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Functional Polymorphisms in the Cyclooxygenase 2 (COX-2) Gene And Risk of Breast Cancer in North Indian Population

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Abstract

Introduction: COX-2 is a rate limiting enzyme involved in carcinogenesis, immunosuppression, inhibition of apoptosis, angiogenesis, tumor cell invasion and metastasis. Enhanced expression of COX-2 has been observed in several forms of cancer such as gastric cancer, breast cancer and esophageal cancer. Single nucleotide polymorphisms (SNPs) in the COX-2 promoter might contribute to differential COX-2 expression and subsequent interindividual variation in susceptibility to cancer. Hence, we assessed the association of COX-2 promoter Single Nucleotide Polymorphisms (SNPs) (-1195G/A, -765G/C and 8473C/T) with breast cancer.

Materials and Method: Genotyping was performed in 82 biopsy proven patients and 49 (34 in case of -765) age and sex-matched healthy control subjects by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis.

Result: Logistic regression analyses revealed that no overall significant associations were detected in the single-locus analysis between the -765, -1195 and 8473 polymorphisms of COX-2 and the risk of breast cancer. However, a significantly increased risk was associated with the combined genotypes containing more than 3 variant alleles (OR= 2.05, 95% CI = 0.816-5.17) compared with the combined genotypes with 0-3 variant alleles. Haplotype frequency analysis suggest that $A_{-1195}G_{-765}T_{8473}$ was more prevalent in patients when compared with the normals whereas $G_{-1195}C_{-765}C_{8473}$, $A_{-1195}C_{-765}C_{8473}$ and $G_{-1195}G_{-765}C_{8473}$ were more in normals as compared to patients though the results were not statistically significant. It appears that $A_{-1195}G_{-765}T_{8473}$ may be related to susceptibility while $G_{-1195}C_{-765}C_{8473}$, $A_{-1195}C_{-765}C_{8473}$ and $G_{-1195}G_{-765}C_{8473}$ may be related to protection in breast cancer.

Conclusion: These findings indicate that these three variants in the regulatory regions of COX-2 may contribute to the etiology of breast cancer.

Keywords: COX-2; SNP; Breast cancer; North Indian

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Introduction

The cyclooxygenase (COX) enzymes, also referred to as prostaglandin end peroxide synthase, catalyze a key step in the conversion of arachidonate to PGH_2, the immediate substrate for a series of cell specific prostaglandin and thromboxane synthases. Prostaglandins play critical roles in numerous biologic processes including the regulation of immune function, kidney development, reproductive biology and gastrointestinal integrity. There are two COX isoforms: The constitutive form, COX-1, is present in many tissues and involved in PG synthesis; and the inducible form, COX-2, is absent from most normal tissues, and rapidly induced by growth factors, cytokines, and various carcinogens [1,2]. COX-2 over expression was shown to increase proliferation, inhibit apoptosis, and enhance the invasiveness of cancer cells resulting in angiogenesis [3-7]. The over expression of COX-2 is found in many tumor types [8-12], including breast cancer [13,14]. Reported that COX-2 over expression was also associated with indicators of breast cancer development, such as lymph-node metastasis, poor differentiation and large tumor size

Transcription regulation is the major mechanism to regulate the expression and stability of COX-2 [15]. The 5' flanking region of the human COX-2 gene, principally involved in regulating gene transcription, contains a canonical TATA box and several putative transcription-factor binding sites, including cAMP-responsive element, nuclear factor-κβ, nuclear factor-IL-6, glucocorticoid response

Table 1: Clinicopathological characteristics in Breast Cancer Cases.

Characteristics	Values
Age (years)	
Range	29-75
Mean	45±10.03
Menopausal status	
Pre	52.44%
Post	47.56%
Tumor status	
T_1	8.57%
T_2	41.43%
T_3	17.14%
T_4	32.86%
Node status	
N_0	27.14%
$N_{+(1-4)}$	72.86%
Clinical stage	
Early (I+II)	52.86%
Late (III+IV)	47.14%

element, polyomavirus enhancer activator 3, activator protein-2, CAAT box/enhancer binding protein, stimulatory protein-1 (Sp1), and a transforming growth factor-β response element suggesting that a complex array of factors is involved in its regulation [16-18]. Found that −1195G/A polymorphism created a c-MYB binding site and induced the higher transcriptional activity of the COX-2. Previous studies suggested that −765G/C polymorphism in 5′UTR, a potentially functional variant, may eliminate an Sp1-binding site but create an E2F binding site, which results in reduced or increased COX-2 expression [19,20]. Furthermore, some studies showed that the 3′UTR of the murine gene for COX-2 contains several regulatory elements altering mRNA stability and translation efficiency [21], which play an important role in degradation, stabilization, and translation of the transcripts. Therefore, polymorphisms in 3′UTR of COX-2 may modify the binding affinity of regulatory factors and alter expression of COX-2, and subsequently influence susceptibility to cancers, including breast cancer [22-24].

