CYCLOOXYGENASE-2 169C>G GENE POLYMORPHISM AND PROSTAGLANDIN E2 LEVEL IN BREAST CANCER

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ABSTRACT

Aims: Cyclooxygenase 2 (COX-2) with the resulting prostaglandin E2 (PGE2) are linked to increased risk of human breast cancer (BC). The aim of this study was to determine COX-2 169C>G gene polymorphism and PGE2 level at various stages of BC clarifying the role of COX-2 gene polymorphism and PGE2 in relation to BC.

Methods: The study population comprised 80 women at different stages of BC and 30 gender- and age-matched healthy control subjects. Plasma PGE2 was measured by enzyme-linked immunosorbent assay, the COX-2 gene polymorphism at position 169C>G was determined using PCR–RFLP method.

Results: The mutated allele COX-2 169G frequency was 46.9% in BC patients and 28.3 % in controls; it was significantly associated with an increased risk of BC (OR: 2.65, 95 % CI: 1.31-5.42; P = 0.003). The homozygous mutant genotype (GG) significantly increased the risk of BC (OR: 7.13, 95% CI: 1.48-46.8; P = 0.004). However, COX-2 gene (GG genotype) was not associated with breast cancer stage. Plasma PGE2 levels were significantly increased in patients compared to the controls. In primary and metastatic BC, there was a significant increase in the plasma PGE2 levels towards the presence of homozygous GG compared with homozygous CC (P< 0.001).

Conclusion: The 169C>G polymorphism of the COX-2 gene was associated with the risk of BC in Egyptian women. Furthermore, individuals with COX-2 GG genotype showed significant increase in plasma PGE2 levels. PGE2 levels may serve as a predictor of poor prognosis in patients with BC.

Key words: Plasma- PGE2, Cyclooxygenase-2 gene, Polymorphism, Breast cancer**.**

INTRODUCTION

Breast cancer (BC) is the most common form of cancer in females. It is the second cause of cancer related mortality after lung cancer [1]. The high prevalence of BC and the limited options for treatment provide a strong rationale for identifying new molecular targets that can be nutritionally or pharmacologically modulated and, thereby, offer a potential for chemoprevention. Among the regulatory molecules that have been characterized as holding great promise for BC treatment is cyclooxygenases-2 (COX-2) [2].

Cyclooxygenases catalyze the first step in the synthesis of prostaglandins (PG) from arachidonic acid. There are two isoforms of COX, designated COX-1 and COX-2. COX-2 gene however is compact and contains a TATA box and several inducible enhancer elements including CEBP/NF-IL6, CRE and NFkB [3]. COX-2 is not detected in most normal tissues but is rapidly induced by a variety of mitogenic and inflammatory stimuli resulting in elevated levels of PGs in neoplastic and inflamed tissues [4]. Multiple lines of evidence suggest that COX-2 plays a significant role in carcinogenesis. In transgenic mice, overexpression of COX-2 led to neoplastic changes in the breast, pancreas, and skin [5].

Tumor formation and growth are reduced in animals that are engineered to be COX-2-deficient or treated with a selective inhibitor of COX-2 [6]. Treatment with selective COX-2 inhibitors has proven efficacy in the prevention and treatment of malignancy in humans [7]. COX-2 derived PGs can stimulate cell proliferation, promote angiogenesis, and inhibit apoptosis and immune surveillance [8-10]. PGE2 secreted from the breast tumor cells stimulates aromatase gene expression in the stromal cells of the surrounding adipose tissue. Aromatase is responsible for the biosynthesis of estrogen in adipose tissue, which is secreted out and serves as a mitogen for the epithelial cells including tumor cells**.** COX-2-derived PGs may also promote metastasis by stimulating cell invasion [11].

The gene for COX-2, designated as PTGS2, carries several polymorphisms, but few studies analyzed the relation between COX-2 gene polymorphisms and BC [12, 13]. A novel SNP at nt 169C > G, in exon 2 in the COX-2 gene is characterized by a substitution of proline to alanine at codon 57 and may be noted to affect the enzyme activity [14]. Li et al. found strong association between169C > G and BC in Chinese patients. No data are available on the frequency of such variant forms in the Egyptian population, either in healthy subjects or in cancer patients [14].

