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Association between *MethylenetetrahydrofolateReductase* (*MTHFR*) GenePolymorphisms and breast cancer in sample of Iraqi women

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Abstract

Folate metabolism is fundamental and essential for DNA structure synthesis and repair. Change in genes that participate in folate metabolism can be linked with different types of malignant tumor, Therefore, this study was conducted to find out the association between *methylenetetrahydrofolatereductaseMTHFR* gene polymorphisms and risk of breast cancer in a sample of Iraqi patients. One Single Nucleotide Polymorphism (SNP) including *MTHFR* C677T was calculated using a tetra primer ARMS PCR_experiment assay. The results explained that *(MTHFR C677T)* consists of three genotype (CC, CT, TT), The CC genotype was the most frequent in patients and control group (40.00%) and(60.00%) ,respectively, while the lowest frequency was for TT genotype(26.25%) in patients group .This study also revealed that the higher frequency 56.88 % for allele C and 43.12 % for T allele in patients group . On the other hand the Odd Ratio (O.R.) for CC, CT , TT genotypes were 1.073, 0.862 and 1.148 respectively, and the genotypes TT show a highest O.R.

Keywords: *Methylenetetrahydrofolatereductase*gene**,** Single Nucleotide Polymorphisms, Breast cancer.

العلاقه بين تعدد الاشكال للجين مثيل تتراهايدرو فوليت المختزل(C677T) وسرطان الثدي في عينة من النساء العراقيات

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الخلاصه

ايض الفوليك ضروري لبناء الحمض النووي واصلاحه . أن التغايرات في الجينات التي تشارك في التمثيل الغذائي لحامض الفوليك يمكن أن تترافق مع عدة أنواع من الأورام الخبيثة، لذلك أجريت هذه الدراسة لمعرفة العلاقه بين تعدد أشكال النوكليوتيدات المفردة لجين مثيل تتراهايدرو فوليت المختزل(*MTHFR*) *MethylenetetrahydrofolateReductase* وخطر الإصابة بسرطان الثدي في عينة من المرضى العراقيين .أختير نوع واحد من تعدد أشكال النوكليوتيدات المفردة Single Nucleotide Polymorphisms (SNPs) وهو (C677T) للجين (*MTHFR*) ، واستعملت طريقة tetra primer ARMS PCR assay لتحديد هذا النوع من تعدد أشكال النوكليوتيدات المفردة للجين.

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اشارت النتائج الى ان (*MTHFR*) يتكون من ثلاثة انماط جينيه (CC,CT, TT)، وان النمط الجينيCCكان اعلى تكرار لدى المرضى ومجموعة السيطرة بنسبة (40.00%) و (60.00%) على التوالي . بينما كان النمط الجيني TT اقل تكرارا في مجموعة المرضى حيث بلغت نسبته(26.25%)، ايضا اشارت هذه الدراسة الى أن اعلى تكرار كان للأليلC وبلغت نسبته % 56.88مقارنة % 43.12 للأليل T في مجموعة المرضى وقد بلغت نسبة الأرجحية للأنماط الجينية(CC,CT ,TT) 1.148 ، 0.862 و 1.073 على التوالي ، وأظهر النمط الجيني TTأعلى نسبة أرجحية .

Introduction

Breast carcinoma is a disease where cells is grow and divided in the tissue out of control [1] There are many danger factors for breast cancer, including genetic factors which account for 25-30% of the tumors in breast tissue [2]. Among them, DNA methylation of different oncogenes or neoplasm suppressor genes may induce selective growth conversion of cells or its repression.

The MTHFR is a key enzyme in the pathway of folate metabolism and detects the intracellular folate pool for synthesis and methylation of DNA [3, 4].The MTHFR have a function in transformation of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteineremethylation to methionine. Natural variance in this gene is prevalent in healthy people [5, 6].Two common allele variants of the *MTHFR* gene have been described, *C677T* and *A1298C* [7].

