Evaluation of the Contribution of Cyclooxygenase 2 Genotypes to Breast Cancer in Taiwan

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Abstract. *Overexpression of cyclooxygenase 2 (COX-2) has been suggested to be associated with breast carcinogenesis. The aim of the present study was to evaluate the contribution of genotypic polymorphisms in COX-2 to breast cancer risk of Taiwanese females. In total, 1,232 breast cancer patients and 1,232 healthy controls were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodology. Six polymorphic variants of COX-2, including G-1195A (rs689466), G-765C (rs20417), T8473C (rs5275), intron 1 (rs2745557), intron 5 (rs16825748) and intron 6 (rs2066826) were examined. The results showed that the GC genotype of COX-2, G-765C was associated with a lower risk compared to the wild-type GG genotype (odds ratio(OR)=0.66, 95% confidence interval(CI)=0.53-0.83, p=0.0005). The C allele of COX-2 G-765C was significantly more frequently found in controls than in cancer patients (p=0.0006). In addition, the OR of the GG/AG+AA, GC/GG and GC/AG+AA at G-765C/Intron 1 combined genotypes compared to wild-type GG/GG genotype were 0.79 (95%CI=0.66-0.96; p=0.0166), 0.61 (95%CI=0.48-0.78; p=0.0001), and 0.71 (95%CI=0.36-1.37; p=0.3040), respectively. As for the combination of G-765C and intron 6, the OR of the GG/AG+AA, GC/GG and GC/AG+AA combined genotypes compared with wild-type GG/GG reference genotype were 0.79 (95%CI=0.62-1.01; p=0.0561), 0.63 (95%CI=0.50-0.81; p=0.0003), and 0.68 (95%CI=0.38-1.21; p=0.1897), respectively. Our results indicate that the C allele of COX-2, G-765C was associated with a decreased risk of breast cancer in Taiwan, and could serve as an early detection and predictive marker for breast cancer risk.*

Breast cancer is currently ranked first among cancers affecting females throughout the world and its incidence rate has increased during the last decades (1). In Taiwan, breast cancer is the fourth leading cancer, presenting high incidence, high mortality, and early onset (2, 3). It is believed that breast cancer is largely multi-causal and its susceptibility is conferred by environmental and hormone exposures in addition to multi-genic variations in the genome. Previous studies have revealed that oriental women affected by breast cancer were significantly younger than white women and had racial/ethnic differences in their survival patterns (4, 5). In recent years, scientists began to explore the mechanisms underlying breast cancer formation at the molecular level. Investigations into these racial/ethnic differences from the cancer genomic angle may enhance the speed of unravelling the genomic and environmental etiology of breast cancer, as well as help towards its early detection and prediction.

Cyclooxygenase, also known as prostaglandin endoperoxide synthetase, plays a crucial role in the cellular metabolism through converting arachidonic acid to prostaglandins. Two isoforms of COX, COX-1 and COX-2 act quite differently. COX-1 is constitutively expressed and is present in various tissues, while COX-2 is not detected in most normal tissues. However, COX-2 can be rapidly induced by inflammatory stimuli, resulting in elevated levels of prostaglandins, which in turn can regulate cell proliferation, apoptosis and angiogenesis, contributing to tumor occurrence and progression (6-9). In recent years,

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mounting evidence has shown that elevated COX-2 in breast tissues is related to the genesis of mammary tumors (10, 11), and is associated with parameters of aggressive breast cancer, including large tumor size, positive axillary lymph node metastases (12), and HER2-positive tumor status (13). On the other hand, targeted inhibition of COX-2 by the selective cyclooxygenase-2 inhibitor celecoxib inhibited the proliferation of breast cancer cell lines *in vitro* (14).

