ISSN: 0067-2904

Evaluation of Oxidative Stress activity, DNA Damage and Global DNA Methylation among Patients with Chronic Kidney Disease

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Received: 8/5/2023 Accepted: 5/8/2023 Published: 30/9/2024

Abstract

Chronic kidney disease (CKD) is a serious threat to global medicine as more than two million get CKD yearly. However, the understanding of DNA damage, DNA methylation in chronic kidney disease (CKD) remains elusive. The study goal was to establish whether homocysteine (Hcy), oxidative DNA damage (ODD), glutathione peroxidase (GPX3) and 5-methylcytosine (5mC) could be used as a potential marker of CKD. Measurements of Hcy, ODD, and GPX3 levels were done by commercial enzyme linked immunosorbent assay (ELISA) kits of 60 patients with chronic kidney disease (age range 20-87 years) and 30 age-matched healthy controls. DNA extracted from blood of patients was used to evaluate the global 5-methylcytosine (5mC) levels by the MethylFlash™ methylated DNA quantification Kit (Epigentek, USA). Results indicated a highly significant rise in Hcy and ODD in CKD patients in contrast to the healthy controls group. Although GPX3 level reduced in CKD patients, there was no significant difference between the patients and healthy controls. This research results also revealed significant rise in global 5mC level in CKD patients in comparison to the healthy controls. There is a potential role of homocysteine, oxidative DNA damage and global 5mC to be used as a biomarker for the progression of CKD.

Keywords: Hcy, Oxidative DNA damage, CKD, GPX3 and DNA methylation.

تقييم نشاط الإجهاد التأكسدي وتلف الدنا ومثيلة الدنا الكلي بين مرضى الكلى المزمن

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الخلاصة

يعد مرض الكلى المزمن تهديدًا خطيرًا للصحة العالمية ، ويصاب أكثر من مليوني شخص بمرض الكلى المزمن سنويًا. مع ذلك ، فإن فهم تلف الدنا ومثيلة الدنا الكلي في مرض الكلى المزمن لا يزال بعيد المنال. كان الهدف من الدراسة هو تحديد ما إذا كان يمكن استخدام الهوموسيستين، تلف الدنا المؤكسد، الغلوتاثيون بيروكسيديز 3 و مثيلة الدنا الكلي كمؤشرات محتملة لمرض الكلى المزمن. تم اجراء قياسات لمستويات الهوموسيستين، تلف الدنا المؤكسد و الغلوتاثيون بيروكسيداز 3 بواسطة عدة الامتزاز المناعي المرتبط بالانزيم (ELISA) لـ60 مريض يعانون من مرض الكلى المزمن (الفئة العمرية 20 – 87 عاما) و 30 شخصا يتمتعون بصحة جيدة. تم استخلاص الدنا من دم المرضى الذين يعانون من مرض الكلى المزمن واستخدمه لتقييم مستويات 5 ميثيل سايتوسين الكلي بواسطة العدة الخاصة بإستخلاص الدنا. اشارت النتائج الى ارتفاع

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كبير في الهوموسيستين و تلف الدنا في المرضى الذين يعانون من المرض الكلوي المزمن على عكس الاشخاص الذين يتمتعون بصحة جيدة. انخفض مستوى الغلوتاثيون بيروكسيداز 3 في المرضى الذين يعانون من المرض الكلوي المزمن و لكن لم يكن هناك فرق كبير بين المرضى و الاشخاص الذين يتمتعون بصحة جيدة. كشفت نتائج البحث ايضا عن ارتفاع كبير في مستوى مثيلة الدنا الكلي في المرضى الذين يعانون من المرض الكلوي المزمن مقارنة بالاشخاص الذين يتمتعون بصحة جيدة. اعتمادا على نتائج هذه الدراسة ، هناك دور محتمل للهوموسيستين ، تلف الدنا المؤكسد ، الغلوتاثيون بيروكسيداز 3 و مثيلة الدنا الكلي ، مما يعزز امكانية استخدامهم كمؤشر حيوي في متابعة تطور مرض الكلى المزمن.