It was showed that two copies of 677C (677CC) have the generality common genotype, beside it was noticed that 677TT (homozygous) have function in decrease the MTHFR performance compared with CC or CT (heterozygous) [8].

There are many evidences that *MethylenetetrahydrofolateReductase (MTHFR)* gene variants increase thymidylate synthase activity in cancer cells, because of increased supply of 5,10-methyleneTHF, the methyl donor for methylation of dUMP to dTMP [9] thereby making *MTHFR* polymorphisms a potential candidate cancer - predisposing factor due to genomic DNA hypomethylation, hyperhomocysteinemia and atherosclerosis [10]. A clear relation between *MTHFR* gene polymorphisms and the risk to expand the tumor in breast tissue has not been established [11, 12]. Many studies have showed that the *MTHFR 677TT* genotype confers higher risk of breast cancer, especially in women in the premenopausal stage [13, 14].

Accordingly, the present investigation aimed to determine the association between *MethylenetetrahydrofolateReductase (MTHFR)* gene polymorphism and the risk of breast cancer in a sample of Iraqi women**.**

Materials and Methods

A study subjects of 80 patient with breast cancer were studied, this sample included women aged from 30 to 80 years with radiotherapy, chemotherapy and hormone therapy . Samples of twenty apparently healthy women were used as control group with age ranged from 25 to 70 years. In all cases and control full history and complete physical examinations were done by using a short structured questionnaire. All samples of patients were collected from the Al– Amal National Hospital for cancer Management – Baghdad. All stages of the research were carried out at Middle East Clinical Laboratories for DNA technical and Al – Musaib Bridge for scientific and lab.

Collection of **blood sample**

Blood samples were collected from patients and healthy control groups .Two and half milliliter of blood collected in EDTA anti-coagulant tubes that used for DNA extraction,

DNA extraction

Genomic DNA was extracted by using kit (ReliaPrep™ Blood gDNAMiniprep System A5081), promega, USA.The concentration and purity of DNA were measured by using the Nanodrop spectrophotometer(Nanodrop2000 – Bioneer, Korea) .

MTHFR genotyping

Genotyping of *C677T* SNP was performed by using a tetra primer ARMS PCR assay, SYBR Green I-based real-time PCR.A tetra primers, as shown in Table-1 were prepared according to the instruction of Alpha DNA company, USA . Real-time PCR was performed in a total volume of 20 µl containing11µl of GreenStarTMqPCR Master mix, 1 µl of each primer per reaction dilution (10pmol/ µl),5µl of the genomic DNA dilution (10 ng/µl), and 5µl sterile distilled water.

The PCR protocol on the thermocycler type(Bioneer ,Korea) was as follows: 7 minutes of initial denaturation at 95^{o}C, followed by denaturationof 35 cycles at 95 ^{o}C for 45 seconds, annealing

temperatures at 60°C for 60 seconds with40 cycles, extension at 72°C for 60 seconds with 30 cycles and a final extension at 72°C for 7 minutes ,this protocol was achieved with some modification and depending on[15].

Melting curves were constructed by lowering the temperature to 65 "C and later increasing the temperature by 0.2 °C /s to 98 °C, and the Tm values calculated from the negative derivation of fluorescence versus temperature (-dF/dT) of the melting curve for amplification products measured at 530 nm.

Table 1-Primers used for amplification of *MTHFR* gene .