The human *COX-2* gene (also known as PTGS2) is located on chromosome 1q25.2-q25.3 and consists of 10 exons spanning 8.3 kb (15). More than 15 single nucleotide polymorphisms for *COX-2* have been identified, but the most extensively studied polymorphisms are the G-765C (rs20417) in the promoter and the C8473T (rs5275) in the 3'UTR of *COX-2*. Genetic polymorphisms at the *COX-2* promoter region have been shown to alter its expression and influence the susceptibility to various carcinomas, including childhood acute lymphoblastic leukemia (16), hepatocellular carcinoma (17), prostate (18) and bladder cancer (19). In 2002, it has been proposed that the G-765C polymorphism on *COX-2* may eliminate a Sp1-binding site but create an E2F binding site and result in altered COX-2 expression (20). Another polymorphism site of *COX-2*, C8473T (rs5275), found on 3'UTR, was suggested to be associated with the alteration of mRNA levels of the gene as sequences within the 3'UTR are important for message stability and translational efficiency (21). In 2010, Yu and his colleagues conducted a meta-analysis on the associations between several COX-2 polymorphisms and breast cancer risk and suggested that no significant association was observed for the G-765C and C8473T polymorphisms (22). However, out of the studies included in their meta-analysis, only two studies have been conducted in Chinese populations and none of the two studies investigated the rs5277 polymorphism (23, 24). In conclusion, the *COX-2* genotype among Chinese has not yet been well-studied.

Therefore, in the present work we aimed to analyze not only the famous polymorphic site of *COX-2*, G-765C (rs20417) and 3'UTR (rs5275), but also four other sites G-1195A (rs689466), Intron 1 (rs2745557), intron 5 (rs16825748) and intron 6 (rs2066826) in a very representative population (control/case=1,232/1,232), and investigated the correlation between COX-2 genotypes and risk of breast cancer among Taiwanese women.

Materials and Methods

Study population and sample collection. One thousand two hundred and thirty-two female cancer patients diagnosed with breast cancer were recruited at the China Medical University Hospital (Taichung, Taiwan). For comparison, equal amounts of age-matched, non-breast cancer healthy volunteers were selected as controls from the Health Examination Cohort of the hospital matched with age (±5 years). Our study was approved by the Institutional Review Board of the China Medical University Hospital (DMR99-IRB-108). Before recruitment, a standard questionnaire was administered through face-to-face interviews by trained interviewers to obtain information on demographic data and related factors.

Genotyping assays. Genomic DNA was prepared using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan), as described previously (25, 26). The polymerase chain reaction (PCR) cycling conditions were: one cycle at 94˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s, and a final extension at 72˚C for 10 min. Pairs of PCR primer sequences and restriction enzyme for each DNA product are listed in Table I.

Statistical analyses. The associations between the genotypes of the *COX-2* polymorphisms and risk of breast cancer and patients' clinical characteristics were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from unconditional logistic regression analysis with the adjustment for age and age at menarche. Pearson's Chi-square test or Fisher's exact test (when any cell was less than 5) was also used to compare the distribution of the genotypes. The data were recognized as significant when the statistical *p*-value was less than 0.05.

Results

The frequency distributions of selected demographic and life-style characteristics of 1,232 breast cancer patients and 1,232 non-cancer controls are shown in Table II. The age-related characteristics of patients and controls were all well-matched ($p>0.05$). About 5% of patients had family history. As for the individual life-style, cigarette smoking and alcoholism were both risk factors for breast cancer in this population ($p<0.05$) (Table II).

The frequencies of the genotypes for the *COX-2* G-1195A, G-765C, 3'UTR, intron 1, intron 5 and intron 6 in controls and breast cancer patients are shown in Table III. The genotype distribution of the COX-2 G-765C was significantly different between breast cancer and control groups ($p=0.0005$), while those for COX-2 G-1195A, 3'UTR, Intron 1, Intron 5 or Intron 6 polymorphisms were not ($p>0.05$) (Table III). Those who carried a GC genotype at *COX-2* G-765C had 0.66-fold OR of breast cancer risk compared to those with GG genotype (95%CI=0.53-0.83). There was no *COX-2* G-765C CC carrier (Table III).

