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Variable R.*Msp*1 fragmentation in genomic DNA due to DNA hypomethylation in CRF patients with *MTHFR* C677Tgene polymorphism: from genetics to epigenetics

Research Article

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Summary

The role of inflammation, hyperhomocysteinemia, germ-line genetic markers and epimutations haven't been understood completely in chronic renal failure (CRF). DNA methylation is a post-replicative modification mechanism that is strongly involved in the physiological control of epimutations and gene expression. In the current study it was aimed to find out the possible role of epigenetic alterations in renal failure due to functional *MTHFR* deficiency in CRF patients requiring long-term haemodialysis. Current cohort includes 228 CRF patients and 212 healthy individuals from same ethnicity. The *MTHFR* C677T SNP analysis was genotyped by real-time PCR analysis. Genomic DNA fragmentation sizes were correlated for wild, heterozygous and homozygous mutated CRF patients after methyl marker cognate enzyme of R.*Msp*1 digestion. The digested DNA fragmentation profiles were also compared by Scion Image histogram plot analysis. Increased T allele frequency was detected in CRF patients, the *MTHFR* 677TT genotype was found 6.1% and the T allele frequency 2.53-fold increased in CRF when compared with healthy individuals. Distinct global DNA methyl patterns that showed variable R.*Msp*1 fragmentations were also detected in current *MTHFR* gene mutated CRF patients. The current results indicate that individuals with germ-line *MTHFR* C677T mutations have a risk for CRF pathogenesis due to the reduced enzyme activity and global DNA hypomethylation that alters the allelic expression of distinct systemic genes. Results needs to be confirmed by a larger scale of sample size.

I. Introduction:

Epigenetics refers to a heritable changes in genomic DNA methyl patterns and gene expression without alterations in the primary nucleotide sequence. Alterations in DNA methylation patterns in randomly genes may lead to abnormal cell function, pathological conditions and tissue development (1-3). Dialysis and chronic renal failure (CRF) has become a serious health problem through the worldwide. CRF is the common disease that caused by a wide range of diseases such as; diabetes mellitus, hypertension, primary glomerulopathies and structural point mutations, epigenetic alterations (4-7). CRF commonly associates with uremic toxins, oxidative stress, inflammation, low folate status and increased plasma levels of homocysteine (Hcy), (8,9). The recent literature findings have also showed that the molecular ethiological parameters such as; chromosomal rearrengements, point mutations, gene polymorphisms, environmental factors, lifestyles, and epigenetic alterations may cause to the CRF(10-12). It has been claimed that Cardiovascular problems are the main reason of mortality in CRF patients (13).

Chronic cardiovascular problems such as; hypertension, hyperlipidemia, hyper-homocystenemia and diabetes mellitus may also cause to CRF progression(14-16).The methylene tetrahydrofolate reductase enzyme that encoded by *MTHFR* gene plays a crucial role in the folate metabolism.Enzyme catalyzes the irreversible reaction of 5,10-methylene-tetrahydrofolate to 5-methyl tetrahydrofolate, which serves as a substrate for the remethylation of homocysteine to methionine, with the subsequent synthesis of S adenosylmethionine(SAM),(17,18).

The substrate of *MTHFR*, 5,10-methylene-tetrahydrofolate, is also required for thymidine synthesis via thymidylate synthase, and indirectly for purine biosynthesis(19). Decreasing the *MTHFR* enzyme function may cause to the global DNA hypomethylation due to lack of the intracelular methyl sources and initiates carcinogenesis and renal dysfuntion process(20, 21) Two common polymorphic SNPs were reported in exons 4 and 7 of *MTHFR* gene; one is defined in codon 677 C<T and the other one is in codon 1298 A<C.The first C677T SNP that studied in the current report, positioned in exon 4 leading to an alanine to valine conversion(22). This polymorphism lies in the C-terminal end of the enzyme, the S-adenosylmethionine regulatory domain, and may result in a decrease of 40% in enzyme activity of the variant genotype. Individuals with homozygous *MTHFR* TT genotype have 30% and heterozygous carriers show 65% loss of enzyme function(22). The TT genotype is also associated with higher plasma homocysteine and reduced plasma folate levels(23-24).

In the current case control study it was aimed to find out the possible role of *MTHFR* C677T SNP and epigenetic alterations due to global DNA hypomethylation in CRF risk in Turkish population

II. Materials and Methods:

A. Study Cohort

Presented results show germ-line mutations for *MTHFR* C677T SNP and R.*Msp*1 fragmentation profiles for high molecular weight genomic DNAs of blood samples from CRF patients that requiring long-term haemodialysis and healthy controls from the same ethnicity. The DNA bank from healthy individuals and CRF patients that optained from our previous project was retrospectively used in the genotyping and epigenetically profiling of target gene in the current study **(Table 1)**, (25). Current cohort was composed of 228 CRF patients [(123 male (54%) and 105 female(46%)] of mean age 57.40±14.3(18-75) and 212 healthy individuals[(152 male (62.0%) and 93 female(38.0%)] of mean age 57.24±9.71(32-68) from the same population.

