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Functional polymorphisms of the cyclooxygenase-2 gene and prognosis of hepatocellular carcinoma - a cohort study in Chinese people

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ABSTRACT. Cyclooxygenase-2 (COX-2) influences carcinogenesis through regulation of angiogenesis, apoptosis, cytokine expression, and immune response suppression. It has been well established that COX-2 is overexpressed in a variety of human cancers, such as hepatocellular carcinoma (HCC). In this study, we aimed to evaluate the association between COX-2 polymorphisms and prognosis of HCC. We genotyped 200 HCC patients of Chinese Han descent for COX-2 gene polymorphisms (-765G>C and -1195G>A) using PCR-RFLP. Data were statistically analyzed using the Kaplan-Meier method and the Cox's proportional hazard regression model. We found that patients with the COX-2 -1195AG and -1195AG + AA genotypes demonstrated significantly decreased disease-free survival (DFS) as compared with those carrying the -1195GG genotype ($P < 0.05$). However, the COX-2 -765G>C polymorphism was not associated with DFS ($P > 0.05$). Moreover, by Cox regression analysis, blood alpha fetoprotein ≤400

ng/mL before the operation and the -1195G>A polymorphism were found to be of prognostic significance ($P < 0.05$), while the -765G>C polymorphism was not ($P > 0.05$). In summary, post-operation progression of HCC is more likely to occur in patients with the -1195AG genotype and the A allele. On the other hand, the -765G>C polymorphism is not an independent influence factor of HCC prognosis.

Key words: Hepatocellular carcinoma; Disease-free survival; COX-2 -1195G>A (rs689466) polymorphism; COX-2 -765G>C (rs20417) polymorphism; Independent influence factor

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer, and the third most frequent cause of cancer-related deaths (Forner et al., 2012). Although early diagnosis and treatment can improve survival, HCC is still rarely cured due to frequent recurrences (Sun et al., 2015). Researchers have found that clinical and pathological diagnosis and classification of HCC are unreliable in predicting patient prognosis and response to therapy (Lee, 2015). HCC arises due to the accumulation of multiple-genetic alterations, and the molecular pathways involved in HCC development have not been fully understood.

As cell proliferation may play a key role in the development of HCC, cyclooxygenases (COXs) have caught scientists' attention for their potential role in HCC. COXs catalyze the conversion of free arachidonic acid into prostaglandin H2, the precursor of prostaglandins, prostacyclin, and thromboxanes (Kristinsson et al., 2009). COXs are also known as prostaglandin endoperoxide synthases or prostaglandin H synthases. Currently, there are 3 known COX isoenzymes: COX-1, COX-2, and COX-3 (Kristinsson et al., 2009; Akkız et al., 2011). COX-1 is expressed constitutively in most tissues, and is in charge of maintaining homeostasis of various physiologic processes; COX-3 is an alternatively spliced product of COX-1, and is believed to be involved in the regulation of pain and fever (Warner and Mitchell, 2002). Normally absent in most cells tissues, COX-2 is stimulated by certain extracellular and intracellular factors such as mitogens, growth factors, hormones, infectious agents, and proinflammatory cytokines (Kristinsson et al., 2009). Aside from cell proliferation, it has been shown that COX-2 participates in many biological and pathological processes such as apoptosis inhibition, inflammation, immune response suppression, tumor cell invasion, metastasis, and angiogenesis (Koki and Masferrer, 2002), all of which are crucial in the development and progression of cancer. Overexpression of COX-2 has been reported in a variety of human cancers, including HCC (Gasparini et al., 2003; Wu, 2006). In addition, tumors with elevated COX-2 expression are usually more aggressive, and patients bearing those aggressive tumors show significantly reduced survival (Buskens et al., 2002).

The COX-2 gene has been demonstrated to be polymorphic, and these polymorphisms may affect the expression or activity of the COX-2 enzyme, leading to variation in susceptibility to cancer through changes in arachidonic acid metabolism (Akkız et al., 2011). Several single nucleotide polymorphisms (SNPs) in COX-2 have been previously reported, but most of these polymorphisms seem to be insignificant, and are not associated with susceptibility to cancer (Humar et al., 2000). However, a few SNPs in COX-2 do exhibit clinical values. Among

them are -765G>C and -1195G>A, which are located in the promoter region (Fritsche et al., 2001). In some studies, the -765G allele has been shown to be associated with heightened COX-2 transcription. This polymorphism creates a transcriptional factor c-MYB binding site, which leads to a significantly higher promoter activity as compared with the -765C allele (Papafili et al., 2002; Zhao et al., 2009). A study by Zhang et al. (2005) has shown significantly increased level of COX-2 mRNA in esophageal tissues containing the -1195A allele, when compared with their -1195G-containing counterparts. Association between these two COX-2 gene polymorphisms and HCC risk has also been investigated by a number of studies (Wu, 2006; Akkız et al., 2011; Nahon and Zucman-Rossi, 2012). Nevertheless, few studies have examined the relationship between SNPs in the promoter region of COX-2 and prognosis of HCC patients. The purpose of this study was to evaluate the association of these two important COX-2 polymorphisms, namely -765G>C and -1195G>A, and HCC prognosis.

MATERIAL AND METHODS

Study subjects and data collection

A total of 200 patients diagnosed with primary HCC between June 2008 and August 2011 were recruited from the First Affiliated Hospital of Guangxi Medical University and Cancer Hospital Affiliated to Guangxi Medical University. All subjects were unrelated Han Chinese treated by the R0 excision procedure (no tumor tissue residues were observed under microscope after excision), and were histologically confirmed as HCC patients after operation. Patients with previous cancers, chemotherapy, and radiotherapy were excluded from the study.