The frequencies of the alleles for *COX-2* polymorphisms in controls and breast cancer patients are shown in Table IV. Neither of the allele of the *COX-2* of the polymorphisms were found to be associated with breast cancer ($p>0.05$) except that of *COX-2* G-765C ($p=0.0006$) (Table IV).

To further investigate the contribution of *COX-2* genotype to breast cancer susceptibility, the two-polymorphic interactions among *COX-2* genotypes were investigated by genotype analysis. The two combinations with statistically significant *p*-values are shown in Tables V and VI. As for the combination of G-765C and Intron 1, the ORs of the GG/AG+AA, GC/GG and GC/AG+AA combined genotypes

Table I. *The primer sequences, polymerase chain reaction and restriction fragment length polymorphism conditions for cyclooxygenase-2 (COX-2) gene polymorphisms.*

Polymorphism (location)	Primers sequences (5' to 3')	Restriction enzyme	SNP sequence	DNA fragment size (bp)
G-1195A	F: CCCTGAGCACTACCCATGAT	*Hha I*	A	273
(rs689466)	R: GCCCTTCATAGGAGATACTGG		G	220+53
G-765C	F: TATTATGAGGAGAATTTACCTTTCGC	*Pvu II*	C	100
(rs20417)	R: GCTAAGTTGCTTTCAACAGAAGAAT		G	74+26
3'UTR	F: GTTTGAAATTTTAAAGTACTTTTGAT	*Bcl I*	T	147
(rs5275)	R: TTTCAAATTATTGTTTCATTGC		C	124+23
Intron 1	F: GAGGTGAGAGTGTCTCAGAT	*Taq I*	G	439
(rs2745557)	R: CTCTCGGTTAGCGACCAATT		A	353+76
Intron 5	F: GCGGCATAATCATGGTACAA	*BsrG I*	T	417
(rs16825748)	R: CAGCACTTCACGCATCAGTT		A	314+103
Intron 6	F: ACTCTGGCTAGACAGCGTAA	*Aci I*	A	327
(rs2066826)	R: GCCAGATTGTGGCATACATC		G	233+94

F and R indicate forward and reverse primers, respectively.

Table II. *Distributions of demographic and life style of breast cancer patients and the age-matched controls.*

Characteristic	Controls (N=1232)			Patients (N=1232)			p-Value
	n	%	Mean (SD)	n	%	Mean (SD)	
Age at onset (years)							
<40	359	29.1%		362	29.4%		0.89[a]
40-55	558	45.3%		547	44.4%		
>55	315	25.6%		323	26.2%		
Age at menarche (years)			12.4 (0.7)			12.1 (0.6)	0.79[b]
Age at first birth of child (years)			29.4 (1.2)			29.8 (1.4)	0.63[b]
Age at menopause (years)			48.8 (1.8)			49.3 (2.0)	0.59[b]
Family history							
First degree (Mother, sister and daughter)				55	4.5%		
Second degree				6	0.5%		
No history				1171	95%		
Personal habits							
Cigarette smokers	86	7.0%		170	13.8%		<0.0001[a]
Alcohol drinkers	91	7.4%		162	13.1%		<0.0001[a]

Statistic results based on [a]Chi-square or [b]unpaired Student's *t*-test.

compared to wild-type GG/GG reference genotype were 0.79 (95%CI=0.66-0.96; p=0.0166), 0.61 (95%CI=0.48-0.78; p=0.0001), and 0.71 (95%CI=0.36-1.37; p=0.3040), respectively (Table V). As for the combination of G-765C and Intron 6, the ORs of the GG/AG+AA, GC/GG and GC/AG+AA combined genotypes compared to the wild-type GG/GG reference genotype were 0.79 (95%CI=0.62-1.01; p=0.0561), 0.63 (95%CI=0.50-0.81; p=0.0003), and 0.68 (95%CI=0.38-1.21; p=0.1897), respectively (Table VI). As for other combinations, there was no significant difference in frequencies of any combined genotypes between the two groups for each combined genotype (data not shown). Joint effects of *COX-2* genotypes and environmental factors, including smoking and alcohol drinking were also analyzed, and no significant interaction was found (data not shown).

