The Association of *Cox-2* Genotypes with Pterygium Formation in Taiwan

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Abstract

The relationship between *cyclooxygenase 2 (Cox-2)* gene and pterygium formation is not well understood. In this study, the association and interaction of genotypic polymorphisms in *Cox-2* gene and pterygium formation was investigated. 113 patients with pterygium and 400 healthy controls (without pterygium) enrolled from the Chang-Hwa hospital in central Taiwan were genotyped by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method. Six polymorphic variants of *Cox-2* were selected from NCBI website and investigated, including G-1195A, G-765C, T+8473C, intron 1, intron 5, and intron 6, and the association of specific genotype with susceptibility to pterygium was analyzed. The data showed that, none of Cox-2 G-1195A, G-765C, T+8473C, intron 1, intron 5, and intron 6 were found to have difference in the distribution between the pterygium and control groups ($p>0.05$). These findings suggest that, Cox-2 may have only post-transcriptional or post-translational regulatory effects on pterygium formation, not at the genetic level.

Keywords: Cox-2, polymorphism, pterygium, ultraviolet radiation, conjunctiva.

Introduction

Pterygium is a common, frequently recurring ocular surface lesions characterized by tissue remodeling, cellular proliferation, angiogenesis, and inflammation (10, 18). Although there is no consensus on its pathogenesis, previous findings have proved that it is a proliferative, rather than degenerative condition strongly correlated with exposure to ultraviolet (UV) radiation of solar light (18). The mechanism by which UV light induces uncontrolled proliferation in pterygial cells is under investigation, but still remains unclear. UV irradiation has a key role in the formation of reactive oxygen species (ROS) (23), which can induce Cox-2 formation via the activation of the NF-kB signaling pathway (4). Both ROS and Cox-2 were found to play the most important role in UV-related cutaneous carcinogenesis (14, 15, 21). Cyclooxygenases (Coxs, also known as prostaglandin endoperoxide synthases) are key enzymes that convert arachidonic acid to prostaglandin H2, a precursor to all of the other prostanoids (12). There are two forms of human Coxs: Cox-1 and Cox-2. It was reported that Cox-2 over-expression may contribute to carcinogenesis via its regulation on apoptosis, immunosurveillance, angiogenesis (17, 29). Moreover, the findings regarding the effects of Cox-2 in cutaneous tumor formation have also been reported in pterygium, including disruption of apoptosis, limbal epithelial proliferation, abnormal *p53* gene expression, and upregulation of basic fibroblast

growth factor, vascular endothelial growth factor, and nitric oxide synthase (13, 24, 35, 37).

In several animal and clinical studies, Cox-2 specific inhibitors have both preventive and therapeutic effects for breast, bladder, lung and pterygium (11, 16, 25, 27, 33). However, the association of *Cox-2* genotypes with pterygium formation has never been investigated. In addition, the mRNA and protein levels of Cox-2 may vary among individuals, and this variability may be partially genetically determined under different molecular mechanisms, which depends on single nucleotide polymorphisms (SNPs) of *Cox-2* (9, 30).

Although Cox-2 over-expression and Cox-2 inhibitor drugs have been studied in cancer, there were very few studies reporting the effects of Cox-2 inhibition in pterygium. Recently, mounting evidences reported that Cox-2 over-expression was frequently found in patients with pterygium (1, 5, 22, 26, 38). The present work is motivated by the biological possibility that genetic variation in the *Cox-2* could alter enzyme expression levels or biochemical function and consequently may have an impact on modifying the individual risk of pterygium. To investigate the hypothesis that the SNP variants of *Cox-2* are associated with the risk of pterygium, the genetic polymorphisms of six *Cox-2* SNPs, including G-1195A (rs689466), G-765C (rs20417), T+8473C (rs5275), intron 1 (rs2745557), intron 5 (rs16825748), and intron 6

(rs2066826), were analyzed in a large Taiwanese pterygium population (control/case=400/113).

Materials and Methods

Study Population and Sample Collection

A total of 113 pterygium patients were enrolled in the study at the Department of Ophthalmology, Chang-Hwa hospital, and Dr. Hsu's Eye Clinic from 2005 to 2010. Patients included in the study had a primary pterygium and an apex of pterygium of at least 1 mm invading the cornea. Non-cancerous and non-pterygium healthy persons were matched by gender and age after initial random sampling from the Health Examination Cohort of the two hospitals. Each participant received an eye examination by ophthalmologists and was given a questionnaire interview at the first time and once per year during the following period during 2005-2010. The eye examination included measurement of the best corrected visual acuity and inspection of the anterior segment. After the eye examination, a well-trained technician would conducted all the personal interviews using a structured questionnaire to collect all the data about demographic lifestyle factors including cigarette smoking (one pack per day for more than five years), alcohol drinking (twice per week for more than five years), eyedrop consumption (half bottle per day for more than one year), occupation and

sunlight exposures (more than six hours per day for more than one year).

