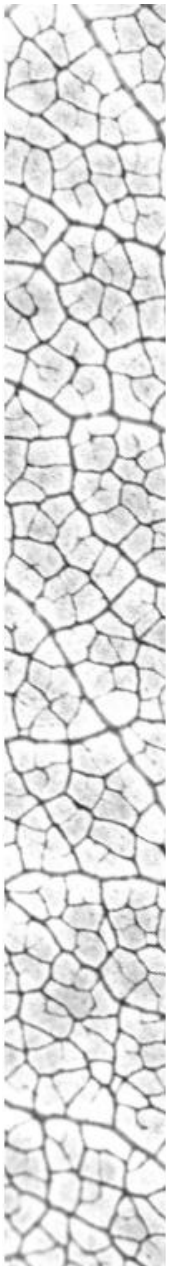

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The relationship between some electrolyte levels and MTHFR C667T gene polymorphism in premenopausal and postmenopausal osteoporosis Iraqi women

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Abstract
Gene polymorphism related to osteoporosis plays a significant role in the development of osteoporosis, therefore, this study aimed to detect the MTHFR C667T gene polymorphism and evaluate its relationship with biochemical parameters in Iraqi women with premenopausal and postmenopausal osteoporosis in Salah Al-din province. Blood samples were collected from one hundred women (53 premenopausal and 47 postmenopausal) with osteoporosis diagnosed by specialist doctors and seventy healthy women with identical ages. Serum calcium and phosphorus concentrations were estimated and genomic DNA was extracted that used to detected MTHFR C667T polymorphism employing Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) technique. The results showed a significant decrease in the calcium and phosphorus concentrations (p-value <0.001) for the osteoporosis group compared with controls. Frequency of T allele and TT genotype were high significant (p-value < 0.001) in osteoporosis patients compared with controls. But there were no significant differences in the frequency of genotypes and alleles between patients group. On the other hand, there was no correlation between the decrease of calcium and phosphorus levels and presence of T allele in the genotypes of patients (p-value > 0.05). Conclude that calcium and phosphorus levels and frequency of normal CC genotype were decreased while the frequency of mutant TT genotype was higher in women with osteoporosis compared to healthy women, this indicates that TT genotype and T allele can be considered as an indicator of osteoporosis in Iraqi women.

Keywords: MTHFR C667T, Iraqi Women, osteoporosis

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INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by low bone mineral density caused by increased in osteoclasts activity, leading to a progressive loss of bone mass and reduction of bone rigidity (Anastasilakis et al. 2018, Weinstein et al. 1998). Risk of osteoporosis increases with aging and around 85% of all osteoporotic fractures occur in women (Reid 2011). In addition to old age, osteoporosis is determined by complex interactions among various factor like; genetics factor, mineral metabolism, environmental condition and lifestyle (Al Anouti et al. 2019, Zhu and Prince 2015). Osteoporosis is less common in premenopausal women than in postmenopausal women (Cohen 2017).

Bone is a living, growing tissue composed of biological active cells inserted into a solid mineralized framework. The main composite of bone is an organic matrix and an inorganic complex crystalline mineral (Reid 2011). Bone mineral metabolism is crucial for Calcium and Phosphorus homoeostasis (Redmond et al. 2014). Calcium ion plays important role in formation, maturation and regeneration of bone, mostly calcium exists in the form of calcium phosphates in bone tissues (Jeong et al. 2019, Peacock et al. 2010). In the skeleton, the majority of phosphate is present in bone (over 80 %) in the form of calcium phosphates salt called hydroxyapatite, phosphate level is important for many bone biological processes including, cell signaling, skeletal development, and bone integrity (Jeong et al. 2019, Penido et al. 2012). Therefore, alternation in serum calcium and phosphorus using a biochemical marker to detect the rate of bone remodelling (Berndt et al. 2005, Li et al. 2019).