Discussion

The abnormal expression of COX-2 has been reported to play an important role in breast carcinogenesis (10, 11). In order to reveal the role of *COX-2* from the genomic viewpoint and to find potential detection markers for breast cancer, up to six polymorphic sites of the *COX-2* gene have been chosen and their association with the breast cancer

Table III. *Distribution of cyclooxygenase-2 (COX-2) genotypes among breast cancer patient and control groups.*

Genotype	Controls	%	Cases	%	*p*-Value[a]	OR (95% CI)[b]
G-1195A (rs689466)						
AA	346	28.1%	355	28.8%	0.9224	Reference
AG	602	48.9%	596	48.4%		0.96 (0.80-1.16)
GG	284	23.0%	281	22.8%		0.96 (0.77-1.20)
AG+GG	886	71.9%	877	71.2%		0.96 (0.81-1.15)
G-765C (rs20417)						
GG	1023	83.0%	1085	88.1%	0.0005*	Reference
GC	209	17.0%	147	11.9%		0.66 (0.53-0.83)*
CC	0	0.0%	0	0.0%		
3'UTR (rs5275)						
TT	823	66.8%	809	65.7%	0.5797	Reference
TC	409	33.2%	423	34.3%		1.05 (0.89-1.24)
CC	0	0.0%	0	0.0%		
Intron 1 (rs2745557)						
GG	901	73.1%	937	76.1%	0.2480	Reference
AG	306	24.8%	272	22.1%		0.85 (0.71-1.03)
AA	25	2.1%	23	1.8%		0.88 (0.50-1.57)
AG+AA	331	26.9%	295	23.9%		0.86 (0.71-1.03)
Intron 5 (rs16825748)						
TT	1183	96.0%	1193	96.8%	0.3286	Reference
AT	49	4.0%	39	3.2%		0.79 (0.51-1.21)
AA	0	0.0%	0	0.0%		
Intron 6 (rs2066826)						
GG	1035	84.0%	1064	86.4%	0.2562	Reference
AG	171	13.9%	145	11.8%		0.82 (0.65-1.05)
AA	26	2.1%	23	1.8%		0.86 (0.49-1.52)
AG+AA	197	16.0%	168	13.6%		0.83 (0.66-1.04)

[a]Based on Chi-square test or Fisher's exact test (when one or more cell was less than 5); [b]OR: Odds ratio, CI: confidence interval; *Statistically significant.

susceptibility in a representative Taiwanese population was investigated. We found that for the promoter site G-765C of *COX-2*, the variant GC genotype and C allele were associated with a decreased risk for breast cancer, compared to the wild-type GG genotype and G allele, respectively (Tables III and IV), while those for other polymorphic sites were not (Tables III and IV). It is reasonable to suggest that the polymorphic site at the promoter region may interact with transcription factors, determining the individual difference at transcriptional and translational levels of COX-2 upon initiation or progression periods of breast carcinogenesis (20). Also, the *COX-2* G-765C site may interact with other polymorphic sites, such as intron 1 (rs2745557) and intron 6 (2066826), to conduct their influences on regulating both COX-2 expression levels and cancer risk (Tables V and VI).