1. Introduction

Chronic kidney disease (CKD) is a persistent illness situation that is getting more common worldwide [1]. It is defined as a steady reduction in kidney efficiency demonstrated by lowering the glomerular filtration rate to sixty mL/min/1.73 m^2, or presence of chronic urinary abnormalities such as albuminuria or structural modifications that have lasted a minimum of three months [2]. According to the Republic of Iraq-Ministry of Health/Environment statistics, the death rate from CKD was approximately 6879 individuals in 2015 [3]. Based on glomerular filtration rate (GFR), CKD is classified into five stages [4]. An imbalance in both antioxidants and reactive oxygen species (ROS) is known as oxidative stress. Excess ROS could result from a rise in ROS production, a decrease in antioxidants that decreases reactive oxygen species, or combination of the two [5]. Homocysteine (Hcy) is an amino acid that contains sulfur in its structure. It results from the conversion of methionine (Met) to cysteine (Cys) [6]. The Hcy is metabolized by 2 different ways: trans-sulfuration and re-methylation. Homocystinuria is an inherited problem in the sulfur-containing amino acid that is described as a heightened level of toxic Hcy in serum [7]. Due to impaired renal metabolism and decreased renal excretionm homocystinuria affects approximately 85% of CKD patients [8].

In human body, the source of Hcy is methionine. With high levels of methionine, Hcy gets converted into cysteine by transsulfuration process. And with a low methionine ratio, homocysteine is re-methylated to methionine which requires the cofactors vitamin B12 and methionine synthase [9]. Glutathione peroxidases (GPXs) are an enzyme family that can convert lipid hydroperoxides to their relevant alcohols and free hydrogen peroxide to H_2O [10]. Glutathione peroxidases perform an important function in protecting cells from free radical damage [11]. This family is composed of eight distinct GPXs that are not only phylogenetically related but are also structurally and antigenically distinct [12]. The plasma GPX, an essential extracellular antioxidant, is primarily produced in the kidney and found in a variety of fluids in humans [13]. The plasma antioxidant enzyme GPX3 protects genomic integrity by inactivating radicals [14]. Oxidative DNA damage (ODD) caused by ROS is a persistent danger to the genome [15]. These could be caused by multiple external and internal sources like endogenous chemical processes, cell metabolism and enzymatic activity [16].

DNA methylation is a covalent modification of cytosine nucleotides that typically happens in the context of CpG. A complex protein system in the cell writes the pattern of DNA methylation via *de-novo* methylation (DNMT3A and DNMT3B) or methyl group removal (TET1, TET2, and TET3), and a collection of factors faithfully duplicate methylation patterns during the replication of DNA (UHRF1D and NMT1) [17]. DNA methylation is an epigenetic process in which a methyl group is transferred to the C5 position of a cytosine to produce 5-methylcytosine (5mC)[18]. Approximately 90% of the cytosines in CpG sites are methylated in somatic human cells. This percentage varies among different tissues and pathological conditions [19]. Global DNA methylation refers to the total methyl bonded to the DNA CpG

sites [20]. Several proofs point to the presence of epigenetic alterations in CKD, most notably changes in DNA methylation, either locally or globally, or both [21]. However, understanding DNA damage, DNA methylation in CKD remains elusive. The study goal was to determine whether homocysteine Hcy, ODD, GPX3 and 5mC global DNA methylation could be used as potential biomarkers for the progression of CKD. Due to the importance of these biological parameters, the disease is increasing in the last years and only few studies have discussed it with the previous mentioned biological parameters.

2. Subjects and Methods

2.1 Study design

This research comprised sixty participants with chronic kidney disease which was conducted at Kidney Diseases and Transplant Center /Medical City/Baghdad/Iraq. The study extended between September 2022 and February 2023. The ages of CKD patients ranged from 20–87 years, while those of 30 healthy subjects who participated in this study ranged from 20 to 70 years.

2.2 Collection of Blood Samples

Venous specimens of blood were gathered from CKD diagnosed patients in the sitting posture using a set of five milliliter disposable syringes. A total of 3 milliliters of blood was gradually squeezed into disposable tubes of serum that contained separating gel, and the remaining two milliliters was placed in EDTA tubes (ethylene diamine tetra acetic acid). After allowing the blood in the gel tubes to coagulate for 15 min. at room temperature, the serum was divided into 3 Eppendorf tubes and stored at -20°C until later use. The blood within the tubes containing ethylene diamine tetra acetic acid (EDTA) was then extracted for deoxyribonucleic acid (DNA) and stored at -20 degrees Celsius up to when the need arises.

