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RESEARCH

Cyclooxygenase-2 Polymorphisms and Breast Cancer Associated Risk in Pakistani Patients

Tariq Moatter · Muniba Aban · Waseem Iqbal · Shahid Pervez

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Abstract Prostaglandins produced by Cyclooxygenase-2 enzyme have been implicated to have a role in breast carcinogenesis. Several single nucleotide polymorphisms (SNPs) linked to COX-2 enzyme are reported to modulate its expression. The aim of the present study was to examine association of these SNPs to breast cancer risk in Pakistani patients. Methods In this case–control study, three sequence variants rs689465, rs689466, rs20417 in the promoter region of COX-2 were screened to evaluate the association with breast cancer risk. A total of 150 breast cancer patients and 101 healthy control genomic DNA were genotyped for rs689456, rs689466, rs20417 and their genotypes distribution in cases and control were compared using Pearson chi square test. Risk association was analyzed through odd ratio calculated by logistic regression. Results A screening analysis of COX-2 SNPs in 101 healthy controls showed distribution of Minor allelic frequency distribution of SNPs as follows : rs689465 (0.12), rs689466 (0.15), rs20417 (0.23). Further analyses revealed that their observed genotype frequencies were consistent with Hardy Weinberg equilibrium and strong linkage disequilibrium was identified between rs20417, rs689465 and rs689466. The Combined allele variants analysis showed that Haplotype rs68965G- 689466A-20417C (OR 2.909; CI 95 %1.3776.327; P=0.007) was significantly associated with breast cancer. Conclusions Our results indicate no strong association between three most frequent COX-2 SNPs rs689465 rs689466, rs20417 studied with breast cancer risk in the single locus analysis. However, our data suggested that combined COX-2 SNP haplotype have a role in breast cancer associated risk in Pakistani patients.

Keywords Cyclooxygenase · Cox-2 · Breast Cancer · Polymorphism · Haplotypes · RFLP

T. Moatter · S. Pervez (✉)
Department of Pathology and Microbiology, The Aga Khan University, Stadium Road, Karachi 74800, Pakistan
e-mail: shahid.pervez@aku.edu

M. Aban · W. Iqbal
Department of Pathology, Baqai Medical University, Karachi, Pakistan

Introduction

Cyclooxygenase isoforms COX-1 and COX-2 are key enzymes in the prostaglandin synthesis pathway. [1]. COX-1 is constitutively expressed in most cell types and is involved in several homeostatic functions in the body [2]. In contrast, COX-2 is undetectable in most normal tissues, but it can be induced by various cytokines, growth factors, hormones and tumor promoting factors [3, 4]. Currently, COX-2 is considered as the classical enzyme whose protein expression is a part of acute response to inflammation and synthesis of prostaglandins from arachidonic acid. Prostaglandins role in breast cancer was first time reported by Bennett et al. in 1977. Today, its diverse role is recognized in various cancer types particularly, colorectal, gastric, esophagus and breast [5–10].

The human COX-2 gene is located on chromosome 1q25.2–3, spanning 8.3 kb in size with tenexons [11]. Its 5' region has several response elements including nuclear factor kB (NF-kB), activation protein-2 (AP-2), stimulatory protein-1(SP1), transforming growth factor and cyclic AMP binding sites [12]. Sequence variation in these regulatory elements could alter gene transcription. For instance, a single nucleotide polymorphism (SNP) in the COX-2 promoter region may modify binding capacity for certain nuclear proteins and indicated to be associated with changes in COX-2 expression level [12, 13]. A previous report showed that COX-2 expression was significantly reduced in the presence of −765C allele compared with the −765G allele [14]. Similarly, Zhang et al.

reported that −1195A-containing COX-2 promoter demonstrated several fold greater expression of reporter gene than the 1195G-containing COX-2 promoter [15]. Breast cancer is a heterogeneous disease involving complex pathways, which are mediated by etiological factors, including environmental, genetics, socio-demographic, reproductive and cultural dynamics. These factors may differently impact on disease pathogenesis and progression. Pakistan has the highest rate of breast cancer among all Asian countries (excluding Ashkenazi Jews in Israel) with an approximately 69.1 per 100,000 age standardized annual rate [16]. Association studies regarding the contribution of SNPs in the COX-2 gene to breast cancer risk have not been previously performed in the Pakistani population. The aim of our study was to determine allelic frequencies of COX-2 SNPs rs20417, rs689465 and rs689466 and their association with breast cancer risk in the Pakistani breast cancer patients.

