Study of Angiotensin converting enzyme (ACE) gene alleles and its effect on DNA damage among chronic kidney disease patients on hemodialysis

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Abstract

Introduction: Angiotensin converting enzyme (ACE) is a key component of Renin-Angiotensin system. ACE I/D polymorphism varies as per individual, Ethnicity, geography and is associated with common diseases like Hypertension, CHD and Nephropathy. The aim of this study was to identify the role of ACE gene alleles and also to find out the effect of its damage in CKD patients.

Material and Methods: The patients diagnosed as Chronic kidney disease (CKD) from department of medicine were included. Patient's written consent was taken. Patients with co existing other illnesses, cancer, on chemotherapy or drugs likely to cause DNA damage were excluded. The peripheral venous blood was used for DNA isolation as per Quigen kit. Isolated DNA was amplified by PCR using primer for the ACE gene as per protocols. PCR product was subjected to electrophoresis for detection of insertion and deletion in ACE gene. Cytokinesis-block micronucleus assay (CBMN) assay and comet assay was performed under alkaline condition to study DNA damage.

Results: Total 47 cases were studied. PCR amplification and gel electrophoresis of DNA sample showed presence of ACE gene DD allele, II allele, D/I allele indicating deletion or insertion in ACE gene. On distribution D/I allele group was on higher 18 (38.29%) side when compared to DD allele 17 (36.17%) and II allele 12 (25.53%). No statistically significant difference (p=>0.05) was observed on comparison with II ACE gene allele with D/I allele and DD allele regarding its effect on DNA damage.

Conclusion: Present study shows higher percentage of patients with D/I ACE gene. The DNA damage difference was not statistically significant on comparison between alleles of ACE gene.

Keywords: Gene, kidney, disease, DNA damage

Introduction

Chronic kidney disease (CKD) is a continuous condition marked by worsening kidney function overtime[1]. CKD patients have signs of widespread DNA damage and prominent risk of developing cancer[2]. DNA lesions may persuade mutations in oncogenes and tumour-suppressor genes that may go ahead to malignancies if mutagenicity is not alleviated by repair mechanisms[3]. In addition to biological inference of renal disease and uraemic milieu on the reliability of DNA, the modality and period of the uraemia treatment may also manipulate the degree of DNA damage and the mechanisms of DNA repair[4]. DNA integrity has been studied by two methodologies, the cytokinesis-block micronucleus (CBMN) assay[5,6] and the comet assay[7]. The CBMN assay identifies the chromosome breakage, loss and rearrangement, in cells that completes nuclear division[5,6]. Wherein comet assay is a precise method for measuring DNA oxidation in an individual cell, and it is a regular technique for estimating DNA damage due to its sensitivity for detecting low levels of DNA damage and is considered as a useful tool to assess DNA damage in peripheral blood lymphocytes (PBL) of CKD patients. High frequencies of micronuclei (MN) are found in cultured PBL of patients before and after renal therapy[3]. MN formation has been authenticated as a biomarker for environmental mutagen, carcinogen

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exposures, genomic instability and is extensively used as a measure of chromosomal damage and early biomarker of cancer probability[8].

The genetic damage can be expressed as the presence of multiple genetic alterations both at the level of DNA sequence, cytogenetic and change in cell cycle checkpoints[9].

It indicates that CKD patients show increased background levels of DNA damage, which specifies reduced DNA integrity in patients with CKD. The studies in India are lesser in number. Due to individual, ethnic, geographical variations in above parameters there is need to be studied in detail in each region of country and world as a whole. This an attempt to correlate the impact of ACE gene I/D polymorphism to DNA damage irrespective of etiology of CKD which has not been done in earlier studies. The aim was to study the role of ACE gene alleles and DNA damage in relation to ACE gene polymorphism in CKD patients. And also to compare the impact of ACE gene polymorphism on DNA damage among CKD patients.

Materials and methods: This present study was carried out at a tertiary care medical college from January 2021 to December 2022. Ethical clearance was taken from the Institutional ethical committee (IEC) and written consent for voluntary participation of participant was obtained.

Patients and sampling criteria:

The subjects diagnosed as CKD patients on haemodialysis from the department of general medicine, Belagavi Institute of Medical Sciences, Belagavi Hospital were selected. With all aseptic precautions in EDTA and heparin vacutainers 5cc venous blood sample was taken from all patients before starting dialysis.

Inclusion criteria: Diagnosed CKD patients on regular dialysis since 12-15 months were included for the study.

Exclusion criteria: Patients with co existing other diseases as cancer, patients on chemotherapy or drugs likely to cause DNA damage were excluded.

Sampling criteria: 5 cc of venous blood in EDTA and heparin containing vacutainers was taken from identified cases and control group with all aseptic precautions. Total 47 samples were collected from CKD patients. Sample size was calculated upto 5% absolute error with the help of the prevalence rate of the disease.