Genotyping Assays

Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and further processed according to previous studies (2, 3, 6-8, 19, 20, 39). The polymerase chain reaction (PCR) cycling conditions were: one cycle at 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 30 sec, and 72^{o}C for 30 sec, and a final extension at 72^{o}C for 10 min. Pairs of PCR primer sequences and restriction enzyme for each DNA product are all listed in Table I.

Statistical Analysis

Only those subjects with both genotypic and clinical data (control/case=400/113) 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 deemed to be significant

when the statistical *p*-value was less than 0.05.

Results

The frequency distributions of selected characteristics of 113 pterygium patients and 400 controls are shown in Table II. The characteristics of patients and controls were all well matched. None of the differences between the groups were statistically significant ($p>0.05$) (Table II). The smoking, alcohol drinking, eyedrop using, and longer working at sun exposure (> 6 hours per day for more than one year) were not associated with risks of pterygium.

The frequencies of the genotypes for the *Cox-2* SNPs in controls and pterygium patients are shown in Table III. 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 III). The frequencies of the alleles for *Cox-2* SNPs in controls and pterygium patients are shown in Table IV. Neither of the allele of the *Cox-2* of the SNPs were found to be associated with pterygium ($p>0.05$).

Discussion

Pterygium is a common, benign, fibrovascular, and infiltrative process of the corneo-conjunctival junction of unknown pathogenesis, and Cox-2 mediates the rate-limiting step in arachidonic acid metabolism. Extensive evidence indicates that the Cox-2 prostanoid pathway is involved in inflammation. In order to know the role of Cox-2 and to find potential biomarkers of pterygium, six SNPs of the *Cox-2* gene were selected from the NCBI website and their associations with the susceptibility for pterygium were investigated in a population in central Taiwan. Studies indicated that, Cox-2 protein overexpression in pterygium specimens while normal conjunctiva and limbus specimens were not (5, 22). However, in this present study, we found that for each single SNP, the variant genotypes of *Cox-2* were not significantly associated with the susceptibility for pterygium (Tables III and IV). This might not be due to small sample size (it is relatively large in pterygium studies worldwide), but more likely *Cox-2* may play a minor role in the aetiology of pterygium in transcription level while in translation level.

Pterygium is now considered to be a result of uncontrolled cellular proliferation, similar to that in tumors, in which there is damage to cellular regulation and control of the cell cycle (31). Mitogenity, construction of a new vascular net and remodeling of the extracellular matrix were observed in pterygia. Altogether they create a new

vascular and fibrotic tissue, which has an aggressive way of growing to and over the cornea (34). In cutaneous tumorigenesis, the mechanism involving Cox-2 induced by UV irradiation was proposed to be via the generation of ROS, so the pathway of UV-ROS-Cox-2 in cutaneous tumor was proposed (21). If this relationship indeed exists in pterygium formation, Cox-2 inhibitor may be become a new target for the therapy of pterygium. In this study, the genetic level evidence showed no difference between the control and pterygium groups (Table III and IV), and the two groups have no difference in their ages, a commonly recognized a risk factor for pterygium formation (Table II). Also, our data showing that sunlight exposure may not be closely related to the ethiology of pterygium in Taiwan (Table II), which is not consistent with those findings that pterygium is more prevalent in tropical and subtropical areas, where the residents have high levels of sunlight exposure (28). In previous studies on general populations, the prevalence of pterygium was 17% in residents on a tropical island in Indonesia (36) and as high as 31.06% in Peru (32). Because sunlight is a major risk factor, differences in sunlight exposure may lead to very different risks in people living in the same area. This may be that in modern cities such as Taichung in Central Taiwan, many people exposed under sunlight wore some eye-protecting hats and anti-UV glasses, while people in the areas of Indonesia and Peru did not. The association of exposure to sunlight needs further confirmation

among the ethic cultures, geographic area, and detail lifestyle habits.

In conclusion, this is the first study which focuses on the SNPs of *Cox-2* and their effects on pterygium. Since none of the six polymorphisms of *Cox-2* investigated were found significant associated with pterygium, the Cox-2 may behave its regulatory effects on pterygium formation at the post-transcriptional and/or post-translational levels. Future studies of the causal relationship between Cox-2 and pterygium, the role of UV-ROS-Cox-2 pathway in pterygium, and the effect of Cox-2 inhibitor in preventing primary or recurrent pterygium are needed.

Acknowledgements

This study was supported by the research grant from Chang-Hua Hospital (CHH-99-09).

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Table I. The primer sequences, PCR-RFLP conditions for *Cox-2* gene polymorphisms.