Materials and Methods

Subject Setting

In this hospital based case–control study 251 females participants were enrolled who were mostly residents of Karachi, a southern city of Pakistan. The age of the women ranged between 30 and 73 years. All participants were divided in two group, healthy control group (N=101) in which subjects have no history of cancer exposure, and patient group (N=150) who received a confirmed diagnosis of breast cancer based on their clinical and histopathological evaluation. This study was conducted at the Department of Pathology and Microbiology, Aga Khan University Hospital, Karachi, Pakistan.

Data Collection

Control and cases for this study were recruited between March 2008 and December 2011. Healthy controls were randomly selected from the general population, whereas paraffin embedded tissue blocks of breast cancer patients were obtained from the Histopathology Section of the Aga Khan university Hospital. Relevant information regarding demographic and clinical data of the breast cancer patients were collected from their medical records.

DNA Extraction and Genotyping

DNA was extracted from blood samples of control subjects collected in EDTA using Blood Mini kit (Qiagen, Hilden, Germany). Furthermore, DNA from paraffin embedded tissue sections of patients was isolated using FPPE DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genotyping of rs689466, rs689465, rs20417 polymorphisms was performed by PCR-based restriction fragment length polymorphism (PCR-RFLP) according to the published protocols [8, 9, 13]. The conditions of the PCR and primers are described in Table 1. Genomic DNA (50 ng/μl) was amplified in a reaction mix containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit *Taq* polymerase (Life Technologies, Grand Island, NY, USA) and 0.4 μM of each oligonucleotide primer. PCR products were then incubated at 37 °C for 16 h with 5 unit of each of the restriction enzyme *AciI* (rs20417), *PvuII* (rs689466), *RsaI* (rs689465) purchased from the New England Biolabs (Hitchin, UK). Next day restriction enzyme digested products were separated by electrophoresis on a 3 % agarose gel.

Statistical Analysis

Observed frequencies of different SNPs were evaluated for the assessment of deviation from the Hardy Weinberg equilibrium. Allele and genotype frequency distribution and differences between patients and control were calculated by Pearson Chi square test. Pairwise linkage disequilibrium analysis was performed by means of Fisher exact test (Haploview, www.broadinstitute.org). Online software SNPStats was used for constructing haplotypes (http://bioinfo.iconcologia.net). Logistic regression was applied for estimation of Odd ratios (ORs) with 95 % confidence interval. All statistical analyses were performed using SPSS version 19.0 for Windows (IBM Inc., Chicago, IL, USA) and p value of <0.05 was set for level of significance.

Results

A total of 251 individuals were enrolled for this study; out of those 101 participants were healthy females (controls), their mean age was 42.82±8.5 years. Similarly, for the 150 breast cancer cases the mean age at diagnosis was 42.71±9.4. Minor allelic frequency distribution of SNPs is as follows: rs689465 (0.12), rs689466 (0.15), rs20417 (0.23). Distribution and association of COX-2 SNPs rs689465, rs689466, and rs20417 with breast cancer risk are shown in Table 2. The frequencies of rs20417GG, GC, CC were, 63.4, 33.7, 2.9 % in control compared with, 48, 36.7 and 7.3 % in breast cancer patients respectively. We observed that the frequency of rs20417 GC and CC were slightly higher in patients in comparison to control group but it was not statically significant rs20417 GC (OR 1.4; P=0.19) and CC (OR 3.2; P=0.079).

Frequency of rs689465 GG, AG, and AA were 75.2, 21.8 and 2.9 % respectively in the control group; additionally it was 68, 24 and 4 % in patients with breast cancer. No significant association with breast cancer risk was observed P=0.581,