2.3 Quantitative Measurements of Serological Biomarkers

Homocysteine, GPX3 and oxidative DNA damage (8-OHdG Quantitation) were quantified in human serum samples using the enzyme-linked immunosorbent assay (ELISA) kits (SunLong Biotech, China), and according to the manufacturer's guidelines. All samples of serum and reagents were thawed and brought to room temperature before use. The wells of a 600 ml wash buffer were then filled with 50 µl of standards (S1, S2, S3, S4, S5 and S6), with a single well left empty to serve as a blank control. Following the addition of 40 µl of sample dilution buffer and 10 µl of serum samples, all wells were rinsed 5 times. After that 50 µl of horseradish peroxidase reagent (HRP conjugate) was added into all wells, mixed and then covered. The plate was incubated at 37 degrees Celsius for a period of thirty minutes before being removed and rinsed 5 times as previously. Then, in the dark, 50 µl of each chromogen solution was gently mixed into all wells. The reaction ended by adding fifty µl of stop solution to all wells which turned the wells yellow. As a result, the reaction was complete. The sample absorbance was determined by using a HumaReader HS (ELISA reader), Human Diagnostic Worldwide, Germany) set to 450 nm wavelength.

2.4 DNA Extraction

The Quick-DNA Blood Miniprep Kit (ZymoResearch, USA) was used to extract DNA from blood samples of CKD patients and healthy controls. The presence and purity of extracted genomic deoxyribonucleic acid were verified by using a NanoDrop spectrophotometer (Nabi, Korea) that evaluates genomic deoxyribonucleic acid concentration (60 - 250 ng/µl) and checks the purity of deoxyribonucleic acid (DNA) by measuring absorbance at 260/280 nm. After 30 minutes of running on 1% agarose gels at 80 V, DNA was stained with red safe nucleic acid staining solution to reveal any DNA damage.

2.5 Global DNA Methylation Procedure

The MethylFlashTM methylated DNA quantification Kit (Epigentek, USA) was used as directed by the manufacturer to determine the total 5mC content in deoxyribonucleic acid gained from samples of blood. The assay employed 100 ng of DNA for each sample. In order to get started, a standard curve for a methylated polynucleotide containing 50% 5mC was established as a positive control. The five concentrations shown in Figure 1 were used for creating this curve. Absorbance was detemined with an ELISA reader at 450 nm. Figure 1 illustrates the standard curve derived using linear regression and the below formula was used for calculating the proportion of 5mC in the entire deoxyribonucleic acid.