We determined 169C > G gene polymorphism of COX-2 gene and PGE2 level at

various stages of BC to clarify the role of COX-2 gene polymorphism and PGE2 in relation to BC.

SUBJECTS AND METHODS

Participants

The study population comprised 110 Egyptian women who were classified into: Group I: included 30 apparently healthy females (mean age 53.3 ± 9.24 years) not suffering from any disease interfering with the study. Group II (primary breast cancer)*:* included 40 newly diagnosed females (mean age 54.60± 6.34 years) in the early stages of BC with no distant metastases (stage I&II). Group III (metastatic breast cancer)*:* included 40 newly diagnosed females (mean age 55.95 ± 6.22 years) in the late stages of BC with invasive behavior and distant metastases (stage III&IV).

All participants gave their written consent before blood sample extraction, and they all underwent a full clinical examination. The patients had a confirmed diagnosis of breast cancer based on histopathological evaluation. Personal and gynecological history was taken. They all have no history of intake of any medications as NSAID interfering with the study during the 3 weeks before clinical evaluation and without any other diagnosed cancer. Histopathological examination and subsequent immunohistochemical (IHC) studies were performed for the detection of estrogen receptors (ER) and progesterone receptors (PR). The clinicopathological features of BC patients were presented in **table l**

Table 1: The clinicopathological features of breast cancer patients

Patient characteristics	Number
Estrogen receptor	
Negative	45
Positive	35
Progesterone receptor	43
Negative	37
Positive	
Tumor stage	
I	20
II	20
III	20
IV	20
Age (years)	
≤ 50	25
> 50	55
Tumor size	44
≤ 2	
> 2	36
Histological grade	
I (Well differentiated)	
II(Moderately differentiated)	29
III (Poorly differentiated**)**	27
	24
Lymph node metastasis	
Negative	36
Positive	44

Blood Sample Collection

Six ml of peripheral venous blood samples were collected on potassium EDTA (1mg/ml) from each subject under complete aseptic condition and were divided into 2 portions: one ml for DNA extraction. Indomethacin (The prostaglandin synthetase inhibitor) with a final concentration of 10 µM was added to five ml of the sample, then they were centrifuged at 3000 rpm for 10 minutes; plasma samples were separated into another set of tubes and kept frozen at -80 ˚C till the time for PGE2 assay. Plasma PGE2 was measured using competitive enzyme linked immunosorbent assay (ELISA) (Cayman Chemical Company, USA).

The ER and PR status was examined by IHC using antibodies provided by Lab Vision (SP1 resp. SP2 monoclonal rabbit antibody, Lab Vision Thermo Fisher Scientific, USA). ER and PR status was considered positive if more than 10% of cells were stained in cell nuclei [15].

Genotyping

Genomic DNA was isolated using the Wizard Genomic DNA Purification Kit purchased from Promega (Madison, Wisconsin, USA).

Genotyping for COX-2 169C>G polymorphism was performed according to [14], and genes were typed using PCR-restriction fragment length polymorphism (PCR RFLP) **(table 2)**.

Table 2: Standard PCR conditions used in genotyping 169C>G of the COX-2 gene

Polymorphic site	Primer sequence	PCR condition	Restriction enzyme and fragment
COX-2 169C>G	Forward, AAACCGAGGTGTATGTATGAGTG Reverse, CAAGAAGACGAAGAAAGGGAGGG	30 s at 94°C, 30 s at 57°C, 60s at 72°C.	MaeII, G, 118+ 88 C, 206