DNA extraction and PCR product amplification from blood samples

By Qiagen kit method DNA was extracted from whole blood containing EDTA. The quantity and quality of the DNA were analyzed by biospectrometer. Determination of ACE genotype of cases from the genomic DNA fragments on intron 16 of ACE gene was amplified by PCR. The condition for amplification was, Initial denaturation: 94°C-5 minutes, Denaturation: 94°C-30 seconds, Annealing: 58° C- 45 seconds, Extension: 72°C-45 seconds, cycling condition: 30 cycles, Final extension: 72°C-7 minutes, Hold at 4°C. Primers for ACE Polymorphism considered for the study were the flanking primer sequences as reported by Rigat et al[10].

Forward Primer: CTG GAG ACC ACT CCC ATC CTT TCT (50nmol)

Reverse Primer: GAT GTG GCC ATC ACA TTC GTC AGAT (50nmol)

All the amplicons were subjected to 2 % agarose horizontal gel electrophoresis with ethidium bromide and bands visualized under UV light. With the help of DNA ladder, Deletion (D allele) and Insertion (I allele), were identified at 191 and 478 bp fragment respectively.

DNA and chromosome damage markers

Cytokinesis-block micronucleus (CBMN) assay and comet assay was performed under alkaline condition. Peripheral blood lymphocytes (PBL) were used to determine DNA and chromosome damage. Lymphocytes were isolated with the use of a Ficoll density gradient (Ficoll-paque PLUS, Amersham, Biosciences, USA), according to standard protocols.

Cytokinesis-block micronucleus assay

The CBMN assay was performed according to Fenech[11] with slight modifications. Briefly, lymphocyte cultures were set up in tubes by adding whole blood to PB-MAX-karyotyping medium containing RPMI-1640 medium, 15% hiFCS, 1% antibiotics, l-glutamine and 1% phytohaemagglutinin, and lymphocytes were incubated for 72 h at 37°C with 5% CO_2. Two cultures per subject were established. Cytochalasin-B (6 µg/ml) was added to the cultures 44 h later. At 72-h incubation, the cultures were harvested by centrifugation (800 rpm/8 min) and treated with a hypotonic solution (0.075 M KCl/4°C). The cells were then centrifuged and a carnoy's fixative was added three times, and the resulting cells were resuspended and dropped onto clean slides. The slides were stained with 10% Giemsa in phosphate buffer (pH 6.8) and scored. A total of 1000 binucleated cells (BNC) with well-preserved cytoplasm and nuclei were scored per subject on coded slides and the frequency of MN/1000 BNC was determined. To avoid differences between observers, the same individual carried out all the microscopic analyses. MN was scored according to the standard recognition criteria.

Comet assay

The alkaline version of the comet assay was performed according to the method of Singh et al[12]. with slight modifications. In brief, 25 µl of the single suspension of the cells of interest are suspended in low melting agarose (LMA) 0.5%, in calcium- and magnesium-free phosphate-buffered saline (fCa/Mg-PBS buffer) and layered onto fully frosted microscope slides pre-coated with 480 µl of standard agarose (SA) 0.75% in fCa/Mg-PBS buffer. A final layer of 100 µl of 0.5% LMA was added on top. Slides were immersed in a jar containing cold lysing solution (1% Triton X-100; 10% DMSO; 10 mM Tris; 2.5 M NaCl; 1 mM Na2EDTA with pH 10 at −4°C for 1 h). Slides were pretreated for 20 min in unwinding buffer (300 mM NaOH; 1 mM Na2EDTA/ pH 13). Electrophoresis was carried out using the same solution buffer for 20 min/25 V and 300 mA (0.8 V/cm). Pre-incubation and electrophoresis were performed in an ice bath. Afterwards, the slides were washed three times in 0.4 M Tris/pH 7.4, and DNA was stained by adding 20 µl of ethidium bromide (10µg/ml). Tail parameters were then calculated using image analysis systems fitted with an Olympus BX53 fluorescence microscope that was equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Images of at least 100 randomly selected cells (50 cells from each of two slides) were analysed from each individual using the software program, Comet Score version 2012. TriTekCorp. Tail parameter used in this study was tail DNA intensity, which covers the widest range of damage and is linearly related to break frequency over most of this range. The percentage of DNA in the tail region and in head of the comet is thought to be directly proportional to DNA damage [13].

Statistical analysis was represented in mean ± SD and percentage. The parametric data was analysed by unpaired't' test and non parametric data was analysed y Mann Whitney 'U' test.

Results

Total forty seven cases were studied. PCR amplification and gel electrophoresis of DNA sample from CKD patients showed presence of ACE gene DD allele, II allele and D/I allele. The distribution of alleles is shown in table 1.