B. DNA Isolation and SNP Analysis

Peripheric blood samples containing EDTA from patients and control group were used for genomic DNA isolation. The total genomic DNA was extracted by the MagnaPure Compact (Roche) and Invitek kit extraction techniques (Invitek®; Invisorb spin blood, Berlin, Germany). Polymorphic C677T SNP for *MTHFR* gene was genotyped in a total of 228 CRF patients and 212 healthy control individuals. Raal-Time PCR technique was used for that target SNP genotyping. The PCR mix and conditions were; Roche Fast Start Master mix (water PCR-grade, Mg+2 stock solution, Primer mix, and HybProbe mix), and DNA template were used for real-time amplification. The multiple PCR consisted of a denaturation step of 10 min at 95^0C, followed by 45 cycles of 5 s at 95^0C,10 s at 55^0C, and 15 s at 72 ^{0}C, and a melting step of 20 s at 95^0C, 20 s at 40^0C, a continuous mode at 85^0C, a cooling step of 30 s at 40^0C.A software program (LightCycler 2.0, Roche) was used for the detection of *MTHFR* C677T mutation profiles that detected in the current cohort. The *MTHFR* C677T analysis was observed in channels 640 for all profiles, the 63^0C wild melting curves were evaluated as wild and the 54.5^0C melting curves were evaluated as mutated profiles as recommended.

C. Methyl - Sensitive Restriction Enzyme Assay and DNA Gel Electrophoresis

DNA methylation patterns can be detected by using several methods, including sodium bisulfite sequencing and restriction digestion using methylation-sensitive endonucleases (methyl marker enzymes) such as R.*Msp*1, *Hpa*II and *Hha*I. The aim of this study was to determine the individual digestion patterns of whole-blood DNA in non-mutated (wild) and mutated CRF patients. The methyl sensitive enzyme R.*Msp*1 (enzyme could not digest the DNA in the presence of 5-methylcytosine – 5mC instead of cytosine in CpG dinucleotides) was used in the current study. In this technique, all genomic DNA samples were first digested with a methylation-sensitive restriction enzyme of R.*Msp*1 and fragmented DNAs were compared by agarose gel electrophoresis based Scion Image plot (USA) analysis technique. Digestion mixture (20 µl) contained 5 µl genomic DNA, 2 µl enzyme buffer for R.*Msp*1, 2 µl RNase (10 µl/ml), 0.1 µl R.*Msp*1 and 11.9 µl distilled water.The reaction mixture was incubated at 37ºC for 2 hours and digested samples were run on 2% agarose gel in TBE buffer at 90 W constant power for 1-1.5 h at room temperature. Gels were stained with ethidium bromide and evaluated at UV supported fluorescence system (DNR, Minilumi type, Germany). DNA methyl patterns were monitored at different times after induction by determining the susceptibility of the DNA to R.*Msp*1 digestion. The agarose jel profiles of the digested and unidigested DNA profiles were also evaluated by Scion Image analysis. Scion Image diagrams were compared for mutated and wild CRF patients in the current results.

F. Statistical Analysis

Association analyses based on binary logistic regression were conducted to determine the odds ratio (OR) and its 95% confidence interval (95%CI) for target mutated *MTHFR* SNP, genotypes and allele frequencies. Statistical analysis was performed using SPSS version 16 (SPSS, Chicago, IL, USA). A value of $P<0.05$ was considered as statistically significant and mutated T allele frequency was discussed in the current report.

III. Results

Some patient demographics such as; hypertension, diabetes, atherosclerosis and polycistic kidney were found in 45/18.6%, 42/17.4%, 33/13.6%, and 15/6.2% for the current CRF cohort respectively. Twenty-eight patients (11.2%) have parental consanguinity and 6 patients (2.5%) have familial FMF history in the current CRF cohort. No apperant demographic findings were detected in ninety (37.2%) patients of current CRF cohort

(Table 1).

Presented results show germ-line C677T SNP polymorphism in *MTHFR* gene and variable genomic methylation patterns of whole-blood DNA that digested by methyl modifying enzyme R.*Msp*1 in CRF patients and healthy controls from the same ethnicity of Sivas population (Tukey). The current results from two hundred twenty- eight CRF patients that requiring long term hemodialysis and two hundred eleven healthy individuals with no history of CRF and nor family history of renal disease were compared in the current article. The germ-line T allele frequency of *MTHFR* 677 C>T SNP has been shown to be a risk factor for the current CRF cohort from Turkish population **(Table 2)**, **(Figure 1)**.