$$5-mC\ (ng) = \frac{Sample\ OD - Negative\ control\ OD}{Slope \times 2 *}$$

$$5-mC\% = \frac{5-mC\ Amount\ (ng)}{S} \times 100\%$$

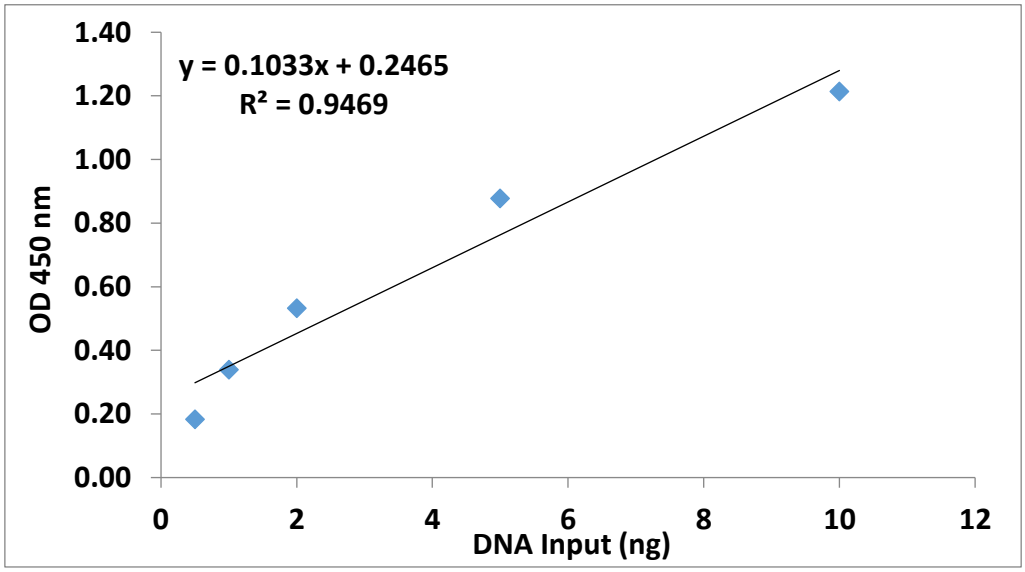

Figure 1: The immunoassay standard curve to determine methylation of deoxyribonucleic acid (DNA). The diagram shows a linear relationship between the amount of 5mC and its absorbance.

2.6 Statistical Analysis

The sStatistical Package for the Social Sciences (SPSS), version 23 was employed for the statistical analysis. The outcome was given as Mean ± SE. ANOVA analysis was applied to compare the statistical differences between groups, and $p \leq 0.05$ was deemed a significant value.

3. Results

Based on the statistical analysis, the result of age was significantly higher in CKD patients (50.08 ±2.28) than the control group (32.76 ±3.02) (Table 1). Also, the results of Table 1 show that the body mass index (BMI) in CKD patients and healthy controls did not differ significantly (26.61 ±0.55 and 25.98 ±1.08 kg/m^2 respectively).

Table 1: Comparison between CKD patients and control group in Age and BMI.

Group	Mean ± SE	
	Age (Year)	BMI (kg/m^2)
CKD Patients	50.08 ±2.28	26.61 ±0.55
Control	32.76 ±3.02	25.98 ±1.08
T-test	7.497 **	2.184 NS
P-value	0.0001	0.567
Data are presented as mean ± Standard Error (SE). ** (P≤0.01), NS: Non-Significant.		

Results of genders showed there was no significant difference between CKD diagnosed male and female patients ((53.33% and 46.67% respectively) in comparison to the healthy control groups' males and females (56.67% and 43.33% respectively) (Table 2).

Table 2: Distribution of sample study according to gender in CKD patients and control group

Group	No	Male No. (%)	Female No. (%)	*P*-value
CKD Patients	50	32 (53.33%)	28 (46.67%)	0.571 NS
Control	30	17 (56.67%)	13 (43.33%)	0.465 NS
P-value	--	0.131 NS	0.0956 NS	---
Data is presented as mean ± Standard Error (SE). NS: Non-Significant				

The results of parameters studied indicated that Hcylevels were significantly (p≤0.05) higher in CKD patients' serum (27.92 ±7.80 μmol/L) than in the control group (9.11 ±0.07 μmol/L) (Table 3). GPX3 levels in the CKD patients (30.04 ±11.14 ng/mL) and control group (36.81 ±2.25 ng/mL) did not differ significantly (Table 3). The ODD level was significantly (p≤0.05) higher in CKD patients (18.01 ±12.25 ng/mL) than in the control group (1.234 ±0.09 ng/mL) (Table 3). Also, the results of Table 3 indicate that deoxyribonucleic acid (DNA) methylation level was significantly (p≤0.05) higher in CKD patients (0.372 ±0.02%) compared to the group of healthy controls (0.257 ±0.02%).

Table 3: Comparison between CKD patients and control group in Hcy, GPX3, ODD and DNA methylation

Group	Mean ± SE			
	Hcy (μmol/L)	GPX3 (ng/mL)	ODD (ng/mL)	DNA Methylation (%)
CKD Patients	27.92 ±7.80	30.04 ±11.14	18.01 ±12.25	0.372 ±0.02
Control	9.11 ±0.07	36.81 ±2.25	1.234 ±0.09	0.257 ±0.02
T-test	15.121 *	20.016 NS	14.337 *	0.0619 **
P-value	0.050	0.652	0.0492	0.0006
Data are presented as mean ± Standard Error (SE). * (P≤0.05), ** (P≤0.01), NS: Non-Significant				

The group of CKD patients were matched based on age (<40, 40-60, and >60 years) (Table 4). Based on the statistical analysis, there was no significant difference in the serum of Hcy level among different age groups of CKD patients (Table 4). The level of GPX3) did not

differ significantly between groups of different age of CKD patients (Table 4). Also, no significant difference in the level of oxidative DNA damage (ODD) among different age groups of CKD patients was observed (Table 4). The results of Table 4 indicate that the global DNA methylation level was non-significant between age groups of CKD patients.