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In premenopausal women, genetic factors have a strong influence on osteoporosis development (Eroglu et al. 2019, Hendrickx et al. 2015, Meier et al. 2010). Postmenopausal osteoporosis is a multifactorial disorder, but the genetics factor and estrogen deficiency are the main factors in postmenopausal osteoporosis development (Ahn et al. 2018, Liu et al. 2018). Recently, data from several studies established that genetic factors contribute a critical role in the development of osteoporosis, such as genetic variants in FGFR2 (Yang et al. 2019), SAA1 gene polymorphisms (Zhou et al. 2019), expressed circulating miRNAs (Pala and Denkçeken 2019) and Methylenetetrahydrofolate reductase (MTHFR) gene polymorphism (Li et al. 2016, Soewarlan et al. 2019). MTHFR is enzyme encoded by MTHFR gene and catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, plays a vital role in provisioning the methyl group for Hcy in Hcy/folate metabolism (Deformity et al. 2018). The MTHFR gene is mapped at chromosome 1 (1p36.3), it has 12 exons and spans 11,785,723 to 11,806,103 base pairs of genomic DNA (Saffroy et al. 2005).

Single nucleotide polymorphism (SNP) is a common form of polymorphism that playing effect role in the structure and/or function of the protein or simply occur in the noncoding regions. Some previous studies suggest an association between SNPs variation and osteoporosis development. Most studies in the field of MTHFR gene and relationships with osteoporosis, it's have focused on common allele C677T (Guan et al. 2014, Saad et al. 2015, Shiraki et al. 2008), and A1298C allele (Auerkari et al. 2017, Saad et al. 2015). Although several published studies between osteoporosis and MTHFR gene polymorphisms, the allele C677T variation with osteoporosis has not studied in the Iraqi population. Therefore, the objective of this study was to detect the MTHFR C667T gene polymorphism and evaluate its relationship with biochemical parameters in Iraqi women with premenopausal and postmenopausal osteoporosis in Salah Al-din province.

MATERIALS AND METHODS

Subjects Selection

Across sectional study was made at Tikrit general hospital, the study population included (170) individuals, one hundred women (53 premenopausal and 47 postmenopausal) with osteoporosis and seventy healthy women used as a control, their ages ranged between 16 and 40 years, all subjects were recruited from Iraqi women population.

Sample Collection

After clinical examination, five ml of peripheral blood were collected from each subject in population study and divided into two parts: the first 1 ml of blood put in EDTA tube for genotyping, while the second part 4 ml was used for serum assessment of biochemical tests.

Determination of Electrolytic in Serum

Serum calcium and phosphorus were determined for patients and control women by the spectrophotometer-based method using kits provided by biomeurx (France) according to the supplier instructions (Asoodeh, & Motlagh 2015).

Determination of C677T MTHFR Gene Polymorphism

Genomic DNA was extracted from blood according to (Ali et al. 2008) method. Amplification Refractory Mutation System-Polymerase chain reaction (ARMS-PCR) technique was used to determine the C677T MTHFR genotypes**.** Three primers were used to amplify a fragment of 277 bp from MTHFR gene according to (Poursadegh Zonouzi et al. 2012), common forward primer 5-TGC TGT TGG AAG GTG CAA GAT-3 and two reverse (Reverse 1 Prime ,bbcxcxxzz\r (C allele) 5-GCG TGA TGA TGA AAT CGG-3 and Reverse 2 Primer (G allele) 5-GCG TGA TGA TGA AAT CGA-3). PCR amplification was performed in a total volume of 20 µL including 10 µL of 2X Go Taq green master mix supplied by Promega company (USA), 100 ng of DNA, 10 pmol of common and reverse C or G allele primer, finally full up 20 µL with DNase/RNase free water. PCR program consists of first denaturation step (95◦C, 2 min) was followed by 10 cycles of denaturation (95◦C, 15 s) and annealing/extension (65◦C, 60 s), and a final 20 cycles of denaturation (95◦C, 10 s), annealing (61◦C, 50 s), and extension (72◦C, 30 s). Then, final extension was carried out in one cycle of 5 min at 72 °C. PCR product was scored by running at a 2% agarose gel electrophoresis.