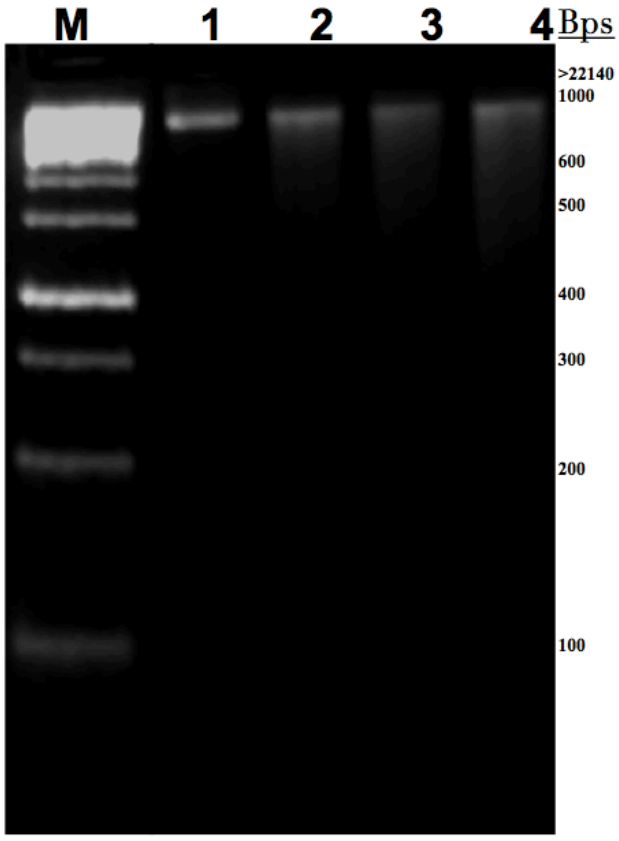

Figure 1. The 2% of agarose gel runs of R.*Msp1* digested and undigested genomic DNA from CRF patients. Variable DNA fragmentations was detected in CRF patients with wild and mutated profiles for *MTHFR* gene after R.*Msp1* digestion.

Lanes

M:GeneRuler Express DNA Ladder, 100 bps

1: Uncut DNA, no fragmentation

2: R.*Msp1* digested genomic DNA from non-mutated (wild) patient

3: Moderate type fragmented DNA from heterozygous mutated patient.

4:Showed increased fragmentations. High molecular weight genomic DNA from homozygous mutated patient showed distinct fragmentation than the wild and heterozygous mutated patients.

The distribution of genotype frequencies of the studied polymorphism between the patients with CRF and control groups was in Hardy–Weinberg equilibrium. The prevalence of genotypes of *MTHFR* gene C677T SNP in patients with CRF (50.0% for CC, 43,9% for CT and 6,1% for TT respectively) was higher than the control group (73.0% for CC, 27.0% for CT and 0.0% for TT respectively), **(Table 2)**. The T allele frequency was 0.281 for CRF patients and 0.130 for health individuals in the current results. *MTHFR* 677TT (homozygous) genotype was found 6.14% and T allele frequency 2.53-fold increased in CRF when compared to the control group form the same population. This difference was statistically significant when compared to the control group **(Table 2)**, (OR: 2.513, CI: 1.778-3.551), p<0.0001.

For establishing the posible role of epigenetic gene regulation in the CRF pathogenesis; the high molecular weight genomic DNA was digested with R.*Msp1* and compared to the wild, heterozygous and homozygous mutated CRF patients. Digested genomic DNAs showed variable fragmentation profiles in both 2% agarose **(Figure 2)** and scoin images plot analyses **(Figure 3)** in mutated and non mutated CRF patiens. The R.*Msp*1 digested high molecular weigth DNA shows variable fregmantation profiles and increased fragmented DNA was detected in homozygous mutated patients. Distinct fragmentations were detected (normal, moderate and high fragmentation) in wild, heterozygous and homozygous mutated in the current CRF patients when digested with methyl marker enzyme R.*Msp*1. There is no fragmentation in un-digested DNA and intact

genomic DNA was in normal apperance (~22000bps), **(Figure 2, Lane 1)**.

Genomic DNA from wild patients for C677T SNP in *MTHFR* gene was in normal fragmentation profile, **(Figure 2, Lane 2)** and DNA from heterozygous patients were moderate **(Figure 2, Lane 3)** and patients with homozygous profile showed different fragmentation profile in the agarose gel runs **(Figure 2, Lane 4)**. The R.*Msp*1 digested genomic DNAs were also compared by scoin image plot analysis technique. Figure 3 shows the plot analysis of digested/non-digested and patients with mutated/non-mutated *MTHFR* gene profiles.