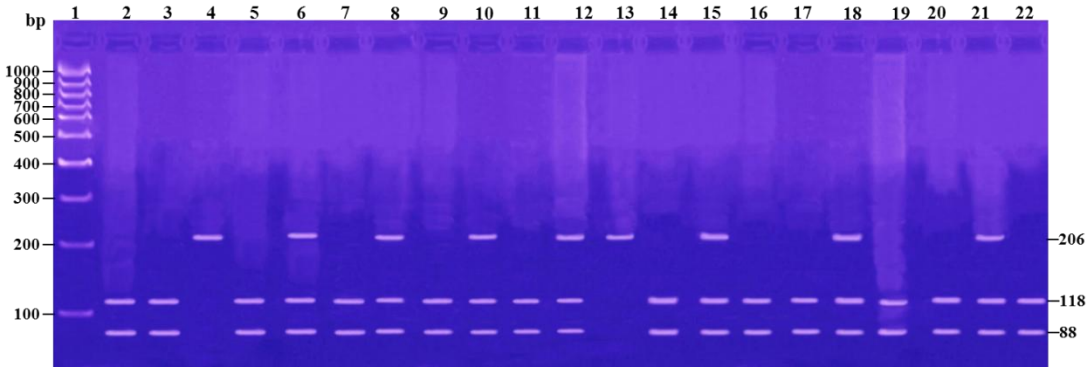

Figure (1): The COX-2 C/G genotyping. The figure shows a 3% agarose gel, stained with ethidium bromide, of C/G PCR genotyping reactions. DNA ladder (lane 1). The CC genotype shows two bands of 118 and 88 bp (lanes 2,3, 5, 7, 9, 11, 14, 16, 17, 19, 20, 22). The GC genotype shows three bands of 206, 118 and 88 bp (lane 6, 8, 10, 12, 15, 18, 21). The GG genotype shows a single band of 206 bp (lanes 4,13).

Statistical Analysis

Numerical values were analyzed by ANOVA and Student's t-test, proportions of groups were compared by the chi square (x^2) test. In addition, the odds ratios (ORs) and 95% CIs were calculated as a measure of the association of the COX-2 alleles with groups. The threshold for significance was $P<0.05$. The appropriate sample size and power of the study were determined using PAWE-3D. PAWE-3D calculations showed that the sample size, together with the specified study

design, allele frequencies and allowable error rates; can give as high as 90% power and can detect variant allele frequency of at least 0.05 and genotype relative risk of ≥ 1.8 at 80% power.

RESULTS

Genetic analysis

The genotype and allele distribution for the COX-2 169C/G polymorphism by study group was shown in **table 3**. Prevalence of PTGS2 169-GG genotypes were significantly higher in BC patients as compared to controls and were associated with a higher risk of BC (OR=7.13, 95% CI 1.48-46.8, P= 0.004). Also, the PTGS2 169-G allele frequency was significantly higher in BC as compared to controls and was associated with a risk of developing BC (OR=2.65, 95% CI 1.31-5.42, P= 0.003).

Breast cancer patients were further stratified into two subgroups: primary and metastatic BC. Again there were significant differences between each BC group when compared with the control group regarding the G allele (OR=2.33, 95% CI 1.06-5.19, P= 0.02 for primary BC group and OR=3.0, 95% CI 1.36-6.67, P= 0.002 for metastatic BC group).

When we compared between primary BC and metastatic BC, there was no significant difference of genotype and allele frequencies between primary and metastatic BC groups (p=0.47, 0.42 respectively).

Table 3: Genotype and allele frequencies of COX-2 169C/G polymorphism in studied groups

	Controls (n=30)	**Group I (n=40)**		**Group II (n=40)**	
	N (%)	**N (%)**	**P***	**N (%)**	**P***
Genotype					
CC	17(56.6)			15(37.5)	0.11
CG	11(36.6)	17(42.5)	0.24	10 (10.0)	0.29
GG	2(6.6)			15(37.5)	0.002
Alleles		11 (27.5)	0.41		
C	45(71.7)			40(50)	
G	15(28.3)	12 (30.0)	0.01	40(50)	0.002
		45(56.2)			
		35(34.7)	0.02		

**when compared with control*

Among patients with breast cancer, the PTGS2 169COG genotypes showed no association with tumour size, histological grade, presence of primary lymph node metastases, or age at diagnosis. Estrogen receptor (ER) positivity was less frequent among patients carrying the GG genotype (22.2%) than among those with the CC and CG genotype (59.4% and 47.6% respectively; P = 0.005), also progesterone receptor (PR) positivity was less frequent among patients carrying the GG genotype (29.6%) than among those with a CC and CG genotype (62.5% and 42.9% respectively; P = 0.03) **(table 4)**.