Statistical Analysis

Results were analyzed using SPSS version 20 PC programming. One-way ANOVA and student's t-test were used for the comparison of mean ± standard deviation (SD) of biochemical parameters of the patients and control group and among the genotypes of MTHFR polymorphism. Alleles and genotypes frequency besides of odds ratios (OR) and their 95% confidence intervals (CI) of the patients and control group were determined to utilize Pearson's chi-square test. $P<0.05$ was viewed as significant and $p<0.01$ as highly significant.

RESULTS

Serum Calcium and Phosphorus Results

Table 1 presents the mean ± SD of calcium and phosphorus serum levels obtained from one hundred Iraqi women with osteoporosis and seventy women as a control group. The cases are further subdivided into two groups, premenopausal and postmenopausal women with osteoporosis. In control group, the calcium serum levels were significantly higher (10.5 ± 1.38 mg/dl) compared to osteoporosis group (8.02 ± 1.18 mg/dl) and phosphorus levels in healthy groups were (3.11 ± 0.547 mg/dl) significantly higher than patient group (2.03 ± 0.598 mg/dl). Also, we found that the similar calcium

Table 1. Shows comparison between biochemical parameters of the study groups

Comparison between women with osteoporosis and control			
Parameter	Mean ± SD		*p* value
	Patients (100)	Control (70)	
Calcium mg/dl	8.02 ± 1.18	10.5 ± 1.38	0.001**
Phosphorus mg/dl	2.03 ± 0.598	3.11 ± 0.547	0.001**
Comparison between premenopausal and postmenopausal women with osteoporosis			
Parameter	Mean ± SD		*p* value
	Premenopausal women (53)	Postmenopausal women (47)	
Calcium mg/dl	8.00 + 1.16	8.04 + 1.21	0.88
Phosphorus mg/dl	2.02 + 0.600	2.05 + 0.601	0.82
Comparison between premenopausal women with osteoporosis and control			
Parameter	Mean ± SD		*p* value
	Premenopausal women (53)	Control (70	
Calcium mg/dl	8.00 + 1.16	10.5 + 1.38	0.001**
Phosphorus mg/dl	2.05 + 0.601	3.11 + 0.547	0.001**
Comparison between postmenopausal women with osteoporosis and control			
Parameter	Mean ± SD		*p* value
	Postmenopausal women (47)	Control (70)	
Calcium mg/dl	8.04 + 1.21	10.5 + 1.38	0.001**
Phosphorus mg/dl	2.02 + 0.600	3.11 + 0.547	0.001**

*p<0.05 significant and **p<0.01 high significant

levels between premenopausal (8.00 + 1.16 mg/dl) and postmenopausal (8.04 + 1.21 mg/dl) women with osteoporosis, also we find that the similar phosphorus levels between premenopausal (2.02 + 0.600 mg/dl) and postmenopausal (2.05 + 0.601 mg/dl) women with osteoporosis, therefore no significant difference between premenopausal and postmenopausal women. On the other hand, results of biochemical parameters (calcium and phosphorus) in **Table 1** shows significant difference (p-value <0.001**) when compared between premenopausal women with osteoporosis and control or when comparison postmenopausal women with osteoporosis and control.

Genotypes and Alleles Frequency

After analysis ARMS-PCR products of the MTHFR polymorphism, three types of genotypes (CC, CT, TT) have been obtained as seen in **Fig. 1**. **Table 2** lists the genotype and allele frequencies in (%) and the number of patients having each genotype of the study population. The distribution of genotypes in women with osteoporosis and control group was an agreement with Hardy–Weinberg equilibrium. We detected a significant difference (p-value <0.001) between the frequency of genotypes and alleles of MTHFR polymorphism in the women with osteoporosis compared with control groups.