Variable fragmentations were also detected in the scion image plot analysis; while the undigested genomic DNA showed similar profiles for both groups (CRF patients and controls), (~ 22000bps), **(Figure 3, Lane A)** the digested high molecular weight DNA showed different fragmentation profiles for CRF patients with wild, heterozygous and homozygous mutated *MTHFR* gene. Digested genomic DNAs showed unique accumulation profiles between ~22000-400bps **(Figure 3, Lane B)** for wild, modarate fragmentation of 600-250bps for heterozygous mutated **(Figure 3, Lane C)** and high fragmentation of 350-150bps for homozygous mutated CRF patients **(Figure 3, Lane D)**.

Gene/Genotypes	**CRF Patients** n=228	**Controls** n=212			
***MTHFR* C677T**	n/%	n/%			
CC	114(50.00)	155(73.0)			
CT	100(43.86)	57(27.0)			
TT	14(6.140)	0			
Alleles					
C	328(0.719)	367(0.870)	**P value**	**Odds ratio**	**CI(95%)**
T	**128(0.281)***	57(0.130)	0.0001	2.513	1.778-3.551

: *Significant

Table 1. Some demographics such as; age, gender and ethnicity of the current CRF patients and healthy controls.

Gene/Genotypes	**CRF Patients** n=228	**Controls** n=212	**P value**	**Odds ratio**	**CI(95%)**
***MTHFR* C677T**	n/%	n/%			
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TT	14(6.140)	0			
Alleles					
C	328(0.719)	367(0.870)			
T	**128(0.281)***	57(0.130)	0.0001	2.513	1.778-3.551

: *Significant

Table 2. The genotypes, allel frequency and odd ratios of *MTHFR* C677T SNP in current CRF patients and

healthy controls.

