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Association of Cyclooxygenase 2 Single-Nucleotide Polymorphisms and Hepatocellular Carcinoma in Taiwan

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Abstract

Hepatocellular carcinoma (HCC) is a worldwide neoplasm for which early diagnosis is difficult and the prognosis is usually poor. Overexpression of cyclooxygenase 2 (COX-2) has been suggested to be associated with hepatocarcinogenesis. Although several COX-2 inhibitors have been used in hepatoma therapy, the genetic background between *COX-2* and HCC remains largely unknown. In this study, the association of genotypic polymorphisms in *COX-2* with HCC was investigated. 298 patients with HCC and 298 healthy controls recruited from the China Medical Hospital in Taiwan were genotyped by a PCR-RFLP method. We have investigated six polymorphic variants of *COX-2*, including A-1195G, G-765C, T+8473C, and variants in introns 1, 5 and 6, and analyzed the association of specific genotype(s) with susceptibility to HCC. The results showed that, for each of the six genotypes, no differences in distribution between the HCC and control groups were found. There was neither obvious joint effect of *COX-2* G-765C/intron 6 haplotype nor genotypes with smoking or alcohol consumption on HCC risk. Environmental factors, other than smoking and alcohol drinking, may affect the postnatal expression of *COX-2* in the etiology of HCC, which is an outcome of complex genetic and environmental interactions. Moreover, our immunohistochemistrical results indicated that the COX-2 protein was significantly over-expressed in well-differentiated HCC, but not significantly increased in expression in poorly-differentiated HCC. We suggest that COX-2 may be a determinant of the differentiation grade of HCC.

Key Words: COX-2, polymorphism, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the leading cause of malignant cancer death in both the world (7) and Taiwan*. Limited choices in treatment and poor prognosis of this disease emphasize the importance in developing an effective chemoprevention strategy. However, the exact molecular mechanism of hepatocarcinogenesis is still unclear (7).

Cyclooxygenases (COX) are key enzymes for

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Table 1. Primer sequences, PCR and restriction fragment length polymorphism (RFLP) conditions for the analysis of *COX-2* gene polymorphisms

Polymorphism (location)	Primers sequences (5' to 3')	Restriction enzyme	SNP sequence	DNA fragment size (bp)
A-1195G	F[a]: 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
T+8473C	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

[a]F and R indicate forward and reverse primers, respectively.

the conversion of arachidonic acid to prostaglandin and other eicosanoids (13). Whereas COX-1 is constitutively expressed, COX-2 is a highly inducible protein. Increased COX-2 expression has been associated with tumorigenesis in various types of human cancer, including the early stages of hepatocarcinogenesis (2, 18, 27, 28). In several animal and clinical studies, COX-2 specific inhibitors have both preventive and therapeutic effects as anticancer drugs for breast, bladder, lung and pancreas cancers (12, 19, 24, 26). However, the association of *COX-2* genotypes with HCC has not been investigated. In addition, the mRNA and protein levels of COX-2 may vary among individuals, and this variability may be partially and genetically determined under different molecular mechanisms, which may depend on single-nucleotide polymorphisms (SNPs) of *COX-2* (11, 25).

To clarify the hypothesis that SNP variants of *COX-2* are associated with the risk of HCC, we analyzed the genetic polymorphisms of six *COX-2* SNPs, namely A-1195G (rs689466), G-765 (rs20417), T+8473C (rs5275), intron 1 (rs2745557), intron 5 (rs16825748) and intron 6 (rs2066826), in a large Taiwanese HCC population (control/case = 298/298).

Materials and Methods

Study Population and Sample Collection

Two hundred and ninety-eight patients diagnosed with HCC were recruited at the Department of General Surgeon at the China Medical University Hospital, Taiwan, in 2004-2010. Each patient and non-cancerous healthy person (matched by gender, age and individual habits, such as smoking and alcohol drinking, from a random sampling from the Health Examination Cohort of China Medical University Hospital) completed a self-administered questionnaire and provided their peripheral blood samples.

Genotyping Assays

Genomic DNA was prepared from peripheral blood samples using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to previous studies (5, 6, 8-10, 15, 16, 32). The polymerase chain reaction (PCR) cycling conditions were: one cycle at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 10 min. PCR primer sequences and the restriction enzyme used for each DNA product are listed in Table 1.