Women patients with CC genotype (24 %) were indicated with low statically difference while both the CT (30%) and TT (45%) genotypes were higher compared with the control. Also, our data noted that there is an increase in the C allele frequency on the contrary T allele in the osteoporosis patient compared to the control group (p-value 0.001), In other hands, no statistical significance was found between premenopausal and postmenopausal women with osteoporosis (p-value 0.695). The results have shown significant differences (p-value 0.001**) between the premenopausal with osteoporosis and control. Also significantly difference (p-value 0.001**) between the postmenopausal women with osteoporosis and controls. According to these results, TT genotype may be considered a potential risk factor for osteoporosis while CC genotype and C allele appear to be protective for osteoporosis disorders in the Iraqi women.

Relationship between MTHFR Polymorphism and Serum Calcium and Phosphorus Levels

The results are given in **Table 3** shows the relationship between MTHFR polymorphism and biochemical levels (calcium and phosphorus) in women with osteoporosis. Calcium levels of CC genotype (8.19 ± 0.828) were higher than CT (7.89 ± 0.930) and TT (7.75 ± 1.49) genotypes but non-significant, also both CT and TT genotype significantly decrease (p-value 0.64) compared to other genotypes. The phosphorus levels of CC genotype (2.01 ± 0.665) were higher than CT (1.98 ± 0.391) and TT (1.93 ± 0.622) genotype but also non-significant, while CC and CT genotype

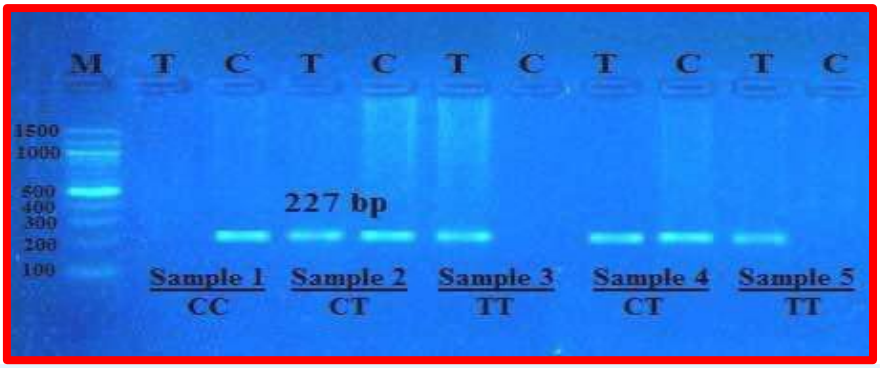

Fig. 1. Represents the ARMS-PCR products on 2 % agarose gel electrophoresis showing genotypes of the MTHFR polymorphism: lane (M) 100 bp DNA ladder, sample 1 (CC) homozygote (one band 227 bp), sample 2 and 4 (CT) heterozygote (2 bands 227 bp), sample 3 and 5 (TT) homozygote (one band 227 bp)

Table 2. Shows comparison between genotypes and alleles frequency of MTHFR C667T polymorphism of the study groups

