation.

Taken together, the proposed mechanism of herbicide namely glyphosate on kidney injury was based on ATP depletion via uncoupling of oxidative phosphorylation, cytochrome C activation, and macromolecular oxidation resulting to induce kidney cell death [28]. Therefore, we assumed that ROS will be generated in kidney cells after glyphosate exposure. Then, the kidney cell biomarker such as KIM-1 and miR-21 will be released from injured kidney cells and could be initially detected in the urine. The objective of this study is to evaluate the KIM-1 level and miR-21 expression to establish a novel biomarker for early kidney injury detection among glyphosate-exposed farmers.

2. Materials and Methods

2.1 Population, questionnaire, and urine collection

This study was conducted in Long District, Phrae Province, Thailand. Ninety-six farmers who practically applied glyphosate herbicide were recruited into our study to conduct face-to-face interview and collect urine. The questionnaire consisted of demographic data, personal and health history, work characteristics (number of years working with herbicides, time to exposure glyphosate, type of work, volume, concentration, and frequency), and personal protective equipment (PPE) use. All full urinary voided spot sample were collected over the exposure assessment period (before herbicide application until after herbicide application).

2.2 Quantification of urinary glyphosate

The urinary glyphosate was determined by a liquid chromatography–tandem mass spectrometry (LC–MS/MS) according to previous publication [29]. The farmer's urine which had urinary glyphosate concentration over limit of quantification (LOQ) by 5 μg/L in period of exposure were further selected to determine kidney injury biomarkers. Two spot urine samples were chosen consisting of pre-work urine sample (before glyphosate application) and post-work urine sample (after glyphosate application 24 hours).

2.3 Determination of urinary creatinine

To normalized urine volume in kidney biomarker determination, urinary creatinine was quantified by ARCHITECT™ clinical chemistry analyzer (Abbott, USA) at Maharaj Nakorn Chiang Mai Hospital Central Laboratory, Faculty of Medicine, Chiang Mai University. The urinary creatinine was expressed as mg/dL.

2.4 Determination of Human Kidney injury molecule 1 (KIM-1)

To determine the kidney injury marker in glyphosate-exposed farmers, the KIM-1 level in pre- and post-work urine samples was measured by ELISA according to the manufacturer's instructions (CUSABIO, China). One-hundred microliters of urine sample were added into human antibody-coated ELISA plates and the plate was then incubated at 37 °C for 2 hours. Next, the biotin-conjugated antibody specific for KIM-1 was added into the plate. After incubation for 1 hour, the reaction color was developed by the chemical reaction between the tetramethylbenzidine (TMB) reagent and streptavidin conjugated with horseradish peroxidase (HRP). The absorbance was measured at a wavelength of 450 nm using a microplate reader (Synergy™ H4, BioTek Instruments, Inc., USA). The concentration of urinary KIM-1 was calculated to compare with standard curve and normalized with urinary creatinine. The urinary KIM-1 level was expressed as μg/g creatinine.

2.5 MicroRNA-21 expression

To evaluate the expression of urinary miR-21 in glyphosate-exposed farmers, the miR-21 expression in pre- and post-work urine samples was quantified by quantitative real-time polymerase chain reaction (qRT-PCR). First, the urine was centrifuged by ultracentrifugation at 200,000 g 4 °C for 1 hour to harvest urinary exosome. Then, the miR-21 in urinary exosome was extracted by Nucleozol reagent (Toyobo, Japan) according to the manufacturer's protocol. Total urinary miRNA was reverse transcribed into cDNA by the Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio Company, USA). The expression of urinary miR-21 was determined by commercial Mir-X miRNA qRT-PCR TB Green Kit (Takara Bio Company, USA). The primer for miR-21 was demonstrated in Table 1.
In brief, the PCR parameters for gene amplification were 40 cycles at 95 ◦C for 5 min for the denaturation, at 95 ◦C for 12 s for annealing, and at 65 ◦C for 50 s for extension. The expression of the target miR-21 level was analyzed by the $2^{-\Delta\Delta Ct}$ method using U6 snRNA as a control according to the manufacturer's protocol. The miR-21 expression was expressed as fold.

Table 1. The sequences of miRNA-21 primers used for qRT-PCR.