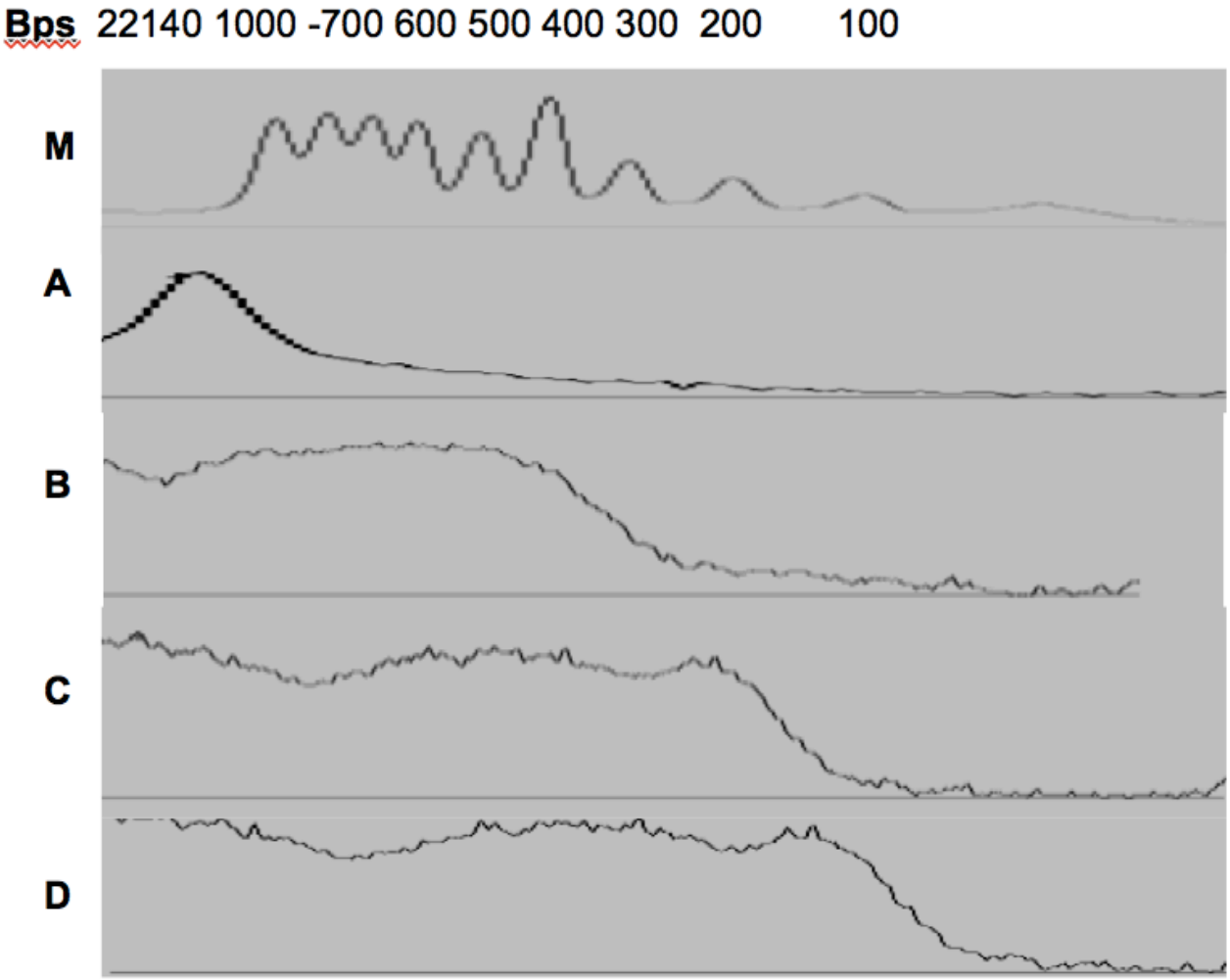

Figure 2. Shows the Scoin Image Plot analysis of high molecular weight genomic DNA from wild and mutated CRF patients.

Lanes

M: GeneRuler Express DNA Ladder, 100 bps

A: Uncut high moleculer weight genomic DNA, no fragmentation

B: R.*Msp1* digested fragment DNA from CRF patient with wild profile for *MTHFR* gene. Normal fragmentation profiles (between 22000-400bps).

C: R.*Msp1* digested fragment DNA from CRF patient with heterozygous mutated profile for *MTHFR* gene. DNA showed moderate type fragmentation profiles and fragmentations were acumulated between 600-250 bps.

D: R.*Msp1* digested fragment DNA from CRF patient with homozygous mutated for *MTHFR* gene. High fragmentation profiles than other patients were detected and fragmented DNAs were acumulated between 350-150 bps.

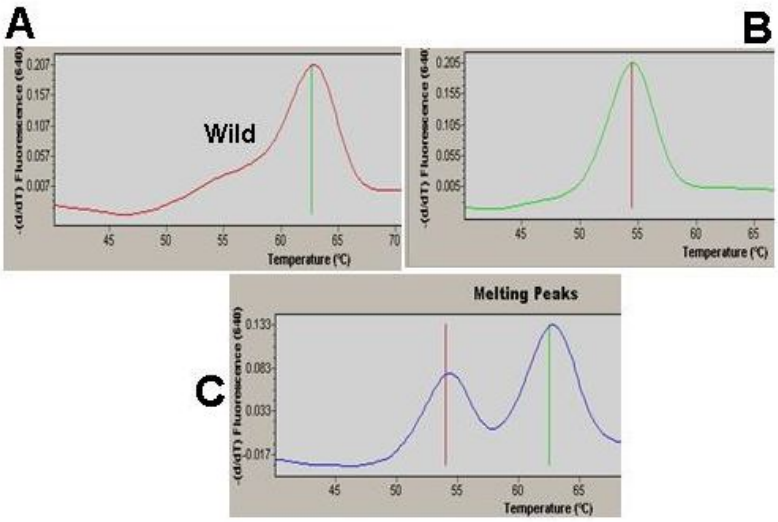

Figure 3. Melting peaks of RealTime-PCR show wild(A) homozygous (B) and heterozygous(C) mutated profiles of *MTHFR* C677T SNP for the presented CRF patients