Gene	primers sequence 5⁻ ------>3⁻	Annealing Temperature	Reference
MTHFR C677T	T: GCACTTGAAGGAGAAGGTGTCTGCGGGCGT F: TGTCATCCCTATTGGCAGGTTACCCCAAA R:CCATGTCGGTGCATGCCTTCACAAAG C poly GGGCGGGCGGCCGGGAAAAGCTGCGTGATG ATGAAATAGG	60°C	Ibrahim, *et al.*[15]

Statistical Analysis

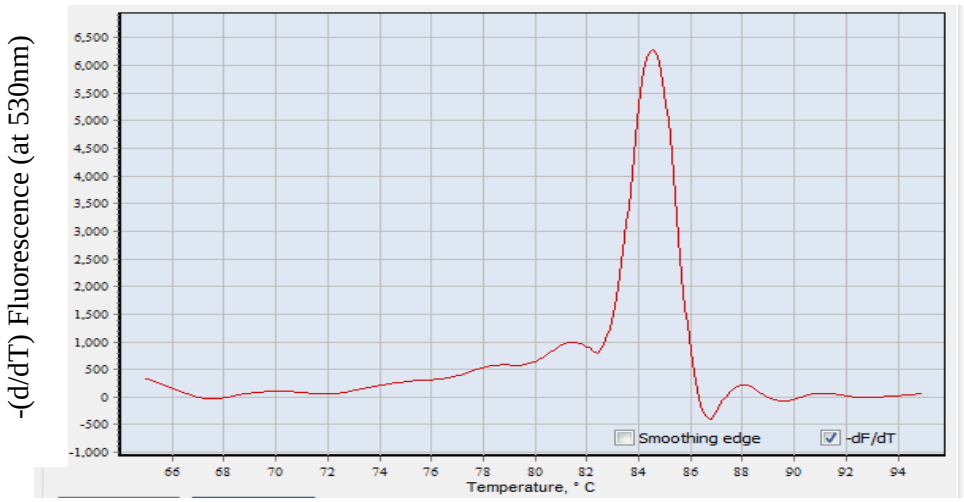
Allele frequency was calculated by using Statistical Analysis System- SAS (2012) program, version 9 for windows [16], and a Chi-square was used to test the significance difference between the patients and control group.

Result and Discussion

Single nucleotide polymorphism (SNP) genotyping is widely used in genetic association studies to characterize genetic factors underlying inherited traits .In this study, it was used a single-tube strategy that combines the tetra-primer ARMS PCR assay, SYBR Green -based real-time PCR, and melting point analysis with primer design strategies to detect the SNP of interest.

Tetra-primer PCR assay generated amplicon with Tm values of 84.50°C for the 677C allele, 81 °C for the 677T allele Figure-1.