Polymorphism (location)	**Primers sequences (5’ to 3’)**	**Restriction enzyme**	**SNP sequence**	**DNA fragment size (bp)**
G-1195A (rs689466)	F: CCCTGAGCACTACCCATGAT	*Hha I*	A	273
	R: GCCCTTCATAGGAGATACTGG		G	220 + 53
G-765C (rs20417)	F: TATTATGAGGAGAATTTACCTTTCGC	*Pvu II*	C	100
	R: GCTAAGTTGCTTTCAACAGAAGAAT		G	74 + 26
T+8473C (rs5275)	F: GTTTGAAATTTTAAAGTACTTTTGAT	*Bcl I*	T	147
	R: TTTCAAATTATTGTTTCATTGC		C	124 + 23
intron 1 (rs2745557)	F: GAGGTGAGAGTGTCTCAGAT	*Taq I*	G	439
	R: CTCTCGGTTAGCGACCAATT		A	353 + 76
intron 5 (rs16825748)	F: GCGGCATAATCATGGTACAA	*BsrG I*	T	417
	R: CAGCACTTCACGCATCAGTT		A	314 + 103
intron 6 (rs2066826)	F: ACTCTGGCTAGACAGCGTAA	*Aci I*	A	327
	R: GCCAGATTGTGGCATACATC		G	233 + 94

*F and R indicate forward and reverse primers, respectively.

Table II. Characteristics of 113 pterygium patients and 113 controls.

Characteristic	*Controls (n = 400)*			*Patients (n = 113)*			p-*value*[a]
	n	*%*	*Mean (SD)*	*n*	*%*	*Mean (SD)*	
Age (years)			*45 (4.8)*			*46.1 (4.4)*	*0.54*
Gender							
Male	*222*	*55.5%*		*63*	*55.8%*		*0.96*
Female	*178*	*44.5%*		*50*	*44.2%*		
Cigarette Smoking							
Yes	*171*	*42.8%*		*59*	*52.2%*		*0.09*
No	*229*	*57.2%*		*54*	*47.8%*		
Alcohol Consumption							
Yes	*145*	*36.3%*		*48*	*42.5%*		*0.58*
No	*255*	*63.7%*		*65*	*57.5%*		
Eyedrop Using							
Yes	*28*	*7.0%*		*5*	*4.4%*		*0.39*
No	*372*	*93.0%*		*108*	*95.6%*		
Under Sunlight Working							
Yes	*117*	*29.3%*		*35*	*31.0%*		*0.73*
No	*283*	*70.7%*		*78*	*69.0%*		

[a] Based on chi-square test.

Table III. Distribution of *Cox-2* genotypes among the pterygium patient and control groups.

Genotype	Controls	%	Patients	%	*p*-value[a]
G-1195A (rs689466)					0.9446
AA	116	29.0%	31	27.4%	
AG	192	48.0%	55	48.7%	
GG	92	23.0%	27	23.9%	
G-765C (rs20417)					0.7691
GG	340	85.0%	95	84.1%	
GC	60	15.0%	18	15.9%	
CC	0	0%	0	0%	
T+8473C (rs5275)					0.6571
TT	255	64.0%	75	66.4%	
TC	145	36.0%	38	33.6%	
CC	0	0%	0	0%	
intron 1 (rs2745557)					0.8032
GG	292	73.0%	86	76.1%	
AG	100	25.0%	25	22.1%	
AA	8	2.0%	2	1.8%	
intron 5 (rs16825748)					0.7776
TT	384	96.0%	110	97.3%	
AT	16	4.0%	3	2.7%	
AA	0	0%	0	0%	
intron 6 (rs2066826)					0.6385
GG	324	81.0%	95	84.1%	
AG	60	15.0%	13	11.5%	
AA	16	4.0%	5	4.4%	

[a] Based on chi-square test.

Table IV. *Cox-2* allelic frequencies among the pterygium patient and control groups.

Allele	Controls	%	Patients	%	*p*-value[a]
G-1195A (rs689466)					0.7436
Allele A	424	53.0%	117	51.8%	
Allele G	376	47.0%	109	48.2%	
G-765C (rs20417)					0.7779
Allele G	740	92.5%	208	92.0%	
Allele C	60	7.5%	18	8.0%	
T+8473C (rs5275)					0.6946
Allele T	655	81.9%	188	83.2%	
Allele C	145	18.1%	38	16.8%	
intron 1 (rs2745557)					0.5250
Allele G	684	85.5%	197	87.2%	
Allele A	116	14.5%	29	12.8%	
intron 5 (rs16825748)					0.7796
Allele T	784	98.0%	223	98.7%	
Allele A	16	2.0%	3	1.3%	
intron 6 (rs2066826)					0.5777
Allele G	708	88.5%	203	89.8%	
Allele A	92	11.5%	23	10.2%	

[a] Based on chi-square test.