Table 1 PCR condition and primers for genotyping of cyclooxygenase-2

Primer	Nucleotide sequence	Identified SNP	restriction enzyme	PCR Condition	Product size	Reference
1,195		rs689465	PvuI	2 min at 95 °C, 35 cycles of 30 seach at 95 °C, 60^0C and 72 °C for 45 s, final extension at 72°C for 5 min	273	[9]
Forward 5'-CCCTGAGCACTACCCATGAT-3'						
Reverse 5'-GCCCTTCATAGGAGATACTGG-3'						
1,290		rs689466	RsaI	2 min at 95 °C, 35 cycles of 30 seach at 95 °C, 60^0C and 72 °C for 45 s, final extension at 72°C for 5 min	173	[13]
Forward 5'-CAGGTTTTATGCTGTCATTTTCC-3'						
Reverse 5'-TAGTGCTCAGGGAGGAGCAT-3'						
765		rs20417	AciI	2 min at 95 °C, 35 cycles of 30 seach at 95 °C, 64^0C and 72 °C for 45 s, final extension at 72 °C for 5 min	284	[8]
Forward 5'-CCATCAGAAGGCAGGAAC-3'						
Reverse 5'-GCTCTATATGCAGCACATAC-3'						

similarly, variant rs689466 was not found to be associated with breast cancer risk. As shown in Table 3, in our study observed and expected genotype frequencies of different SNPs were consistent with Hardy Weinberg equilibrium and our result showed strong linkage disequilibrium between rs689465, rs689466 and rs20417 in the two possible combinations (Table 3).

Table 4 lists a total of eight haplotype combinations observed among control and cases (Table 4). Haplotype rs689465G: rs689466A: rs20417C (OR 2.9 CI 95 %1.377–6.327, P=0.007) was more frequent in breast cancer patients versus controls and it was statistically significant. In the present study, one rare haplotype AAC was observed in only control samples, whereas haplotype AGC was not detected in any of the samples. Moreover, all other haplotype were not associated with breast cancer risk.

Discussion

The role of SNPs and their possible associations with cancer risk has been a topic of much discussion. Most studies have evaluated functionally important COX-2 SNPs rs689465, rs689466, rs20417 in several cancers including colorectal, gastric, esophagus, and breast cancer. Overall, these studies showed a lack of consensus [8, 9, 17–19]. A plausible explanation for this discrepancy may be differential role of COX-2 polymorphisms in different cancers. For instance, approximately 19 studies have been published on the role of rs20417 polymorphism and risk of cancer including colorectal, esophageal, oral and breast cancer but much controversy has emerged from their observations. According to a meta-analyses published in 2010 by Ke-DaYu, the effect of a single gene might have limited impact on breast cancer [20]. Another

Table 2 Distribution of COX-2 SNPs in healthy controls and breast cancer patients

Genotype		db SNP id	Control	Case	OR (CI95%)	Case	OR (CI95%)	P value
	1,195	rs689465						
GG			76(75.2)	102(68)	1	102(68)	1	0.523
AG			22(21.8)	36(24)	1.219(0.664–2.231)	36(24)	1.219(0.664–2.231)	
AA			3(2.9)	6(4)	1.490(0.361–6.149)	6(4)	1.490(0.361–6.149)	0.581
Unknown			.	6(4)		6(4)		
	1,290	rs689466						
AA			77(76.2)	112(74.7)	1	112(74.7)	1	
AG			21(20.8)	19(12.7)	0.622(0.314–1.234)	19(12.7)	0.622(0.314–1.234)	0.174
GG			3(2.9)	4(2.7)	0.917(0.200–4.21)	4(2.7)	0.917(0.200–4.21)	0.911
Unknown			.	15(10)		15(10)		
	765	rs20417						
GG			64(63.4)	72(48)	1	72(48)	1	
GC			34(33.7)	55(36.7)	1.435(0.834–2.478)	55(36.7)	1.435(0.834–2.478)	0.191
CC			3(2.9)	11(7.3)	3.259(0,870–12.20)	11(7.3)	3.259(0,870–12.20)	0.079
Unknown			.	12(8)		12(8)		

Table 3 Pairwise linkage disequilibrium between SNPs

SNP	*D*'	r^2	*P*
rs6894566 &rs689466	53.1	8	0.0783
rs689465 &rs20417	45	11	0.017
rs689466 &rs20417	25	16	Infinity

D' Degree of imbalance in module, r^2 Degree of correlation

meta-analysis reported by Wei Zhu et al. found an association between COX-2 rs20417 polymorphism and increased cancer risk among Asians in comparison to European and Africans, suggesting a possible role of ethnic differences and genetic background. Other important reasons include masking effect of unidentified genes and or other genetic variant involved in tumorogenesis [21].