Table 4: Effects of age groups in parameters study of CKD patients.

Age Groups	Mean ± SE			
	Hcy (µmol/L)	GPX (ng/mL)	ODd (ng/mL)	5mC (%)
20-40 yr.	21.35 ±1.21	19.96 ±1.64	6.52 ±0.59	0.330 ±0.06
40-60 yr.	19.38 ±0.51	18.18 ±0.82	5.58 ±0.50	0.377 ±0.02
>60 yr.	46.25 ±25.98	54.29 ±35.58	50.20 ±44.97	0.400 ±0.06
P-value	0.311 NS	0.350 NS	0.282 NS	0.590 NS
Data are presented as mean ± Standard Error (SE). NS: Non-Significant				

Based on the statistical analysis, the correlation between DNA methylation & homocysteine (Hcy) was not significant (0.374) in CKD patients (Table 5). The correlation between methylation & GPX3 was also not significant (p=0.606) in CKD patients (Table 5). Also, the results in Table 5 indicate that the correlation between methylation & oxidative DNA damage (ODD) was not significant (p=0.784) in CKD patients. The correlation between Hcy & GPX3 in CKD patients was however significant (Table 5). The correlation between Hcy & ODD was significant ($P\leq0.0001$) in CKD patients (Table 5). The correlation between Hcy & ODD in CKD patients was significant ($P\leq0.0001$) (Table 5). Also, the results in Table 5 indicate that the correlation between GPX3 & oODD was significant ($P\leq0.0001$) in CKD patients.

Table 5: Correlation coefficients-r between different parameters in CKD patients' group

Parameters	Correlation Coefficients-r	*P*-value
5mC & Hcy	-0.21 NS	0.374
5mC & GPX3	-0.14 NS	0.606
5mC & ODD	-0.08 NS	0.784
Hcy & GPX3	0.99 **	0.0001
Hcy & ODD	0.99 **	0.0001
GPX3 & ODD	0.99 **	0.0001
Data are presented as mean ± Standard Error (SE). ** (P≤0.01), NS: Non-Significant		

4. Discussion

CKD is a complex disorder impacting around 10-15% of the world's population. The results of this study showed a rise in the age of CKD patients compared to the group of healthy control and this result corresponds to a study that found a strikingly high CKD prevalence in people aged 20yr. and above. And that the rising prevalence of poor kidney function in older people could be attributed to a rise in age-related risk factors for CKD development [22]. Another study found that in older patients with CKD, the relative risk of death varies significantly from that in younger CKD patients [23]. Renal mass declines throughout age ranges of 30 and 80 yr., with the sharpest drop taking place after reaching the age of 50 yr. Scarring from fibrosis and fat occur mostly in the renal cortex, damaging the nephrons that are crucial for maximum concentration of urine. Even in typical aging kidneys,

by the age of 75 yr., 30% of the glomeruli got damaged and showed diffused glomerular sclerosis, and the remaining glomeruli decreased filtering function [24]. For decades, it has been observed that estimated glomerular filtration rate (eGFR) decreases with age [25]. Another study found that many patients demonstrated a steady decline in the rate of glomerular filtration and renal blood flow as they aged, with significant variation between individuals [26]. However, this study suggested that the BMI did not differ significantly between CKD patients and the healthy control group. This result corresponds to another study that found that raised BMI was an independent risk factor for chronic kidney disease [27], however it opposes another study that found that being overweight or obese were linked to an increased risk of CKD as compared to those of normal weight [28].