Comparison between women with osteoporosis and control							
Genotypes	**Patients (100)**		**Control (70)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
CC	24	24 %	50	72 %	0.001**	1 Ref.	-
CT	30	30 %	10	14 %		6.25	2.63 - 14.85
TT	46	46 %	10	14 %		9.583	4.14 - 22.19
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
C	78	39 %	110	78.6 %	0.001**	1 Ref.	-
T	122	61 %	30	21.4 %		5.735	3.50 - 9.40
Comparison between premenopausal and postmenopausal women with osteoporosis							
Genotypes	**Premenopausal women (53)**		**Postmenopausal women (47)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
CC	13	24.5 %	11	23.4 5	0.695	1 Ref.	-
CT	14	26.4 %	16	34.0 %		0.74	0.25 - 2.17
TT	26	49.1 %	20	42.6 %		1.10	0.41 - 2.97
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
C	40	37.7 %	38	40.4 %	0.697	1 Ref.	-
T	66	62.3 %	56	59.6 %		1.12	0.63 -1.97
Comparison between premenopausal women with osteoporosis and control							
Genotypes	**Premenopausal women (53)**		**Control (70)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
CC	13	24.5 %	50	71.4 %	0.001**	1 Ref.	-
CT	14	26.4 %	10	14.3 %		5.38	1.95 - 14.86
TT	26	49.1 %	10	14.3 %		10.0	3.86 - 25.88
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
C	40	37.7 %	110	78.6 %	0.001**	1 Ref.	
T	66	62.3 %	30	21.4 %		6.05	3.44 -10.62
Comparison postmenopausal women with osteoporosis and control							
Genotypes	**Postmenopausal women (47)**		**Control (70)**		***p* value**	**OR**	**95% IC**
	No.	**%**	**No.**	**%**			
CC	11	23.4 %	50	71.42	0.001**	1 Ref.	
CT	16	34.0 %	10	14.29		7.27	2.61- 20.2
TT	20	42.6 %	10	14.29		9.09	3.34 - 24.47
Allele Frequency	**No.**	**%**	**No.**	**%**	***p* value**	**OR**	**95% IC**
C	38	40.4 %	110	78.6 %	0.001**	1 Ref.	-
T	56	59.6 %	30	21.4 %		5.403	3.03 - 9.62

*p<0.05 significant and **p<0.01 high significant.

Table 3. Shows comparison between biochemical parameters of the patients group according to MTHFR C667T genotypes

Parameter	**Mean ± SD**		***p* value**
	CC No. 24	**CT No.30**	
Calcium	8.19 **±** 0.828	7.89 **±** 0.930	0.22
Phosphorus	2.01 **±** 0.665	1.98 **±** 0.391	0.82
Parameter	**Mean ± SD**		***p* value**
	CC No. 24	**TT No.46**	
Calcium	8.19 **±** 0.828	7.75 **±** 1.49	0.18
Phosphorus	2.01 **±** 0.665	1.93 **±** 0.622	0.60
Parameter	**Mean ± SD**		***p* value**
	CT No. 30	**TT No.26**	
Calcium	7.89 **±** 0.930	7.75 **±** 1.49	0.64
Phosphorus	1.98 **±** 0.391	1.93 **±** 0.622	0.69

significantly decrease (p-value 0.82) compared to other patient genotypes.

DISCUSSION

Multifactor include food availability, lack of exercise, low sun exposure, gender, age, genetic polymorphism and the ethnic group associated with osteoporosis. Therefore, many investigated tried to understand the etiology of osteoporosis or at least recognize its risk factor (Kanis et al. 1997, Selvapandian et al. 2016).

In the current study levels of serum calcium is maintained within normal limits. But our data also show a significant difference when a comparison between osteoporosis group (8.02 ± 1.18) and control groups (10.5 ± 1.38), this means decrease calcium serum level in patient, so the few hardness of bones. Where, calcium is a key component in myeloid mass (Arnaud et al.1990, Almayahi et al. 2016), this result was an agreement with a recent study (Shakoor et al. 2014, Li et al. 2019) and disagreed with other studies (Rana 2013, Ali 2018). Although, serum phosphorus showed a significant difference between the osteoporosis group (2.03 ± 0.598) and control group (3.11 ± 0.547), serum phosphorus concentration still with normal range. This result agreed with previously published work revealed that there was of no noticeable consideration for the analysis of osteoporosis (Omrani 2006, Mutlu 2007). The results of this study did not show any significant difference in the levels of both serum calcium and phosphorus between premenopausal and postmenopausal women osteoporosis group. Also, the results of the calcium and phosphorus serum in premenopausal women with osteoporosis support evidence from previous observations (Pandey et al. 2013). Also, calcium hemostatic controlled by multifactor factor including, dietary intake, vitamin D and parathyroid hormone (Martins et al. 2017, Wasilewski et al. 2019).