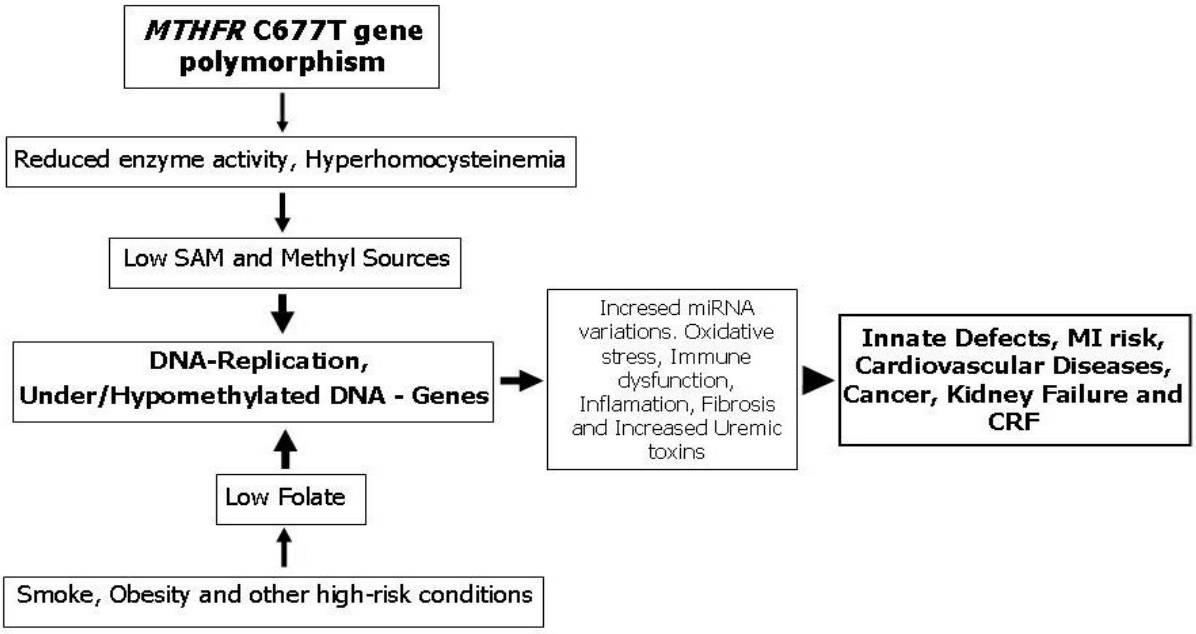

Figure 4.Hypothetically presentation of external and/or epigenetical ethyolological parameters such as; *MTHFR* polimorphism, low methyl sources and high risk conditions that caused to epimutations, DNA hypomethylation, randomly gene reactivation and variable miRNA expression that could play crucial role in the pathogenesis of CRF and renal diseases

IV. Discussion

It is well known that the disruption and alteration of genomic methylation patterns in eukaryotic cells is a hallmark of tissue-specific gene expression, transposable element repression, genomic imprinting, X chromosome inactivation, cancer and some other chronic diseases in human. As asserted by same authors CRF is a disease that is caused by a wide range of parameters such as diabetes, hypertension, primary glomerulopathies and commonly associates with uremic toxins, oxidative stress, inflammation, low folate status and levels of homocysteine. Moreover, several molecular parameters such as; chromosomal rearrengements, point mutations, gene polymorphisms, environmental factors, lifestyles, and epigenetic alterations may contribute to cause the CFR.The role of inflammation, hyperhomocysteinemia, familial genetic markers and epimutations remains incompletely understood in CRF ethiopathology. As claimed by Satta et al., Hcy is toxic for the endothelium, it may enhances vascular smooth muscle cell proliferation, increases platelet aggregation and fibrinolysis that progresses to ESRD (8). Some researchers were indicated that homocysteine levels are significantly elevated at a relatively early stage of CRF patients (14).

As claimed by Deltas et al., the familial hematuric diseases are a genetically heterogeneous group of monogenic conditions, caused by mutations in one of several genes (26). Bomsztyk et al.,have also claimed that epigenetics represents a new paradigm for understanding of pathophysiology, serves the valuable guiding development of new diagnostic tools and alternative therapies in acute kidney disease (27, 28). As claimed by Zeisberg et al., the aberrant hypo and/or hypermethylation of some target genes plays an important role in facilitating fibrotic fibroblast activation and in driving fibrogenesis (29). The epigenetic alterations by genomic DNA hypomethylation, histon protein asetilation are associated with miRNA variation, cardiovascular disease, inflammation hypertension, fibrosis, heart failure, renal fibrosis, kidney failure, and end-organ damage(30). *MTHFR* gene encodes a protein that plays a key role in folate metabolism and methyl source in eukaryotic cells that essential for epigenetic gene regulation. The different results regarding the effects of *MTHFR* C677T polymorphism on CRF risk can be ascribed to the differences in racial origin of the population, the lifestyle, and the pattern of diet, mehyl sources and folat methabolism in distinct populations and/or sub-populations.

Here we report the hypomethylated global whole-blood DNA in CRF patients that requering lon-term hemodialysis. Presented cohort includes 228 CRF patients and 212 healthy individuals from the same ethnicity. The *MTHFR* C677T SNP was genotyped by real-time PCR and the genomic DNA fragmentation sizes were correlated for wild, heterozygous and homozygous mutated CRF patients after methyl marker cognate enzyme of R.*Msp*1 digestion. Increased T allele frequency was detected in CRF patients when compared to the health individuals in the current results. The T allele frequency 2.53-fold increased in CRF when compared with control group. Since the *MTHFR* is an important enzyme involved in folate metabolism, in this study, we examined whether polymorphism and haplotype of *MTHFR* C677T is correlated with the risk for CRF in the presented results. Variable R.*Msp*1 fragmentation profiles due to DNA hypomethylation were detected in CRF patients with mutated and non-mutated *MTHFR* gene. The current results showed that the mutated individuals with germ-line *MTHFR* C677T have a risk for CRF pathogenesis due to the reduced enzyme

activity and global DNA hypomethylation. It is possible to hypothesis that the redused enzyme activity alters the allelic expression of distinct systemic genes due to disruption and alteration of genomic methylation patterns in mutated patients.