A



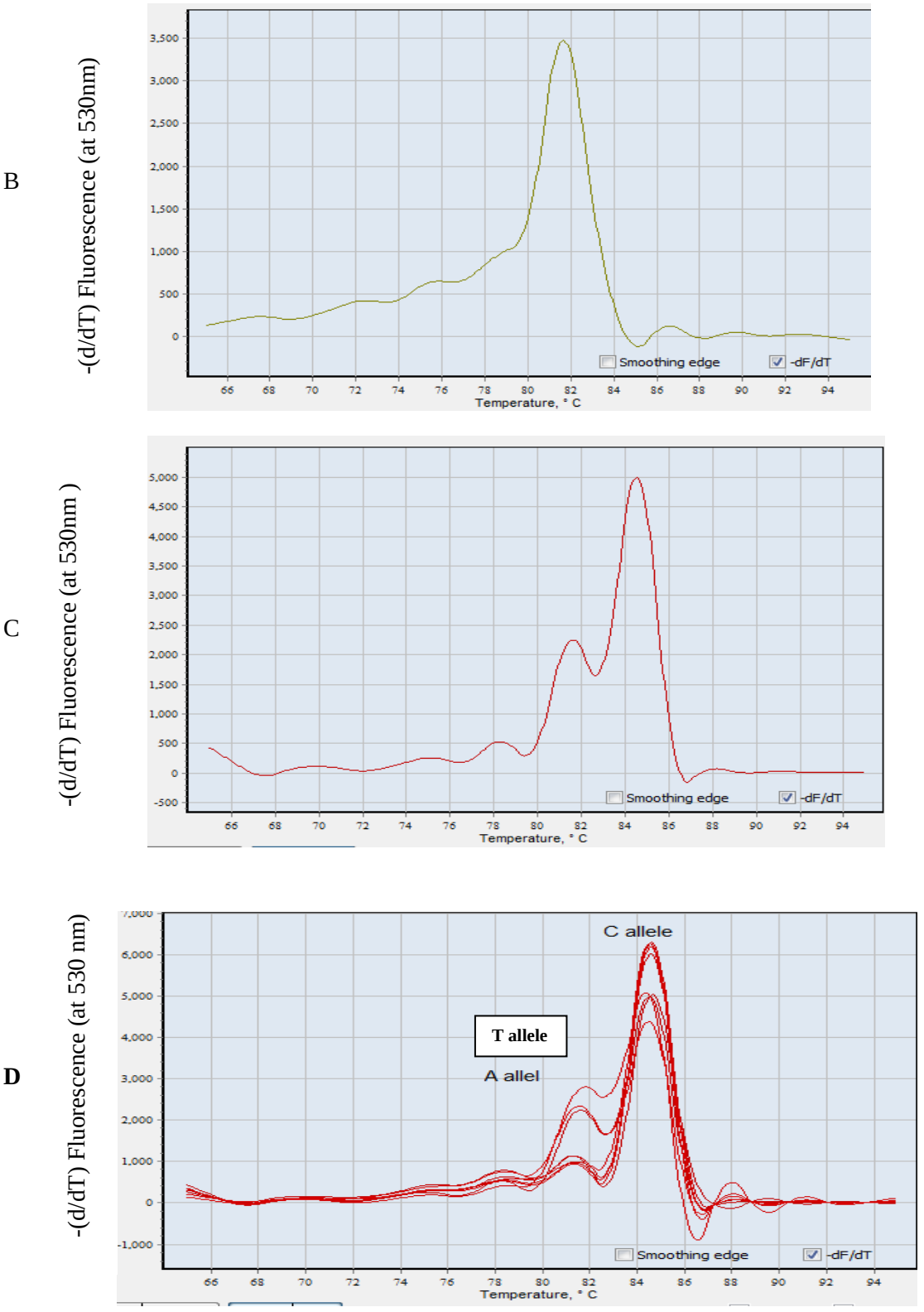


Figure 1- Real-time PCR assay for *MTHFR 677* genotyping:(**A)** CC genotype , (**B)** TT genotype, (**C)** CT genotype, (**D)** 8 samples screening for the C677 indicated to T and C allele position depending on Tm temperature(-df/dT fluorescence at wavelength 530nm) .

Because the T_m values are close to one another, a short GC tail was added to the inner *677C* allele-specific primer. With the addition of a short GC tail. Then, because the melting peak of the non-allelic

gene- specific product interfered with the *677T* allele peak, the primer concentration of the outer primers were reduced to 1 pmol per reaction to decrease the non-allelic gene- specific product melting-point peak.

Genotype and allele frequencies of *MTHFR C677T* polymorphism were compared between patients group and control group . The frequencies of the CC, CT, and TT genotypes were 40.00% , 33.75% and 26.25% respectively in patient group and 60.00% , 40.00% and 0.00% respectively in control group, Figure-2 . The allele frequency, which for C and T alleles were56.88 % and 43.12 % respectively in patients group, 60.00% and 40.00% respectively in control group.