Gene target	Sequence	Reference
miR-21-5p	5′-CGGCGGTAGCTTATCAGACTGA	[30]

2.6 Statistical analysis methods

Statistical analysis was performed using the SPSS for Windows, Version 16.0. (Chicago, SPSS Inc; 2007) and the GraphPad Prism version 8.3.0 for windows (GraphPad Software, San Diego, California USA, www.graphpad.com). All demographic data (gender, age, number of years working with herbicides, time to exposure glyphosate, type of

work, volume, concentration of glyphosate in urine, and personal protective equipment usage) were described and analyzed by descriptive statistics. The levels of urinary KIM-1 and miR-21 expression in pre-work and post-work urine samples were expressed as median (minimum – maximum) and compared using the Wilcoxon signed-rank test. Spearman's rank correlation test was used to evaluate a relationship between urinary biomarkers and demographic data.

3. Results

The urinary glyphosate level was analyzed in 96 participants. The results showed that the urinary glyphosate level which was greater than limit of quantification (LOQ) by 5 μg/L were observed in 17 participants. Therefore, these participants were selected for urinary biomarker quantification. The demographic characteristics were represented in Table 2. Most participants were male (64.7%) and aged between 37 – 60 years old. The average of farming experience of all subjected was 28 ± 13 years and the average exposure time to glyphosate was approximately 5 hour per day. All of participants had the responsibility to spray glyphosate on the agricultural area. Regarding behavior in using PPE, participants usually wore gloves (52.9%), masks (47.1%), boots (100%), and long-sleeved shirts (100%). The concentration of urinary glyphosate was 37.9 ± 37.5 (10.26 – 170.43) μg/g creatinine.

Table 2. Demographic data of the subjects

Characteristics	N (%) / mean ± SD
Gender	
Male	11 (64.7)
Female	6 (35.3)
Years working with herbicides (years)	28 ± 13
Time to exposure glyphosate (hours)	5 ± 2.8
Type of work	
Mixing and loading glyphosate	13 (76.5)
Spraying pesticides	17 (100)
Cleaning and collecting equipment	14 (82.4)
Use of Personal Protective Equipment (PPE)	
Mask	8 (47.1)
Gloves	9 (52.9)
Boots	17 (100)
Long-sleeved shirt	17 (100)
Concentration of glyphosate (μg/g creatinine)	37.9 ± 37.5

The comparison of urinary KIM-1 level and miR-21 expression between pre- and post-working urine samples are shown in Figure 1. The average urinary KIM-1 level in the pre-working and post-working urine samples were 1.34 (0.60 – 17.82 μg/g Cr) and 1.26 (0.26 – 9.69 μg/g Cr), respectively. However, no statistical significance was observed between pre- and post-working urine samples. The expression of urinary miR-21 was compared between pre- and post-working urine samples. The average miR-21 expression was 1.4 (0.07 -12.97) fold in the pre-working urine sample and 1.21 (0.04 -7.67) fold in the post-working urine sample. There were no significant differences of the urinary miR-21 expression between the pre-working and post-working urine sample.

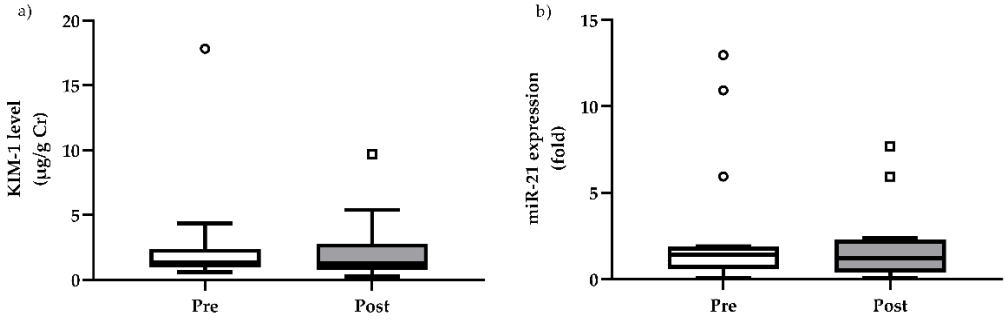

Figure 1. Two urinary biomarkers for kidney injury detection were investigated in glyphosate-exposed farmers. (a) The urinary KIM-1 level and (b) miR-21 expression of glyphosate-exposed workers in the pre-work (open bar) and post-work (solid bar) urine sample. The data are represented as median (minimum – maximum).

To evaluate the correlation between kidney injury markers and demographic data, the ΔKIM-1 level and ΔmiR-21 expression were calculated to represent the changes of biomarker during glyphosate application. The biomarker level in pre-working urine sample was subtracted from the post-working urine sample to generate ΔKIM-1 level and ΔmiR-21 expression. The result showed that ΔmiR-21 expression was to be a moderate positive correlation with volume of glyphosate (r = 0.499).