Data from previous studies identified that genetic factors (almost SNPs polymorphism) contribute a critical role in the development of osteoporosis. The MTFHR is a gene encoding for an important enzyme in the methionine cycle (Reilly et al. 2014). According to the

results in **Table 2**, we observed that the CC genotype was significantly higher (72 %) in the control group than in the patient's group (22 %). A possible explanation the possible protective role of this genotype. On the contrary, we note a high frequency of TT genotype (46 %) in the patient's group compared with the healthy group (14 %). This study confirms that genotype is associated with the genetic capability of the osteoporosis, especially that the frequency of allele T was (61 %) and the odds ratio value (5.735) of the patients compared to frequency (21.4 %) in the healthy group. The substitution of 677 C/T results in an amino acid change from alanine to valine in the 225 codons. This substitution diminishes the enzyme's activity in 35% of patients having the CT variant and in 50% to 70% of patients having the TT variant (Zetterberg et al. 2002). There are controversial results of C677T for the association with osteoporosis in different populations (Saad et al. 2015). Prior studies that have noted the important role of race and ethnic on the epidemiology of osteoporosis (Al Anouti et al. 2019, Cauley et al. 2011). Therefore, our results agree well with osteoporosis study in the Danish population (Abrahamsen et al. 2003), Mexican patients (Brambila 2014) and women china population (Li and Wu 2010). In contrast, our study did not agree with another study on premenopausal north Indian women (Pandey et al. 2013), postmenopausal Indonesian women (Soewarlan et al. 2019) and china women population (Guan et al. 2014). In general, several researchers have shown that white and Asian women are high risk to develop osteoporosis than women of other ethnic backgrounds (Du et al. 2017, Redmond et al. 2016).

In the other hand, our results show no significant difference in the allele frequency between premenopausal women (allele C 37.7% and allele T 62.3%) and postmenopausal women (allele C 40.4% and allele T 59.6%) with osteoporosis. The relationships between premenopausal women with osteoporosis and MTHFR C667T polymorphism are an agreement with previous studies. (Pandey et al. 2013). In the case of premenopausal osteoporosis, secondary causes are responsible for at least 50% of cases (Martínez et al. 2012), therefore, our results proved the role of C677T in osteoporosis development.

Results in **Table 3** shown biochemical parameters of the patient's group according to MTHFR C667T genotypes, the women with CC genotype had high Mean ± SD of calcium level (8.19 ± 0.828) compare to CT (7.89 ± 0.930) and TT (7.75 ± 1.49) genotypes. Also, the women with CC genotype had high Mean ± SD of phosphorus level (2.01 ± 0.665) compare to CT (1.98 ± 0.391) and TT (1.93 ± 0.622) genotypes, no statistical significance was found between calcium and phosphorus levels with osteoporosis. Therefore, in the osteoporosis patients, these results refer to that the MTHFR C667T polymorphism may play a weak role in the levels of calcium and phosphorus. Absent clear effect of MTHFR C667T polymorphism on biochemical levels calcium and phosphorus in osteoporosis women, this because serum calcium and phosphorus homeostasis affected by a variety of factors, such as a complex interactions among organ systems, primarily the skeleton, intestine, and kidneys, hormones like parathyroid hormone, fibroblast growth factor 23, and calcitonin and vitamin D also help maintain calcium and phosphorus mineral homeostasis (Alien et al. 2014, Berndt et al. 2009).

In conclusion, our study indicates that calcium and phosphorus levels and frequency of normal CC genotype were decreased while the frequency of mutant TT genotype was higher in women with osteoporosis compared to healthy women, this indicates that TT genotype and T allele can be considered as an indicator of osteoporosis in Iraqi women. Further research should be undertaken to confirm these results by suggesting more studies should be using a larger number of samples in different cities of the Iraq.

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