Table 1: shows Distribution of cases as per the alleles of ACE gene

Total cases	DD allele (%)	II allele (%)	D/I allele (%)
47	17 (36.17)	12 (25.53)	18 (38.29)

Distribution of ACE genotype alleles was DD alleles 17 (36.17%), II allele 12 (25.53%) and I/D alleles was 18 (38.29%). The percentage of I/D allele observed in CKD individuals was higher when compared to II allele as shown in table 1.

The mean ± SD was calculated for number of dialysis, micronuclei per 1000 Bi nucleated cells by CBMN assay and % tail DNA density in comet assay as shown in table 2, figure 1 and 2.

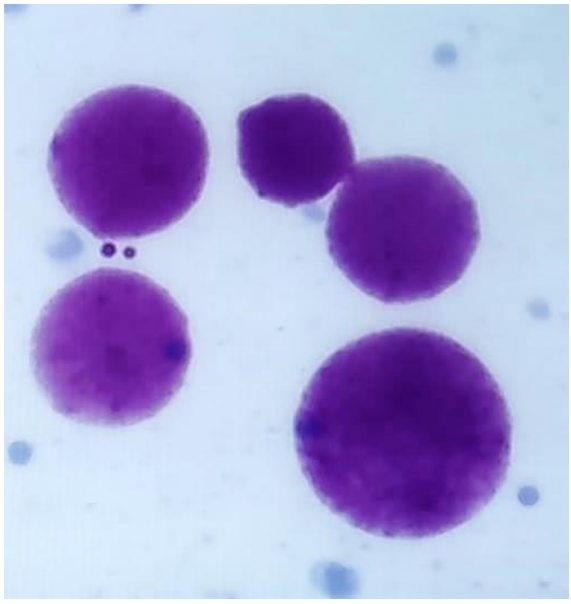
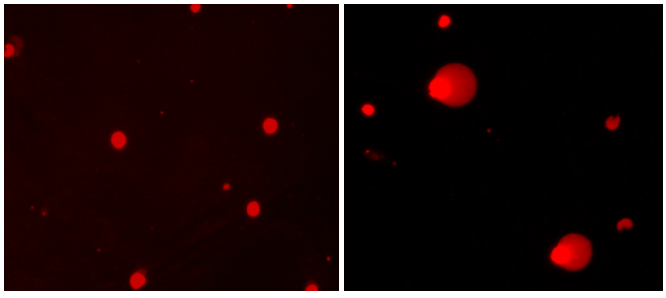


Figure 1: Shows- representative slide image of CBMN assay with bi-nucleated cells and micronuclei.



No DNA damage Moderate DNA damage

Figure 2: Shows- representative slide image of comet assay stained with Ethidium bromide

Figure 1 shows micronuclei image of bi-nucleated cells. By cytokinesis-block micronucleus assay (CBMN) the broken chromosome in the anaphase of mitosis which is due to DNA double- strand breaks are seen as MN. Figure 2 shows no DNA damage as well as moderate DNA damage as seen by comet assay.

Table 2: Shows mean ± SD of ACE gene alleles with respect to various parameters studied.

ACE gene alleles	No. of Dialysis	CBMN assay - micronuclei per 1000 Bi nucleated cells	Comet assay (% of tail density)
	Mean ± SD	Mean ± SD	Mean ± SD
DD	185.82±131.69	19.94±15.67	11.32±4.97
II	339.33±261.59	16.92±14.58	14.52±5.19
D/I	183.33±182.93	18.67±16.76	13.12±8.34

Values indicated mean ± SD of CKD participants in each gene group.

In case of DD allele, the mean ± SD for number of dialysis done was found to be 185.82±131.69 and in the same group CBMN assay showed 19.94±15.67 micronuclei per 1000 Bi nucleated cells while the mean % tail DNA density by comet assay was 11.32±4.97. In case of II allele, the mean ± SD for number of dialysis done was found to be 339.33±261.59 and the CBMN assay showed 16.92±14.58 micronuclei per 1000 Bi nucleated cells whereas the mean % tail DNA density by comet assay was 14.52±5.19. On analysis of D/I allele, the mean ± SD for number of dialysis done was found to be 183.33±182.93 and CBMN assay was 18.67±16.76 micronuclei per 1000 Bi nucleated cells although the mean % tail DNA density by comet assay was 13.12±8.34.

Nevertheless no statistically significant difference (p=>0.05) was noted between II ACE gene allele on comparison with DD and D/I alleles group as shown in table 3.

Table 3: Shows statistical comparison of II ACE gene allele with other alleles (p=value) for various parameters.