Table 4: The cyclooxygenase-2 polymorphism and clinicopathological features in breast cancer patients

	CC N=32	**CG** N=21	**GG** N=27	***P*** GG **vs** (CC+CG)
Estrogen receptor				
Negative	13(40.6%)	11(52.4%)	21(67.9%)	0.005
Positive	19(59.4%)	10(47.6%)	6(22.2%)	
Progesterone receptor				
Negative	12(37.5%)	12(57.1%)	21(67.9%)	0.03
Positive	20(62.5%)	9(42.9%)	8(29.6%)	
Age at diagnosis (years) (mean± SD)	53 ±12	54 ±11	52 ± 11	0.28
Tumor size				
≤ 2	17(53.1%)	12(57.1%)	15(55.5%)	0.87
> 2	15(46.9%)	9(42.9%)	12(44.5%)	
Histological grade				
I(Well differentiated)	10(31.2%)	9(42.8%)	10(37%)	0.90
II(Moderately differentiated)	11(34.3%)	7(33.3%)	9(33.3%)	
III(Poorly differentiated)	11(34.3%)	5(23.8%)	8(29.6%)	
Lymph node metastasis				
Negative	13(40.6%)	9(42.8%)	14(51.8%)	0.52
Positive	19(59.4%)	12(57.2%)	13(48.2%)	

Biochemical analysis

The mean values of plasma PGE2 levels were 111.60± 46.19 pg/ml in controls, 549.60±181.62pg/ ml in BC patients as a whole, 397.90±109.06 pg/ml in primary BC group and 701.22±87.99 pg/ml in metastatic BC patients. Plasma PGE2 levels were significantly increased in BC patients when compared with control group (P < 0.001). Metastatic BC group showed significantly higher PGE2 levels as compared with primary BC group (P < 0.001). Higher plasma PGE2 levels were associated with negative ER than positive ER (608.82± 173.63 vs 473.37± 164.32 respectively, p= 0.001) and with negative PR than positive PR (629.0± 132.56 vs 457.24± 188.64, P< 0.001 respectively).

Regarding association between the COX-2 gene polymorphism and plasma PGE2 levels, individuals with COX-2 GG genotype showed significant increase in plasma PGE2 levels as compared with those having CG or CC genotypes in each studied group **(table 5)**.

Table 5: Effect of COX-2 gene genotype on plasma PGE2 (pg/ml) in each studied group

	CC	CG	GG	*P*
Group I				
Range	60.4-199.6	100.3-186.7	202.4-219.6	<0.001
Mean± SD	84.35±31.80	135.63±26.08	211.01±12.72*	
Group II				
Range	242.3-533.7	265.6-515.4	323.3-572.7	<0.001
Mean± SD	346.0±90.24	388.18±107.49	480.33±90.28*	
Group III				
Range	530.1-789.9	628.5-780.5	620.1-873.9	<0.001
Mean± SD	630.53±67.41	701.10±42.19	211.01±12.72*	

**p< 0.05 when GG compared with CC or CG*

DISCUSSION

COX-2 gene encodes the COX-2 enzyme, which has been in ruled in the pathogenesis of BC development [16]. We found significant increase in the frequency of the G allele of COX-2 in BC patients compared with controls. Consequently, the frequency of the homozygous PTGS2 169GG genotypes found to be significantly higher in patients compared with controls. The homozygous GG genotype was associated with an increased breast cancer risk by seven folds more than controls. There was no significant difference in the frequencies of COX-2 GG genotypes between primary and metastatic BC. These results are in agreement with the results of many studies who reported that polymorphisms in the PTGS2 gene, which encodes for the COX-2 enzyme, have been, in particular, related to cancers of the breast [12-14].

As many reports found that higher COX-2 activity was associated with an increased tumour risk and poor prognosis [7, 17], others reported overexpression of COX-2 in BC [18-20]. In the COX-2 169C>G substitution, there is substitution of proline to alanine in the hydrophobic core of the protein component of the enzyme, destabilizing the protein (unfolding). This exhibit changes in the thermodynamic properties that induce thermodynamic stability effect and increasing COX-2 activity [13]. This hypothesis was confirmed by our results that individuals with COX-2 GG genotype showed significant increase in plasma PGE2 levels as compared with those having CG or CC genotypes in all studied groups. On the other hand, Dossus et al. and Piranda et al. found no strong association between other PTGS2 SNPs and the risk of BC [22, 23]. A high prevalence in Brazil and that their occurrence may lead to haplotypes with different potentials for changes in COX-2 expression [23].