Pakistan is a multicultural country with considerable ethnic diversity. The main ethnic groups include, Punjabi, Pathan, Sindhi, Seriki, Mohajir, Baloch, Kashmiri and Afghan refugees [22]. The social norms are different from most European and other Asian countries. It is possible that the ethnic background may contribute to a high prevalence of COX-2 sequence variants. However, there is no data of COX-2 SNPs distribution for the Pakistani population available.

In this study, the 1.2 kb region of COX-2 promoter was selected which contains an important regulatory site of COX-2. Moreover, previous in vitro studies have shown functional effects of SNPs rs689465, rs689466, rs20417 on promoter activity. Therefore, potentially functional SNPs rs689465, rs689466, and rs20417 were selected for this study. However, our results showed that none of the three polymorphisms was significantly associated with breast cancer risk in the single locus analysis. These findings are consistent with other investigators who have also reported no association between rs20417, rs689465, rs689465 and breast cancer. [19, 23, 24] On the other hand, some of the haplotypes, constructed from the three SNPs examined were found to be

Table 4 COX-2 SNPs haplotype distribution in controls and patients and their association with breast cancer

SNPs Haplotype	Controls *N*=101(%)	Patients *N*=150 (%)	OR(95%CI)	*P* value
GAG	64(63.37)	77(51.51)	1	
GAC	10(10)	34(23)	2.909(1.337–6.327)	0.007
AAG	10(9.7)	23(15.5)	1.912(0.848–4.310)	0.118
GGG	7(6.4)	7(4.37)	0.831(0.277–2.494)	0.742
GGC	6(6.3)	7(4.37)	0.970(0.310–3.031)	0.958
AAC	3(3.43)	.	0	0.99
AGG	1(0.007)	2(1.1)	0.83(0.51–13.55)	0.897
AGC				

*rs689465-rs689466-rs20417

associated with breast cancer risk specially the haplotype G1195-A1290-C765 (OR 2.909 CI 95 % 1.377–6.327, P0.007). This data was in contrast to Piranda et al. studies, who have reported no significant difference in the haplotype distribution was observed between patients and controls (*P*=0.99) [19]. However, our findings were in agreement with Gao J et al.; in their studies genotypes in combination containing more than 3 variant alleles were found associated with a significant increased risk of cancer and have suggested that polymorphisms in the regulatory regions of COX-2 may interact in a combinatorial manner in breast cancer development [23].

Several published studies on COX-2 polymorphism and association with cancer risk seem to vary from population to population as well as differences are reported in the ethnic groups of the same population. According to these studies, rs20417C allele is more common in western countries like Europe, America, and Australia compared to Asian countries [20, 25]. In the present study, we observed that rs20417G allele was more common in the general population, whereas the C allele was more frequent in breast cancer patients. However, this association was not statically significant. Consistent with the published studies, our study found no significant association between breast cancer risk and COX-2 rs689465 and rs689466 polymorphisms. [24, 26].

This is the first report regarding the genotype and haplotype distribution of COX-2 linked SNP variants in the Pakistani population and their association with breast cancer risk. Our study has some limitations. First, the sample size was small that led to relatively lower statistical power and inability to detect small effect of SNPs. Secondly, controls were randomly selected from a similar population therefore selection bias cannot be excluded, even though allelic distribution of the three SNPs in control still complies the Hardy Weinberg equilibrium. In conclusion, this is the first case–control study in Pakistani breast cancer patients that examined COX-2 SNPs, rs689465, rs689466 and rs20417 with breast cancer risk. We report that combination of SNPs containing three variant alleles are associated with increased breast cancer risk in Pakistani population. Further studies are necessary to determine the role of ethnic background in the observed differences between the control and case study subjects. Elevated COX-2 expression has been linked to increased cancer risk and poor prognosis, whereas inhibition of COX-2 if found to reduce the incidence in certain cancer types. If our data are confirmed in a larger study-format, this breast cancer risk associated haplotype (G1195-A1290-C765) found in our study may be used for early diagnosis of patients and for incorporating COX-2 targeted therapy. Moreover, along with treatment, protective cover by nonsteroidal anti-inflammatory drugs to inhibit COX-2 could also be effectively used for the prevention of breast cancer in patients who carry this high risk COX-2 haplotype.

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