Immunohistochemical Staining for COX-2

For liver specimens, tissue sections (5 μm) mounted on silanized slides (DAKO Japan, Kyoto, Japan) were deparaffinized with xylene and dehydrated in a graded series of ethanol. After rehydration in absolute ethanol for 15 s, the slides were heated by microwave in 10 mM citrate buffer, pH 6.0 (Zymed Lab Inc., San Francisco, CA, USA) for 8 min. After being washed in ice-cold phosphate-buffered saline (PBS), sections were pre-blocked for 10 min in an autoblocker (Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK). Then, they were incubated overnight with a mouse monoclonal anti-human COX-

Talbe 2. Characteristics of the HCC patients and the controls

Characteristic	Controls (n = 298)			Patients (n = 298)			*P*-value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			54.1 (4.6)			52.3 (4.5)	0.68
Gender							1.00
Male	213	71.5		213	71.5		
Female	85	28.5		85	28.5		
Habit							
Smoking	213	71.5		224	75.2		0.35
Alcohol drinking	198	66.4		206	69.1		0.54

[a]Based on *Chi*-square test.

2 antibody at 1:100 dilution (Transduction Lab Inc., Franklin Lakes, NJ, USA). After three washes in PBS, the sections were incubated with a horseradish peroxidase (HRP)-conjugate anti-mouse IgG antibody (Santa Cruz, CA, USA) at room temperature for 1 h. Finally, 3, 3'-diaminobenzidine (Sigma, Missouri, USA) was added. Counter-staining was done with hematoxylin (Sigma, St. Louis, MO, USA). The image capture was done by an Olympus BX 50 fluorescence microscope (Olympus, Optical, Tokyo, Japan) and a Delta Vision disconsolation microscopic system operated by SPOT software (Diagnostic Instruments Inc., Twinsburg, Ohio, USA).

Western Blotting Analysis

The liver specimens were homogenized in RIPA lysis buffer (Upstate Inc., Lake Placid, NY, USA), the homogenates were centrifuged at 10,000 g for 30 min at 4°C, and the supernatants were used for Western blotting. Samples were denatured by heating at 95°C for 10 min, separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat milk and incubated overnight at 4°C with a mouse monoclonal anti-human COX-2 antibody at 1:1000 (Transduction Lab Inc.), then with the corresponding HRP-conjugated goat anti-mouse IgG secondary antibody (Chemicon, Temecula, CA, USA) for 1 h at room temperature. After reaction with ECL solution (Amersham, Arlington Heights, IL, USA), bound antibody was visualized using a chemiluminescence imaging system (Syngene, Cambridge, UK). Finally, the blots were incubated at 56°C for 18 min in a stripping buffer (0.0626 M Tris-HCl, pH 6.7, 2% SDS, 0.1 M mercaptoethanol) and re-probed with a monoclonal mouse anti-β-actin antibody (Sigma) used as the loading control. The optical density of each specific band was measured using a computer-assisted imaging analysis system (Gene Tools Match software; Syngene, Nuffield, Cambridge, UK).

Statistical Analyses

Only those with both genotypic and clinical data (control/case = 298/298) were selected for final analysis. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *COX-2* SNPs in the controls from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's *Chi*-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the genotypes between cases and controls. Data were recognized as significant when the statistical *P*-value was less than 0.05.

Results

The frequency distributions of selected characteristics of 298 HCC patients and 298 controls are shown in Table 2. These characteristics of patients and controls are all well matched. None of the differences between both groups were statistically significant ($P > 0.05$) (Table 2).

The frequencies of the genotypes for the *COX-2* SNPs in the controls and the HCC patients are shown in Table 3. The genotype distributions of the genetic polymorphisms of *COX-2* of the six polymorphisms investigated were not significant between the two groups ($P > 0.05$) (Table 3). The frequencies of the alleles for *COX-2* SNPs in the controls and the HCC patients are shown in Table 4. None of the allele of the *COX-2* of the SNPs was found to be associated with HCC ($P > 0.05$).

To further investigate the association of genotype *COX-2* and HCC, the two-SNP *COX-2* interactions among SNPs were investigated by genotype analysis. There were no significant differences in the frequencies of any combined genotypes between the two groups for each combined genotype (data not

Table 3. Distribution of *COX-2* genotypes among the HCC patient and the control groups

Genotype	Controls	%	Patients	%	*P*-value[a]
A-1195G (rs689466)					0.9220
AA	81	27.1	84	28.2	
AG	145	48.7	144	48.3	
GG	74	24.8	70	23.4	
G-765C (rs20417)					0.1951
GG	250	83.9	262	87.9	
GC	48	16.1	36	12.1	
CC	0	0	0	0	
T+8473C (rs5275)					0.6645
TT	201	67.4	195	65.4	
TC	97	32.6	103	34.6	
CC	0	0	0	0	
intron 1 (rs2745557)					0.7953
GG	226	75.8	219	73.5	
AG	67	22.6	73	24.5	
AA	5	1.6	6	2.0	
intron 5 (rs16825748)					0.5768
TT	290	97.3	293	98.3	
AT	8	2.7	5	1.7	
AA	0	0	0	0	
intron 6 (rs2066826)					0.3192
GG	248	83.2	236	79.2	
AG	44	14.8	51	17.1	
AA	6	2.0	11	3.7	

[a]Based on *Chi*-square test.