The present work is motivated by the possibility that genetic variation in the *COX-2* gene could alter enzyme expression levels or biochemical function and consequently have an impact on prostaglandin biosynthesis. Therefore, polymorphisms might modify the individual risk of inflammatory disease, tumor incidence, or tumor malignancy. A second possibility is that *COX-2* polymorphisms could change the response to NSAIDs resulting in decreased or increased sensitivity to selective or nonselective COX inhibitors. We hypothesized that potential genetic polymorphisms in *COX-2* that result in altered expression and/or activity of the protein may modulate the inflammatory response, modifying overall breast cancer risk or risk for subtypes of breast cancer.

Materials and Methods

Study subjects

This study included 82 breast cancer patients and 49 cancer-free controls. Patients were recruited from the Breast cancer clinic, All India Institute of Medical Sciences, New Delhi, India. All cancer subjects were histopathologically diagnosed with breast cancer. Exclusion Criteria for normal subjects included persons with malignancies, recent operations, trauma, infection and with genetic abnormality infections. Exclusion criteria for patients included persons with other associated malignancies, radiation therapy, any other chronic diseases, malnutrition, pregnancy and child birth. After informed consent was obtained, each subject was personally interviewed by using a structured questionnaire to obtain study related information. The clinicopathological characteristics of the patients are tabulated in Table 1. After the interview, a 5-ml venous blood sample was collected from each subject. The study was approved by the Ethical committee.

Genotyping

Genomic DNA was extracted from the peripheral blood leukocytes pellet by standard procedures using Sodium perchlorate method. The genotyping assays for three SNPs of COX-2 (−1195G/A, −765G/C, and 8473C/T) were described previously [25,26] Briefly, the PCR primer pairs were: −1195G/A F, 5′-ccctgagcactacccatgat- 3′, R, 5′-gcccttcataggagatactgg-3′; −765G/C F, 5′ tattatgaggagaatttacctttcgc-3′, R, 5′-gctaagttgctttcaacagaagaaat-3′; and 8473C/T F, 5′-gtttgaaattttaaagtacttttgat-3′, R, 5′-tttcaaattattgtttcattgc- 3′. The 20-μl polymerase chain reaction (PCR) mixture contained approximately 50 ng DNA (100ng DNA for -765), 12.5 pmol of each primer, 0.1 mM of each dNTP, 10 X MgCl2 free PCR buffer and 2 U Taq polymerase. The concentration of MgCl2 was 1.5 mM for 8473C/T and 1 mM for −1195G/A and 2 mM for -765G/C. The PCR profile consisted of an initial melting step of 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 s, 61˚C (for −1195 G/A) or 54˚C (for −765 G/C) or 48˚C (for 8473 C/T) for 40 s and 72˚C for 45 s, and a final extension step of 72˚C for 10 min. Restriction enzymes *PvuII, HhaI and BclI* (MBI fermentas) was used to distinguish the −1195G/A, −765G/C, and 8473C/T genotypes, respectively.

Finally, in total, 82 cancer cases and 49 controls (34 controls in case of -765) were successfully genotyped for all three polymorphisms of COX-2.

Stastical analysis

Statistical analysis was performed by using statistical program GraphPad Software. The differences in the frequencies of various alleles and genotypes between breast cancer patients and healthy controls were performed by chi-square test (χ^2 test). The P-values obtained were further corrected (Pc) by multiplying with the number of alleles tested. The Pc value <0.05 was considered as significant. The odds ratio and confidence interval was calculated by the following website:

Results

The genotype distributions and allele frequencies of COX-2 −1195G/A, −765 G/C, and 8473C/T in the cancer cases and controls are shown in Table 2. In this study, a significant difference (Pc = 0.018) was found in *COX-2* -1195 GA genotype, where GA heterozygous was more frequent in normals than in cancer patients suggesting a protective role of this genotype against breast cancer. *COX-2* -1195 AA and *COX-2* -1195 GG were more in patients as compared to normals though Pc value was not significant. At *COX-2* -765 site, the GG genotype was slightly more while GC was less in patients as compared to normals (76.8% *vs* 67.7% and 19.5% *vs* 29.4%; Pc>0.05 respectively). There was no difference in the *COX-2* 8473CT and TT genotype frequencies in breast cancer patients when compared to

Table 2: Frequency (%) of *COX-2* genotypes and alleles in breast cancer patients and controls.