This study indicated there was no significant difference in genders between CKD patients and the group of healthy control. This opposes a study that discovered women have a slower progression of chronic kidney disease [29]. This study also opposes another study that stated that CKD was more prevalent in males than the females [30]. However, the reason for our results might return to the number of samples of CKD patients. Gender differences in lifestyle have been proposed as a possible reason for the influence of gender on CKD. A high protein and caloric dietary intake which is more common in males than in women, has been linked to the development and progression of kidney problems. High LDL, triglyceride, and uric acid levels, as well as low HDL levels, are linked to faster renal disease progression. These patterns are more prevalent in men and are impacted by diet and lifestyle [31].

This study reported thatHcylevel was significantly higher in CKD patients in comparison to the group of healthy control and that Hcy had a positive correlation with GPX3 and ODD. Our results of Hcy are in line with the results of many other studies that showed that Hcy can be employed as disease prognostic indicator [32] and also correspond to another study that indicated that the kidney performs an essential role in homocysteine metabolism, and it is known that plasma total homocysteine rise in proportion to the degree of renal function loss [33]. Homocysteine concentrations are known to be regulated by folate and vitamin B12 concentrations, with low concentrations of these vitamins increasing homocysteine levels. Comorbidities and multi-drug therapy in CKD patients might result in malnutrition and, as a result, vitamin B12 and folic acid insufficiency. Furthermore, uremic individuals have poor folic acid metabolism, and functional vitamin B12 insufficiency could be detected due to raised transcobalalmin losses in the urine and decreased absorption in the proximal tubule [34].

This study showed a decrease in GPX3 level in chronic kidney disease patients in comparison to the group of healthy control and it had a positive correlation with ODD . This result agrees with another study that indicated that in the primary stages of CKD, GPX3 was discovered to be impacted. In CKD patients, this enzyme was deficient because the kidney is the primary source of plasma GPx synthesis, plasma GPx activity may decrease as the kidney's functions fall [35, 36]. Also, the result of this study found an increase in oxidative DNA damage (ODD) in chronic kidney disease patients in comparison to the group of healthy control. This finding corresponds to a study that indicated that six studies involved pre-dialysis (pre-D.) patients, and five of them discovered higher DNA damage as compared to healthy control group [37] and also corresponds to a study that indicated that CKD patients have higher levels of genomic damage than healthy controls. Raised DNA damage levels might be regarded as risk factor for chronic kidney disease progression. Reduced capacity for antioxidants in CKD patients may result in oxidative damage [38]. Also, the results of this study agree with another study that indicated that the kidneys are extremely metabolically active organs, receiving 25% of total cardiac output and using 7% of daily energy expenditure

to support their numerous tasks. They are, in consequence, subject to extreme oxidative damage [39]. The difference between the excessive production and insufficient clearance of highly reactive molecules (RNS and ROS) in response to behavioral or environmental stress is known as oxidative nucleic acid damage. Damage to nucleic acids or DNA, for instance; base and sugar alterations, covalent crosslinks, and single- and double-strand breaks, can be caused by oxidative stress. The deoxyribonucleic acid bases, particularly guanine (G), are subject to oxidation which results in oxidized guanine products. One of the most prevalent oxidative byproducts of nucleic acids, 8-hydroxy-2-deoxyguanosine (8-OH-dG), is involved in nucleobase alterations most commonly. The increased development of oxidative nucleic acid damage in CKD patients is due to compromised antioxidant system performance and an imbalance between endogenous antioxidant forces and free radicals which might raise the risk of subsequent cancer development [40]. In this study the levels of global DNA methylation significantly increased in CKD patients in comparison to the group of healthy control and it agrees with another study that reported that alterations of 5mC level can precede modification in kidney functions or the development of CKD, but also be their consequence.

An impacted metabolic state, like uremia in CKD, could have a role in altering epigenetics-mediated gene expression. Hyperhomocysteinemia may cause glomerular damage by impairing DNA methylation, inducing oxidative stress among other adverse effects [41].

Conclusion

Depending on the results of this study there are potential roles of Hcy, ODD, GPX3 and 5mC in the usage of them as biomarkers in the progression of CKD.

Ethical Clearance

This study was approved by ethical committee of Department of Biology, College of Science, University of Bagdad, Baghdad, Iraq with authorization reference number CSEC/0922/0110 in September 29, 2022.

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