Recent studies provide evidence that genetic polymorphisms in *MTHFR* gene and epigenetic variations that contributed by that polymorphism may determine the individual susceptibility of patients to develop chronic progressive kidney disease as postulated in the current results. Some epigenetical biomarkers were reported by Sapienza et al., (4) for chronic kidney disease progression. External high risk conditions such as; oxidative stress, inflammation, immune dysfunction, hyperhomocysteinemia, infections and uremic toxins may induce epimutations and /or unexpected gene reactivations – increase miRNA expression by DNA hypomethylation in CRF (Figure 4). Aberrant DNA methylation may cause to the variable RNA interference (iRNA) in some pathological tissues. MicroRNAs are critical in the maintenance of glomerular homeostasis and hence RNA interference may be important in the progression of renal disease (2).The kidney function was recovered by epigenetically based clinical therapy for re-methylation of TGF-beta1 promoter gene in Wistar rats with CRF(9).

In recent years, the *MTHFR* gene has been emerged more and more attention by some researchers. *MTHFR* can regulate DNA methylation, maintain the integrity and stability of DNA, modify chromosomes, as well as influence the development of mutations, and its polymorphisms have been believed to be associated with DNA hypomethylation, gene reactivation variable miRNA expression and other epigenetics alterations by low methyl sources and randomly gene reactivation. It is well known that, many parameters such as; oncogenes, epigenetic alterations in tumour suppressor genes, viruses and many other intrinsic and/or extrinsic environmental factors may directly play crucial role in CRF progression. The current results with some of previous literature findings have pointed out the importance of the functional gene mediated CRF progression. Presented results indicate that *MTHFR* 677TT homozygous individuals are more likely to develop CRF than those with wild-type genotype. In this study of germ-line homogenous *MTHFR* TT individuals had 2.53-fold increased risk of developing CRF due to the increased T allele frequency when compared to the control group from the same population. This difference was statistically significant (OR: 2.513, CI: 1.778-3.551), $p<0.0001$, **(Table 2)**.

V. Conclusion

Current results suggest evidence that the global DNA hypomethylation plays crucial role in the pathogenesis of CRF. Results also suggest that the *MTHFR* C677T SNP indicates susceptibility to CRF and germ-line homozygous mutated individuals has an additional risk factor for renal failure. It is also possible to assumed that both genetics and epigenetics may be helpful in the discussion and understanding of CRF pathogenesis.

Competing interests

All authors declare that they have no competing interests.

Authors' contributions:

M.K.,O.O.; acquisition of data, performed the bioinformatical work, analyzed the clinical data and designed the clinical experiments, O.O., F.S and M.U; designed the experiments, performed PCR, analyzed the data, M.K., E.A. and A.U.; interpretation of data, performed the statistical analysis, fine redaction of the article and O.O.; supervised the project and wrote the manuscript. All authors read and approved the final manuscript.

References:

Aydin B, Ipek MS, Ozaltin F, Zenciroğlu A, Dilli D, Beken S, Okumuş N, Hoşağasi N, Saygili-Karagöl B, Kundak A, Renda R, Aydog O.A novel mutation of laminin β-2 gene in Pierson syndrome manifested with nephrotic syndrome in the early neonatal period.Genet Couns. 2013;24(2):141-147

Bomsztyk K, Denisenko O. Epigenetic Alterations in Acute Kidney Injury. Seminar in Nephrology. 2013; 33(4): 327-340

Bostom AG, Lathrop L. Hyperhomocysteinemia in end-stage renal disease: Prevalence, etiology and potential relationship to arteriosclerotic outcomes. Kidney Int 1997; **52**(1):10-20

Botezatu A, Socolov R, Socolov D, Iancu IV, Anton G. Methylation pattern of methylene tetrahydrofolate reductase and small nuclear ribonucleoprotein polypeptide N promoters in oligoasthenospermia: a case-control study. Reprod Biomed Online. 2013 [Epub ahead of print]

Deloughery TG, Evans A, Sadeghi A, McWilliams J, Henner WD, Taylor LM Jr. Press RD. Common mutation in methylenetetrahydrofolate reductase. Correlation with homocysteine metabolism and late-onset vascular disease. *Circulation*. 1996; **94**: 3074–3078

Deltas C, Pierides A, Voskarides K. Molecular genetics of familial hematuric diseases. Nephr. Dial. Transplan. 2013; 28(12): 2946-2960

Deltas C, Pierides A, Voskarides K.Molecular genetics of familial hematuric diseases.Nephrol Dial Transplant. 2013;28(12):2946-2960

Dwivedi RS, Herman JG, McCaffrey TA, Raj DS.Beyond genetics: epigenetic code in chronic kidney disease. Kidney Int. 2011;79(1):23-32