Table 4. *COX-2* allelic frequencies among the HCC patient and the control groups

Allele	Controls	%	Patients	%	*P*-value[a]
A-1195G (rs689466)					0.6824
Allele A	307	51.2	312	52.4	
Allele G	293	48.8	284	47.6	
G-765C (rs20417)					0.1745
Allele G	548	92.0	560	94.0	
Allele C	48	8.0	36	6.0	
T+8473C (rs5275)					0.6419
Allele T	499	83.7	493	82.7	
Allele C	97	16.3	103	17.3	
intron 1 (rs2745557)					0.4989
Allele G	519	87.1	511	85.7	
Allele A	77	12.9	85	14.3	
intron 5 (rs16825748)					0.4028
Allele T	588	98.6	591	99.2	
Allele A	8	1.4	5	0.8	
intron 6 (rs2066826)					0.1130
Allele G	540	90.6	523	87.8	
Allele A	56	9.4	73	12.2	

[a]Based on *Chi*-square test.

Table 5. Frequencies of combined *COX-2* G-765C and intron 6 genotype polymorphisms among the HCC and the control groups

COX-2 G-765C/intron 6	Controls		Patients		OR (95% CI)	*P*-value[a]
	n	%	n	%		
All	298	100.0	298	100.0		
GG/GG	208	69.8	207	69.5	1.00	
GG/AG+AA	42	14.1	55	18.5	1.31 (0.84-2.05)	0.2595
GC/GG	40	13.4	29	9.7	0.73 (0.44-1.22)	0.2437
GC/AG+AA	8	2.7	7	2.3	0.88 (0.31-2.47)	1.0000

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

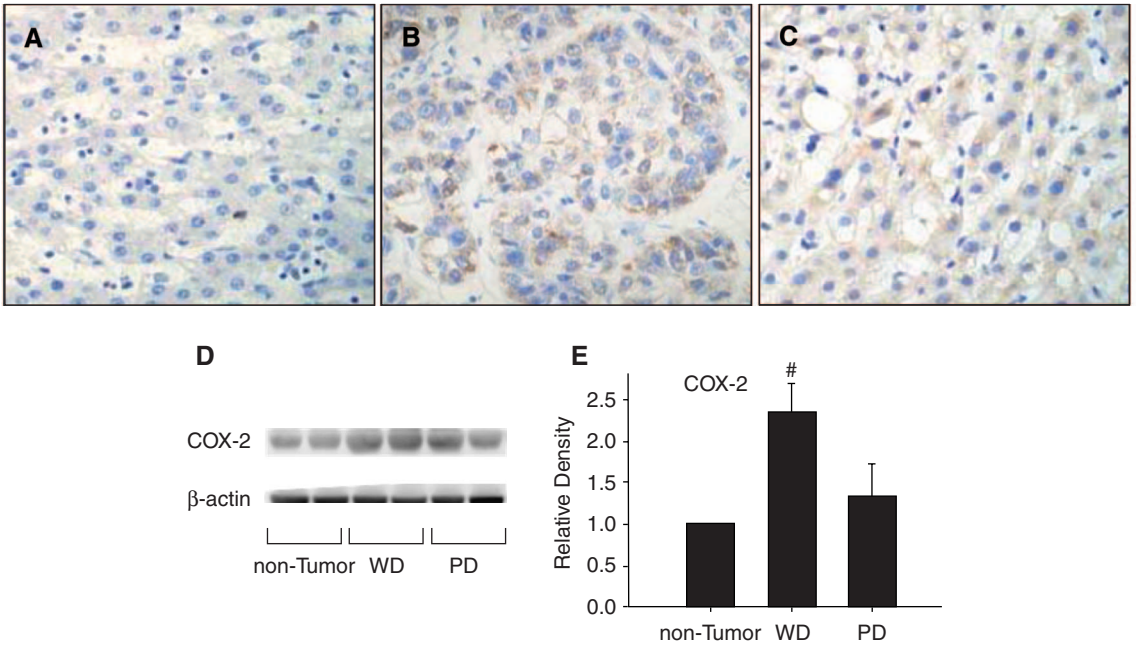

Fig. 1. Expression levels of COX-2 in different differentiation grades of HCC (×400). A, non-tumor portion. B, tumor portion in well-differentiated (WD) HCC. C, tumor portion in poorly-differentiated (PD) HCC. D, Western blot analysis of COX-2 expression. E, Quantification of the Western blot data from the above group. β-actin was used as the loading control. Data are averaged from six tissues per group with 15 μg total sample protein for each lane. $^{\#}P < 0.05$ compared to the non-tumor portion tissues.