Table 3. Spearman's correlation (r) between urinary biomarkers and demographic data *$p < 0.05$

	ΔmiR-21 Expression	Age (years)	Years to working with pesticides	Exposure assessment period (hours)	Volume of glyphosate use (L)
ΔKIM-1 Level	0.471	0.409	0.478	0.104	0.259
ΔmiR-21 Expression	-	0.345	0.362	0.185	0.499*

4. Discussion

Many studies have been reported the association between glyphosate exposure and kidney injury [1, 31, 32]. The quantification of urinary biomarkers was performed in our study to establish suitable biomarker for early kidney injury monitoring in glyphosate-exposed farmers. The amount of urinary KIM-1 level and miR-21 expression was not significant different between pre-work and post-work urine samples of farmers. Previous study demonstrated that the significant level of urinary KIM-1 was found in herbicide-exposed farmers. De Silva et al., 2016 showed that urinary KIM-1 level in agricultural workers living in chronic kidney disease endemic areas was significantly higher than this level in control subjects from the same locations. The urinary KIM-1 was suggested to be early kidney damage markers for identify CKD suspected cases [33]. However, the urinary KIM-1 level was not significantly increased in patients with AKI causing from acute PQ poisoning [34]. In addition, Abdul et al., 2021 demonstrated that the urinary glyphosate or paraquat level did not correlate with the urinary KIM-1 level [32].

The study of miR-21 expression was performed to identify cancer, kidney, and cardiovascular diseases [35, 36]. Regarding kidney disease, the urinary KIM-1, miR-21, miR-200c, and miR-423 in both acetaminophen overdose patients with AKI and without AKI

diagnosis were significantly higher than those level in healthy controls [37]. Although the study of urinary miR-21 expression in herbicide-exposed workers have been less reported, many publications have been focused the response of other microRNAs in pesticide intoxication. The expression of miR-223, miR-518d-3p, miR-517b, miR-597, and miR-28-5p were found to be significant increase in farmer during post-harvest seasons of agricultural activity. Moreover, the positive dose-response trend of the miRNAs in the class of miR-223, miR-518d-3p, miR-597, miR-517b, and miR-133b with total dialkylphosphates (organophosphate insecticide metabolites) were remarkably observed [23]. Also, the expression of miRNAs (miR-29a-3p, miR-23b-3p, miR-19b-3p, miR-130a-3p, miR-125a-5p, and miR-106b-5p) were significantly up-regulated in paraquat-treated lung cell lines. These up-regulated miRNAs mediated the protein insertion into mitochondrial membrane involved in apoptotic signaling pathway [38]. Our finding demonstrated the significant positive correlation between ΔmiR-21 expression and volume of glyphosate use (L). It was the first to report the relationship of miR-21 biomarker with the glyphosate usage. It was assumed that high volume of glyphosate usage in farms was key parameter demonstrating the high risk of intake exposure. The high dose of glyphosate could induce kidney injury as described above. Subsequently miR-21 was early detected and could be used for kidney injury prediction. However, many factors probably influenced on urinary biomarker levels in our study leading to insignificant results between pre- and post-urine samples. These could be explained with following reasons. First, the exposure dose during work-task was relatively low in comparison with the previous study of Abdul et al., 2021. They found the significant of renal biomarkers such as serum cystatin C and neutrophil gelatinase-associated lipocalin (NGAL) in glyphosate users who showed the urinary glyphosate level between 33.1 – 827.3 μg/g Cr (median 224.3 μg/g Cr) which was higher than glyphosate level in our study [32]. Moreover, all farmers used PPE for instance mask, gloves, boots, and long-sleeved shirt to reduce the risk of exposure [31, 39, 40].

The limitation of this study was no biomarker data of a healthy control group (normal population), who do not apply herbicides. The sample size for biomarker quantification is limited. Although two biomarkers were not significant between pre- and post-work urine sample, it might be concluded that the miR-21 expression related with the dose of glyphosate application in farmers.

5. Conclusions

In this work, there was no significant differences of selected biomarkers between pre-work and post-work. The ΔmiR-21 expression positively correlated with volume of glyphosate used with the spearman's rank correlation analysis. The miR-21 could be used as a marker to predict early renal injury in occupational exposure. However, more study needs to be done.

Supplementary Materials: None

Author Contributions: KW and SK conceived the present idea. KW, SK and RS were involved in planning and supervised the work. KW, SK, KK and UI contributed to sample collection and management. KK and UI contributed to interviewing of the participants. KK and SK carried out the experiments. KK, SK, and KW contributed to the interpretation of the results and data analysis. KK, SK, and KW contributed to original draft preparation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: None

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