COX-2 SNPs	Patient	OR (95% CI)
-1195 G/A		
GG	05 (6.1)	3.1(0.35-27.49)
GA	13 (15.8)	0.29(0.13-1.67)
AA	64 (78.1)	2.45(1.31-5.31)
Alleles		
G	142 (86.6)	1.38(0.64-2.98)
C	22 (13.4)	0.72(0.34-1.56)
-765 G/C		
GG	63 (76.8)	1.58(0.66-3.83)
GC	16 (19.5)	0.58(0.23-1.46)
CC	03 (3.7)	1.25(0.12-12.49)
Alleles		
G	23 (14.0)	0.60(0.31-1.15)
A	141 (86.0)	1.67(0.87-3.21)
8473 C/T		
TT	33(40.2)	0.98(0.48-2.01)
TC	48(58.5)	1.06(0.52-2.17)
CC	1(1.3)	0.59(0.36-9.69)
Alleles		
C	50(30.5)	1.04(0.60-1.80)
T	114(69.5)	0.96(0.56-1.66)

normals. Though, the frequency of these genotypes was higher than *COX*-2 8473CC genotype.

When analyzed for association of *COX-2* genotypes with risk of breast cancer incidence using unconditional logistic regression analysis, *COX-2* -1195GA and AA genotypes did not show association (OR = 0.14, 95% CI = 0.014 to 1.31 and OR = 0.44, 95% CI = 0.049 to 3.95 respectively) with risk of breast cancer when compared with the GG genotype taken as referent. *COX-2* -765CC and GC showed no significant association (OR = 1.09, 95% CI = 0.11 to 11.07 and OR = 0.58, 95% CI = 0.23 to1.47 respectively) with breast cancer when compared to the referent -765GG genotype. Results of the present study suggest *COX-2* 8473 CT and CC genotype not to be significantly associated (OR = 0.96, 95% CI= 0.47-1.99 and OR=1.65, 95% CI = 0.098-22.9 respectively) with risk of breast cancer when compared with the TT genotype taken as referent (Table 2).

The combined effect of these three variants on breast cancer was significantly increased in the presence of "more than 3 variant alleles" compared with the combined genotypes with "0-3 variant alleles" (Table 3).

Haplotype analysis was also performed and eight haplotypes were derived from the observed genotypes of these three COX-2 polymorphisms. Haplotype frequency analysis suggested that $A_{-1195}G_{-765}T_{8473}$ was more prevalent in patients when compared with the controls whereas $G_{-1195}C_{-765}C_{8473}$, $A_{-1195}C_{-765}C_{8473}$ and $G_{-1195}G_{-765}C_{8473}$ were more in controls as compared to patients though the results were not statistically significant. We may suggest that $A_{-1195}G_{-765}T_{8473}$ may be related to susceptibility while $G_{-1195}C_{-765}C_{8473}$, $A_{-1195}C_{-765}C_{8473}$ and $G_{-1195}G_{-765}C_{8473}$ may be related to protectiveness against breast cancer (Table 4).

Genotypes $A_{-1195}G_{-765}C_{8473}$, $A_{-1195}C_{-765}T_{8473}$ and $G_{-1195}C_{-765}T_{8473}$ were found to be approximately equal in both patients and controls. Thus, it may be suggested that these may not be contributing factors for breast cancer development in North Indian population (Table 4).

In addition, the associations of three polymorphisms of COX-2 with breast cancer risk stratified by age, menopausal status and stage of cancer were analyzed but no significant associations found (Table 5).

Discussion

Increased concentrations of PGE_2, a major product of COX-2, have been reported in human breast cancer and in experimental murine mammary tumour models [25,26]. Mammary tumorigenesis can be suppressed by both genetic and pharmacologic ablation of COX-2, thus clearly identifying a role for COX-2 in breast neoplasia.

The expression and stability of COX-2 is subjected to complex mechanisms regulated by various elements in both the 5′UTR and 3′UTR of the transcript. Therefore, polymorphisms in the promoter region and 3′UTR of the COX-2 gene may potentially influence gene expression and then modulate the individual's susceptibility to cancers. To investigate the impact of functional SNPs of COX-2 on tumor development, molecular epidemiological studies have been conducted for several cancer types, including esophageal, lung, colon and breast [28-31].