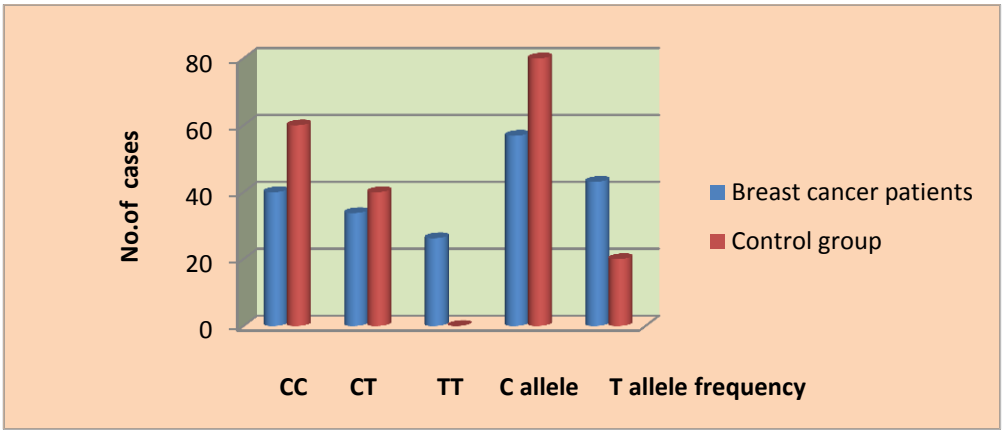


Figure 2- Genotype distribution and allele frequencies of *MTHFR C677T* polymorphism for patients and control group.

Polymorphisms in genes encoding enzymes of folate metabolism are a focus in breast cancer risk studies due of the role of these enzymes in DNA methylation, synthesis, and reform. *MTHFR*, encoding for 5, 10-methylenetetrahydrofolate reductase, is one of the most studied genes in this regard. There is indicator that the *677T* allele increase thymidylate synthesis performance in carcenoma cells because of an increased supply of 5,10-methylenetetrahydrofolate, the methyl doner for methylation of dUMP (Deoxyuridylate) to turn it into dTMP (Deoxythymidylate) [17]. This increase of dTMP pathway may come at the cost of a decrease methylation for DNA because the *677T* allele (C/T or T/T) in cancer patients have constitutively minimal levels of 5-methylcytosine in their neoplasm and encirclement normal tissue [18].

It was found [19] that the *MTHFR* SNPs *C677T* was linked with several types of cancers, especially breast cancer, more over it was noticed [20] that MTHFR677C > T was linked with an increased risk while 1298A > C polymorphism was associated with a decreased risk for breast cancer. It was reported [21] that the *MTHFR 677T* allele increased the risk for breast cancer in premenopausal than postmenopausal women from an American inhabitance .It was demonstrated [22] that the *MTHFR C677T* genotype may augment the risk of early-onset cancer in breast tissue before the age of 40years in an English population.

The results in Table-2 show the SNP of *MTHFR C677* have three genotypes (CC,CT, TT) with significant differences in the genotype and allele frequencies for both patients and control group and the value of Odd Ratios for CC, CT , TT genotypes were (1.073, 0.862 and 1.148), respectively .

Table 2 -Genotype and allele frequencies of *MTHFR C677T* polymorphism for patients and control group.

Genotype	Patients (No.= 80)	control (No.=20)	O.R.	Chi-square (χ^2)
CC	32 (40.00%)	12 (60.00%)	1.073	8.250 **
CT	27 (33.75%)	8 (40.00%)	0.862	6.593 **
TT	21 (26.25%)	0 (0.00%)	1.148	8.75 **
Total	80	20	---	---
Chi-square (χ^2)	5.028 *	12.631 **	---	---
Allele freq.				
C	56.88 %	60.00%	---	---
T	43.12 %	40.00%	---	---
*($p < 0.05$) , **($p < 0.05$)				

In this study , it was found a relationship between a commonly occurring polymorphism of *MTHFR C677* and breast cancer risk in sample of Iraqi woman ,this was agree with many studies[15,23,24,25,26,27,28],but in other studies noticed there were no association between *MTHFR C677T* and breast cancer risk [29,30].There are many factors that could explain the discrepant results from different studies, including different population characteristics(sample size and ethic differences), different family history that may modify breast cancer risk such as *BRCA1 / 2* mutation , [31] steroid hormone administration, reproductive history, especially, menopausal status and folate intake .

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