In the present study estrogen receptor (ER) and progesterone receptor (PR) positivity were less frequent among patients carrying the GG genotype than among those with the CC and the CG genotype. This may be in line with data from Wülfing et al. and Boland et al. who reported an inverse relationship between COX-2 activity and ER and PR content of BC tissue [24, 25]. By contrast, others found no significant correlations between COX-2 and ER or PR as COX-2 has no correlation with established prognostic markers [26, 27]. Tumour size, histological grade, presence of primary lymph node metastases, or age at diagnosis were not associated with COX-2 genotypes and this agrees with Li et al. [14].

Regarding plasma PGE2 level, significant increasement in plasma PGE2 levels were observed in breast cancer patients when compared with control group. This agreed with studies by Thill et al. [28] and Cordes et al. [29] and disagreed with Malachi et al. [30] who found no association between plasma PGE2 levels and breast cancer as plasma PGE2 did not reflect the tissue levels. On

comparing PGE2 levels between groups, primary and metastatic breast cancer showed significantly higher PGE2 levels as compared with control group. Metastatic breast cancer showed significantly higher PGE2 levels as compared with primary breast cancer. These results agreed with the results of Howe [19] who demonstrated that high PGE_2 concentrations have been associated with high metastatic potential in breast cancer patients. Cordes et al. [29] detected an elevated COX-2 expression in breast and ovarian cancer patients compared to healthy women and that PGE_2 serum levels were higher in both types of cancer. The reason for the observed elevation in PGE2 levels in BC is not clearly understood. Not only increased enzyme synthetic activity but also decreased catabolic activity or increased availability of precursor polyenoic acids meight be causative causes for higher PGE2 levels [29].

In the present study high plasma PGE2 was associated with negative estrogen and progesterone receptors. This corresponds to the results of Davies [31] and Howe [19] who demonstrated that high PGE2 concentrations had been associated with both high metastasis potential and a lack of estrogen and progesterone receptors. On the other hand other study reported that synthesis of PGE2 by estrogen-positive tumors was significantly higher than by estrogen-negative [32]. There are few limitations of our study. Identifying significant associations of genetic variants with diseases as BC may need a larger sample size. However, the present study fulfills most of the criteria of a good genetic association study [33].

In conclusion, the 169C>G polymorphism in exon 2 of the COX-2 gene was associated with the risk of BC in Egyptian females. The current results support that COX-2 169C>G polymorphism was associated with elevated plasma PGE2 levels in females carrying GG genotype. Moreover, PGE2 levels may be closely related to tumor progression and may serve as a predictor of poor prognosis in patients with BC.

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دراسة التباين الجينى لإنزيم السيكلواكسيجيناز ٢ و مستوى البروستاجلاندين هـ ٢ فى سرطان الثدى

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يعتبر سرطان الثدى أكثرأنواع الأورام الخبيثه إنتشارا فى السيدات ، هناك العديد من العوامل التى يعتقد أنها تساعد على حدوث هذا النوع من الأورام ، من هذة العوامل ما يتعلق بالعمر، الحالة الأنجابية، أستخدام الأدوية التى تحتوى على الهرمونات، التاريخ العائلى ونمط الحياة مثل نوعية الأكل و السمنة، التعرض للأشعاعات قد يلعبان دور هام فى حدوث سرطانات الثدى.

يعتبر انزيم سيكلواكسيجيناز هو المسئول عن تحويل حمض الاراشيدونيك الى البروستاجلاندين. يتكون هذا الانزيم من سيكلواكسيجيناز ١ و سيكلواكسيجيناز ٢. ولقد ظهرت العديد من الفرضيات التى تربط بين زيادة انزيم سيكلواكسيجيناز ٢ مع زيادة البروستاجلاندين هـ ٢ وحدوث سرطانات الثدى. كما وجد أن زيادة نشاط هذا الانزيم مع البروستاجلاندين هـ ٢ قد يلعب دورا هام فى تكاثر الخلايا السرطانية وإنتشارها فى جميع أنحاء الجسم.