Because of the role that COX-2 plays in breast cancer development and progression and their aberrant expression in various types of cancer, we hypothesized that these polymorphisms in COX-2 may be associated with an increased risk of breast cancer attributable to the abnormal expression of this gene. In this study, we recruited 82 breast cancer patients and 49 age, sex and ethnicity matched healthy control subjects and genotyped COX-2 for three polymorphic sites to test the above hypothesis. In this study, a significant difference (Pc = 0.018) was found in COX-2 -1195 GA genotype, where GA heterozygous was more frequent in normals than in cancer patients suggesting a protective role of this genotype against breast cancer [32]. Conducted a case-control study of 1026 esophageal cancer cases and 1270 controls in a population of north China and found that COX-2 -1195AA and −765GC genotypes were associated with a significantly 1.72- fold (95% CI 1.35-2.20) and 2.24-fold (95% CI 1.59-3.16) increased risk of developing esophageal cancer compare with their wild-type genotypes. However, a nested case-control

study in a Caucasian population showed that the −1195G/A polymorphism (assigned as −798A/G) in the promoter of COX-2 was not significantly associated with risk of advanced colorectal adenomas [33]. For the -765G/C polymorphism [34], reported that -765C allele was associated with an increased risk of prostate cancer in African Americans (assigned as −899G/C) [35]. showed an elevated risk of colon cancer in a Singapore Chinese population.

There was no change in prostate cancer in Bini Nigerians [36] and in non-small-cell lung cancer in a Norwegian population (assigned as 926G/C) [37]. For the molecular epidemiological studies on the associations between COX-2 8473C/T polymorphism and cancer susceptibility, the results were also conflicting [38,39].

In the present study, no overall significant associations were found between the -1195G/A, -765G/C and 8473 polymorphisms and risk of breast cancer in the single-locus analyses in this population. Analysis of the predicted mRNA secondary structure indicated

that the 8473T>C exchange interrupts a 25 bp stem and creates an additional loop. This suggests a potential effect on the mRNA stability and expression, but the results of this in silico analysis remain to be proven by in vitro data. Thus, further in vitro analyses of the genetic regulation of COX-2 expression will be necessary before a conclusion on the functionality of the PTGS2 8473 polymorphism can be drawn.

The combined genotypes containing "more than 3 variant alleles" were associated with a significantly increased risk of breast cancer (OR= 2.05, 95% CI = 0.816-5.17), suggesting that polymorphisms in the regulatory regions of COX-2 may conjointly play a role in the development of breast cancer as reported in a study conducted.

The frequencies of genetic polymorphisms often vary between ethnic groups. In this study, the −1195G/A genotype frequencies were 6.1% for GG, 15.8% for GA, and 78.1% for AA which differed greatly from those reported in Chinese population (23.3% for GG, 50.9% for GA, and 25.8% for AA) and those reported in a Caucasian population (3.5% for GG, 30.8% for GA, and 65.7% for AA, respectively). Similarly, the frequencies of −765G/C genotypes in the present study were 76.8% for GG, 19.5% for GC, 3.7% for CC which differed greatly from those reported in Chinese population (90.5% for GG, 9.2% for GC, 0.3% for CC) and in a U.S. Caucasian population (69.4% for GG, 27.2% for GC, 3.4% for CC). For 8473 C/T polymorphism, the frequencies of 8473C/T in the present study were 40.2% for TT, 58.5% for CT, 1.3% for CC which differed from those in Chinese population (67.2% for TT, 29.8% for CT, 3% for CC). Ethnic variation in the COX-2 genotype distribution warrants additional comparative studies with more patients to confirm our results. Several limitations in our study need to be addressed. First, the sample size of the malignant breast cancer cases was not large enough to detect a small effect from low penetrating genes or SNPs. Second, inherent selection bias cannot be completely excluded, because patients were enrolled from the cancer hospitals and random controls were selected from a similar population. Third, it has been well documented that regular intake of NSAIDs may protect against breast cancer. Unfortunately, in the present study, no data are available on personal factors such as NSAID use and diet that potentially affect the COX-2 genotype.

In conclusion, our study demonstrated that COX-2 polymorphisms may conjointly contribute to risk of breast cancer development in a North Indian population. Validation of these findings with functional parameters and larger studies with more rigorous study designs of other ethnic populations are needed.

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