shown). We can take the two SNPs with the lowest *P*-values, G-765C and intron 6, as an example: the odds ratios (ORs) of the GG/AG+AA, GC/GG, and GC/AG+AA combined genotypes compared with the common GG/GG reference genotype were 1.13 (95% confidence interval, CI = 0.84-2.05; $P = 0.2595$), 0.73 (95% CI = 0.44-1.22; $P = 0.2437$), and 0.88 (95% CI = 0.31-2.47; $P = 1.0000$), respectively (Table 5). We have also investigated the joint effects of *COX-2* genotypes and environmental factors, including smoking and alcohol drinking; no significant interaction was found (data not shown).

Although, the six genetic polymorphisms of *COX-2* were not significant between HCC and the control group, in immunohistochemistrical assays and Western blot analysis (Fig. 1). We found that the COX-2 protein was significantly over-expressed in well-differentiated HCC, and slightly but not significantly more increased in poorly-differentiated HCC than in non-tumor portions.

Discussion

Abnormal expression of COX-2 has been reported to play an important role in hepatocarcinogenesis (21, 27). In order to elucidate the role of COX-2 and to uncover potential tumor markers for HCC, we chose six SNPs of the *COX-2* gene and investigated their association with HCC susceptibility in a Taiwan population. We found that for any single SNP, the variant genotypes of *COX-2* were not significantly associated with the susceptibility for HCC

(Tables 3 and 4). This may not be explained by the limited sample size (for this was a relatively large cohort for HCC population studies), but it is more likely that *COX-2* may play a minor role in the etiology of HCC, which is an outcome of complex genetic and environmental interactions. Since we could not find a direct association of *COX-2* genotype with hepatocellular carcinoma risk, transcriptional, translational and post-translational levels could be involved in hepatocarcinogenesis. Therefore, factors involved in *COX-2* expression need more attention for it is known that transcriptional modulation of *COX-2* is cell type-specific (3). It has been reported in literature that the expression level of *COX-2* is mainly regulated by C/EBPs (14) and reactive oxygen species (ROS) in hepatocytes (1, 20). Therefore, the involvement of C/EBPs and ROS in the regulation of *COX-2* expression in hepatocytes may also be a direction of future investigation.

The supporting evidence comes from the study that documented that increased COX-2 expression is associated with inhibition of apoptosis (4, 23), increased angiogenesis (29) and enhanced metastatic ability (17). Moreover, overexpression of COX-2 is associated with tumorigenesis in a number of human cancers, including HCC (18, 28). Regarding tumorigenesis, COX-2 contributes to tumor formation or growth, although the *in vivo* mechanism by which COX-2 affects tumor growth has not been determined. In addition, both tumor and stroma-derived COX-2 could influence tumor angiogenesis and/or immune function (31). Also, studies have indicated that COX-2 over-expression is important in mediating drug resistance to apoptosis in HCC (2). Pharmacological suppression of COX-2 induced apoptosis of hepatoma cells (2, 30), and over-expression of COX-2 mRNA (17) and protein (27) was closely related to a poor survival rate. According to the histological grade, we found that COX-2 expression was well correlated with the differentiation grade of HCC. COX-2 was up-regulated in well-differentiated HCC, whereas it was down-regulated in poorly-differentiated HCC. Such a close relationship between COX-2 expression and the differentiation grade of HCC has been previously reported (18, 27). These and our reports suggest that modulation of COX-2 expression may be a determinant of cellular differentiation in HCC. Such a biological role of COX-2 is supported by a recent observation that when epithelial cells are transfected with the *COX-2* gene, adhesion to the extracellular matrix increases and apoptosis is inhibited (22).

To sum up, this is the first study which focuses on the SNPs of *COX-2* and their effects on HCC risk. Further investigations of multiple SNPs of other cancer-related genes, gene-gene and gene-environment interactions, and phenotypic assays of the HCC-associated SNPs are needed. Moreover, COX-2 might play a role in the advancement as well as the early stages of hepatocarcinogenesis.

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