Fang JY, Xiao SD. Folic acid, polymorphism of methyl-group metabolism genes, and DNA methylation in relation to GI carcinogenesis. J Gastroenterol. 2003; **38**: 821–829

Friedman G, Goldschmidt N, Friedlander Y, Ben-Yehuda A, Selhub J, Babaey S, Mendel M, Kidron M, Bar-On H. A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations. *J Nutr*. 1999; **129**: 1656–1661

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP, Rozen RA. Candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase [letter]. Nat Genet. 1995; **10**: 111–113

Garibotto G, Valli A, Anderstam B, Eriksson M, Suliman ME, Balbi M, Rollando D, Vigo E, Lindholm B.The kidney is the major site of S-adenosylhomocysteine disposal in humans. Kidney Inter. 2009;76(3): 293-296

Goyette P, Aditya P, Renate M, Frosst P. Chen Gene structure of human and mouse methylenetetrahydrofolate reductase (MTHFR). Mammalian Genome. 1998; **9**: 652–656

Halimi S, Zmirou D, Benjamou PY, et al. Huge progression of diabetes prevalance and incidence among dialysed patients in mainled France and overseas French territories: a second national survey six years apart. Diabetes Meta 1999, **25**:507-512

Heggermont WA, Heymans S. MicroRNAs Are Involved in End-Organ Damage During Hypertension. Hypertension. 2012;60(5):1088-1093

Izmirli M, Inandiklioglu N, Abat D, Alptekin D, Demirhan O, Tansug Z, Bayazit Y. MTHFR gene polymorphisms in bladder cancer in the Turkish population. Asian Pac J Cancer Prev. 2011;**12**(7):1833-1835

Kawamura M, Fijimoto S, Hisanaga S, Yamamoto Y, Eto T. Incidence, outcome and risk factors of cerebrovascular events in patients undergoing maintenance hemodialysis. Am J Kidney Dis 1998;**31**(6):991-896

Miao XH, Wang CG, Hu BQ, Li A, Chen CB, Song WQ.TGF-beta1 immunohistochemistry and promoter methylation in chronic renal failure rats treated with Uremic Clearance Granules. Folia Histochem Cytobiol. 2010;48(2):284-291

Ozen F, Polat F, Arslan S, Ozdemir O. Combined Germline Variations of Thrombophilic Genes Promote Genesis of Lung Cancer. Asian Pac J Cancer Prev. 2013; **14** (9): 5449-5454

Parle-McDermott A, Mills JL, Molloy AM, Carroll N, Kirke PN, Cox C, Conley MR, Pangilinan FJ, Brody LC, Scott JM. The MTHFR 1298CC and 677TT genotypes have opposite associations with red cell folate levels. Mol Genet Metab. 2006; **88**: 290–294

Sapienza C, Lee J, Powell J, Erinle O, Yafai F, Reichert J, Siraj ES, Madaio M.DNA methylation profiling identifies epigenetic differences between diabetes patients with ESRD and diabetes patients without nephropathy. Epigenetics. 2011;6(1):20-28

Satta E, Perna AF, Lombardi C, Acanfora F, Violetti E, Romano MM, Capasso R, Pisano M,

Paduano F, De Santo NG.[Hyperhomocysteinemia in chronic renal failure]. G Ital Nefrol. 2006;**23**(5):480-489

Sezgin I, Koksal B, Bagci G, Kurtulgan H, Ozdemir O. CCR2 polymorphism in chronic renal failure patients that requiring long-term haemodialysis. Intern Med. 2011;**50**(21):2457-2461

Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers Prev. 2000; **9**: 849–853

Tampe D, Zeisberg M.A primer on the epigenetics of kidney fibrosis. Minerva Med. 2012;103(4):267-278

Tylicki L, Fodinger M, Puttinger H, et al. Methylenetetrahydrofolate reductase gene polymorphisms in essential hypertension relation: with the development of hypertensive end-stage renal disease. Am J Hypertens 2005; 18(11):1442-1448

United States Renal Data System; USRDS 1997 Annual Data Report. VI. Causes of death. Am J Kidney Dis 1997; **30** (suppl 1): 107-117

Weisberg IS, Jacques PF, Selhub J, Bostom AG, Chen Z, Curtis Ellison R, Eckfeldt JH, Rozen R. The 1298A > C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine. Atherosclerosis. 2002; **156**: 409–415.

Whyte MP, Leelawattana R, Reinus WR, Yang C, Mumm S, Novack DV.Acute severe hypercalcemia after traumatic fractures and immobilization in hypophosphatasia complicated by chronic renal failure. J Clin Endocrinol Metab. 2013;98(12):4606-4612

Zeisberg EM, Zeisberg M. The role of promoter hypermethylation in fibroblast activation and fibrogenesis. J Pathol. 2013; 229(2): 264-273