COX-2 played a role in the etiology of breast cancer, which is an outcome of complex genetic and environmental interactions. In the analysis of the synergistic effects of genotypes together with smoking and alcoholism life-style on breast cancer, no significant findings were found. Although we could not find a positive interaction of *COX-2* genotype with these environmental risk factors, the environmental factors could not be excluded for their influence on the transcriptional, translational and post-translational levels of COX-2. For instance, the expression levels of COX-2 are reported to be regulated by reactive oxygen species (ROS) in hepatocytes (27), and intracellular ROS may be elevated by various environmental stimuli, including smoking and alcohol drinking. Since it is known that transcriptional modulation of COX-2 is cell-specific (28), the correlation between ROS and COX-2 expression in breast carcinogenesis may be investigated in breast cancer cell models. In 2014, Gao and his colleagues found that GC/CC genotypes at *COX-2* G-765C were associated with a higher cancer risk and larger tumor size, suggesting that variant genotypes of *COX-2* promoter polymorphism may not only participate in cancer susceptibility determination, but also in the progression of breast carcinogenesis (29).

In conclusion, the present study focused on the single or combined *COX-2* genotypes and their effects on breast cancer risk in Taiwan. Further investigations on multiple genotypes of other cancer-related genes, gene-gene and gene-

Table IV. *Distribution of cyclooxygenase-2 (COX-2) allelic frequencies among breast cancer patient and control groups.*

Allele	Controls	%	Patients	%	*p*-Value[a]
G-1195A (rs689466)					
Allele A	1294	52.5%	1306	53.0%	0.7320
Allele G	1170	47.5%	1158	47.0%	
G-765C (rs20417)					
Allele G	2255	91.5%	2317	94.0%	0.0006*
Allele C	209	8.5%	147	6.0%	
3'UTR (rs5275)					
Allele T	2055	83.4%	2041	82.8%	0.5945
Allele C	409	16.6%	423	17.2%	
Intron 1 (rs2745557)					
Allele G	2108	85.6%	2146	87.1%	0.1152
Allele A	356	14.4%	318	12.9%	
Intron 5 (rs16825748)					
Allele T	2415	98.0%	2425	98.4%	0.2821
Allele A	49	2.0%	39	1.6%	
Intron 6 (rs2066826)					
Allele G	2241	90.9%	2273	92.2%	0.1003
Allele A	223	9.1%	191	7.8%	

[a]Based on Chi-square test; * Statistically significant.

Table V. *Frequencies of combined COX-2 G-765C and Intron 1 genotype polymorphisms among breast cancer patient and control groups.*

COX-2 G-765C/ Intron 1	Control		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
All	1232	100.0	1232	100.0		
GG/GG	712	57.8	806	67.6	1.00 (reference)	
GG/AG+AA	311	25.2	279	20.5	0.79 (0.66-0.96)*	0.0166*
GC/GG	189	15.4	131	8.4	0.61 (0.48-0.78)*	0.0001*
GC/AG+AA	20	1.6	16	3.5	0.71 (0.36-1.37)	0.3040

[a]Based on Chi-square test. OR, Odds ratio; CI, Confidence interval; *Statistically significant.

Table VI. *Frequencies of combined COX-2 G-765C and Intron 6 genotype polymorphisms among breast cancer patient and control groups.*

COX-2 G-765C/ Intron 6	Control		Patients		OR (95% CI)	*p*-Value[a]
	n	%	n	%		
All	1232	100.0	1232	100.0		
GG/GG	854	69.3	938	76.1	1.00 (reference)	
GG/AG+AA	169	13.7	147	12.0	0.79 (0.62-1.01)	0.0561
GC/GG	181	14.7	126	10.2	0.63 (0.50-0.81)*	0.0003*
GC/AG+AA	28	2.3	21	1.7	0.68 (0.38-1.21)	0.1897

[a]Based on Fisher's exact test. OR, Odds ratio; CI, Confidence interval; *Statistically significant.

environment interactions, and phenotypic assays of the cancer-associated polymorphic sites are urgently needed in the future. The presence of C allele at G-765C of *COX-2* was not only associated with a lower cancer risk, but was also involved in early breast carcinogenesis.

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