ACE gene alleles	No. of Dialysis	CBMN assay - micronuclei / 1000 Bi nucleated cells	Comet assay (% of tail density)
DD vs II	0.152	0.647	0.080
D/I vs II	0.087	0.819	0.230
D/I vs DD	0.509	0.791	0.667

Non parametric Mann Whitney U test was applied, *p value=<0.05

Discussion

Various biological parameters have been measured to estimate the burden of DNA damage, like single and double strand breaks and alkali-labile sites, detectable with the comet assay, which have been found to be significantly increased in peripheral blood lymphocytes (PBL) of patients who are undergoing dialysis[7]. In present study there are three genotypes: Distribution of ACE genotype alleles in case of DD alleles was 17 (36.17%), II allele was 12 (25.53%) and I/D alleles was 18 (38.29%). Our results show that genetic damage of DNA measured by comet assay and CBMN assay, do not show any significant impact in CKD patients among ACE gene alleles. Studies have shown that an insertion (I)/deletion (D) polymorphism exist in the angiotensin-converting enzyme (ACE) gene, and that this polymorphism affects the serum ACE level.

Previous studies using different techniques for measurement of DNA and chromosome damage have noted high levels of genetic damage which are more common in CKD patients on comparison with populations with normal renal function[3,7,14,15]. H Stopper et al investigated spontaneous genomic damage in peripheral lymphocytes of 19 patients with severe end-stage renal disease not enrolled onto a maintenance hemodialysis (MHD) program (creatinine level, 5.4 to 10.5 mg/dL) and 16 long-term MHD patients (111 to 282 months on MHD) and the possible association of genomic damage with the degree of renal insufficiency and duration of MHD.A significant increase in MN frequencies (28.2 +/- 9.4 MN/1,000 BN) was also seen in patients with advanced renal failure. H Stopper et al in another study showed that there was a significant increase to 14.7% +/- 3.5% in cells of 23 patients with chronic renal failure, and a further increase to 17.1% +/- 3.5% in the subgroup of 12 patients with serum creatinine values greater than 6 mg/dL. Damage was 16.7% +/- 4.2% in cells of the MHD group (26 patients) and 20.1% +/- 3.0% in the subgroup with MHD therapy longer than 10 years (8 patients). Cellular DNA damage in the group of 15 maintenance hemodiafiltration patients was 15.6% +/- 2.1%, ranging between predialysis and MHD patients, and did not seem to increase with treatment time. Maria J Puchades et al using high-resolution liquid chromatography columns (HPLC), enzyme assays like the superoxide dismutase (SOD), glutathione oxidized/reduced ratio (GSSG/GSH), and nuclear, as well as mitochondrial 8-oxo-dG (8-oxo-dG mit) showed that CKD patients had significantly higher levels of products derived from molecular oxidation with a significant decrease in antioxidant enzymes which can cause DNA damage. Silvia Berenice Sandoval et al proved that the frequency of MN in CRF patients was significantly higher than in controls and correlated with the progression of the disease and

to the glomerular filtration rate also. In addition, they observed a significant association between genetic damage and serum creatinine levels. They concluded that genetic damage, measured as frequency of MN, increases when renal function decreases. This is in contrast with present study where DNA damage was not significant which may be due to the duration of illness in the patients, ethinicity, geographical area difference as well as degree of kidney damage.

Micronuclei frequency derived from the CBMN assay is widely used for in vitro genotoxicity testing and population biomonitoring[16]. MN are DNA particles that take place during mitosis which are formed due to unrepaired DNA double strand breaks, thereby causing chromatin fragments or incorrect distribution of whole chromosomes[17]. The phase of CKD, dialysis modality and co-morbidities have major impact to influence the background levels of DNA effects in PBL. Based on degree of impairment of renal function the degree of chromosome damage is associated[7,10] 10 and also in patients receiving dialysis; the residual renal function have an effect on the magnitude of this damage. Nevertheless, possibility of DNA damage is mainly caused by other factors among CKD such as oxidative stress[14] and not directly related to dialysis modality[18,19].

The role of non-genetic factors like suppression of immune system, chronic inflammation, viral-linked factors, raised levels of oxidative stress, decreased antioxidant levels and accumulation of uraemic toxins are likely to be the chief contributing factor for high morbidity in patients with end stage renal disease (ESRD), this could conceivably be intervened by genetic damage. The occurrence of a low-degree chronic inflammatory state has been extensively predictable in CKD patients[20].

Conclusion

In summary this study reveals that the DNA damage difference was not statistically significant on comparison between the alleles of ACE gene in CKD patients. However higher values of D/I alleles was found when compared with DD alleles and II alleles of ACE gene in CKD patients. Future scope for the study insists conduction of longer follow up studies to add on and to study the potential genetic damage in future.

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Conflict of interest: Nil
Source of funding: Nil

Date received: May 05, 2023
Date accepted: Aug 16, 2023