ولقد أثبتت بعض الدراسات ان هناك العديد من العوامل التى تؤثر على بعض الجينات التى يعتقد أنها قد تلعب دورهام فى حدوث سرطانات الثدى ، ومن هذه الجينات وجود تباين فى شكل چين سيكلواكسيجيناز ٢.

الهدف من البحث :

- تحديد النوع الجينى لجين سيكلواكسيجيناز ٢ و قياس مستوى مادة البروستاجلاندين هـ ٢ بالدم فى السيدات الذين يعانون من سرطان الثدى.
- إيضاح دور التباين الجينى لجين سيكلواكسيجيناز ٢ و مستوى مادة البروستاجلاندين هـ ٢ بالدم فى التطور المرضى لحالات مرضى سرطان الثدى.

المرضى وطرق البحث:

تمت هذه الدراسة فى قسمى الكيمياء الحيوية والبيولوجيا الجزيئية والاورام بكلية الطب جامعة الزقازيق.

ولقد إشتملت هذه الدراسة على ١١٠ سيدة مقسمات الى ثلاثة مجموعات :-

المجموعة الأولى : تشمل ٣٠ سيدة أصحاء لا يعانون من أى أمراض كمجموعة ضابطة.

المجموعة الثانية : تشمل ٤٠ سيدة يعانون من سرطان الثدى بالمرحلة الأولى والثانية.

المجموعة الثالثة : تشمل ٤٠ سيدة يعانون من سرطان الثدى بالمرحلة الثالثة والرابعة.

وقد تم قياس مستوى البروستاجلاندين هـ ٢بالدم بأستخدام ELISA. كما تم فحص جين سيكلواكسيجيناز ٢ بأستخدام التفاعل التسلسلى عديد البلمرة لاختبار حدوث التحور الشكلى فى هذا الجين .

النتائج:

وجد أن معدل تكرار الشطر الجينى G لجين سيكلواكسيجيناز ٢ فى السيدات اللاتى تعانين من سرطان الثدى ٤٦,٩% وهى أعلى من معدل التكرار فى السيدات الأصحاء ٢٨,٣%. بالتالى أدت زيادة الشطر الجينى G لجين سيكلواكسيجيناز ٢ إلى زيادة فى معدل تكرار الشكل الجينى (GG) لجين سيكلواكسيجيناز ٢ فى السيدات اللاتى تعانين من سرطان الثدى سواء فى المراحل الأولى أو المتأخرة ، مما يؤدى إلى زيادة مخاطر الأصابة بسرطان الثدى لسبعة مرات فى السيدات اللاتى تحملن الشكل الجينى (GG).

كما وجد أن نسبة وجود مستقبلات الإستروجين و البروجيسترون كانت أقل فى السيدات اللاتى تعانين من سرطان الثدى و اللاتى تحملن الشكل الجينى (GG).

و اظهرت النتائج وجود زيادة فى مستوى البروستاجلاندين هـ ٢ فى بلازما الدم فى السيدات اللآتى تعانين من سرطان الثدى و كانت نسبة الزيادة أكبر فى المراحل المتأخرة من المراحل الأولى. كما وجد أن نسبة وجود مستقبلات الإستروجين و البروجيسترون كانت أقل مع وجود زيادة فى مستوى البروستاجلاندين هـ ٢.

ووجد أن مستوى البروستاجلاندين هـ ٢ أعلى فى السيدات اللآتى تحملن الشكل الجينى (GG) من السيدات اللآتى تحملن الشكل الجينى (CC، CG).

الخلاصة:

أكدت نتائج البحث العلاقة بين التعدد الشكلى لجين سيكلواكسيجيناز ٢ و كلا من ارتفاع نسبة البروستاجلاندين هـ ٢ فى الدم و زيادة خطر الإصابة بمرض سرطان الثدى. و بالتالى يكون تحديد هذا التعدد الشكلى لجين سيكلواكسيجيناز ٢ أداة لمعرفة السيدات اللاتى يزيد لديهن خطر الإصابة بسرطان الثدى.

كما أن البروستاجلاندين هـ ٢ يلعب دوراً هاماً فى حدوث المرض و بالتالى تكون الوسائل المستخدمة لخفض مستوى البروستاجلاندين هـ ٢ ذات فاعلية فى تقليل نسبة الإصابة بسرطان الثدى و حدوث المضاعفات.