At the time of inclusion, detailed information of all the patients, such as age, gender, smoking and drinking habits, family history of cancer, were recorded; these are listed in Table 1. Heavy alcohol intake was defined as weekly minimum consumption of 50 mL pure alcohol for at least 1 year, while smoking history was defined as daily minimum consumption of 10 cigarettes for at least 1 year. In addition, approximately 2 mL peripheral blood from each patient was collected into a test tube containing the anticoagulant acid citrate dextrose. Whole blood samples were stored at -80°C until analysis. Written informed consent to the study was given by all participants, and study protocols have been reviewed and approved by the Institutional Ethics Committee at both participating hospitals.

DNA extraction and polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis

DNA was extracted from whole blood samples using the Qiagen Tiangen DNA Isolation Kit (Tiangen Biochemical Technology Ltd., Beijing, China) according to manufacturer's instructions. The extracted DNA was stored at 4°C until use.

PCR-based RFLP was performed to determine COX-2 gene polymorphisms at -765G>C and -1195G>A, as previously described (Li et al., 2009). The primers, length of amplified fragments, restriction pattern, and restriction enzymes used in this study are listed in Table 2. The 20-μL PCR mixture contained approximately 50 ng DNA, 12.5 pmol of each primer, 0.1 mM of each dNTP, 1X PCR buffer, and 1 U Taq polymerase. The concentration of $MgCl_2$ was 1.0 mM for both -765G>C and -1195G>A analysis. The following PCR cycling conditions were used: an initial melting step at 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C

(for -1195G/A) or 54°C (for -765G/C) for 30 s, and 72°C for 45 s; and a final elongation at 72°C for 10 min. The PCR products were ran on a 2% agarose gel, and the restriction enzymes *Pvu*II and *Aci*I (Fermentas Inc., Canada) were used to distinguish the -765G/C and -1195G/A genotypes, respectively. To ensure quality control, 15% of the samples were genotyped twice by different people. Furthermore, 10 samples were randomly selected for confirmative DNA sequencing.

Table 1. Characteristics of the patients enrolled.

Characteristic	Patients [N (%)]
Age (years)	
≤50	112 (56.00)
>50	88 (44.0)
Gender	
Male	175 (87.50)
Female	25 (12.50)
Family history of cancer	
Yes	14 (7.00)
No	186 (93.00)
Smoking status	
Smoker	83 (41.50)
Non-smoker	117 (58.50)
Drinking status	
Drinker	72 (36.00)
Non-drinker	128 (64.00)
Blood serum HBsAg	
(+)	168 (84.00)
(-)	32 (16.00)
Child-Pugh classification	
A	196 (98.00)
B	4 (2.00)
C	0 (0)
AFP before surgery (ng/ml)	
≤400	127 (63.50)
>400	73 (36.50)
Histological grade	
Highly and moderately differentiated	117 (58.50)
Poorly differentiated	83 (41.50)
Tumor size (cm)	
≤5	79 (39.50)
>5	121 (60.50)
Number of tumors	
Single	159 (79.50)
Multiple	41 (20.50)
Portal vein tumor thrombus	
Yes	30 (15.00)
No	170 (85.00)
Clinical stage	
I	139 (69.50)
II	19 (9.50)
III	42 (21.00)
IV	0

Statistical analysis

Qualitative variables are reported as frequencies and percentages. Data analysis was performed using the Statistical Package for Social Sciences (SPSS) software for Windows (version 20.0). Deviations from Hardy-Weinberg equilibrium were analyzed using the χ^2 test. The disease-free survival (DFS) was calculated from the time of the first date of surgical

treatment to the first observation of disease progression. If the disease did not progress, DFS was censored at the time of the last follow-up. Survival curves for DFS were estimated by the Kaplan-Meier method and compared using the log-rank test. Univariate Cox's regression analysis was used to assess the association between each potential prognostic factor and DFS. Multivariate Cox's regression analysis was applied to evaluate the effect of different variables on DFS with adjustments for age, gender, smoking or drinking status, family history of cancer, HBV infection, Child-Pugh classification, clinical stage, blood serum alpha fetoprotein (AFP) level before surgery, histological grade, portal vein tumor thrombus, tumor size and number. The values of relative risk [hazard ratio (HR)] and their 95%CI were calculated from the Cox model, starting from the time of the first date of surgical treatment to the first observation of disease progression (event). For all tests, a two-sided P value of less than 0.05 was considered statistically significant.

Table 2. Primer sequences, amplicon size, restriction enzyme, and restriction pattern for COX-2 polymorphisms.

Polymorphism	Primer sequences	Length (bp)	Restriction enzyme	Restriction pattern (bp)
-1195G>A	F: 5′-tatctcaccctcacatctc-3′ R: 5′-tggttactagcccttcatag-3′	304	*Pul*II	AA 304 GG 270, 34 AG 304, 270, 34
-765G>C	F: 5′-ccgcttcctttgtccatcag-3′ R: 5′-ggctgtatatctgctctatatgc-3′	309	*Aci*I	CC 309 GG 189, 120 GC 309, 189, 120

RESULTS

Characteristics of study population

Demographic, clinical, and tumor-related characteristics of patients in the study are summarized in Table 1. There were 175 males (87.5%) and 25 females (12.5%, male/female ratio 7/1) with ages ranging from 27 to 78 years (median age = 48 years old).

Identification of SNPs

After confirmation of successful PCR amplification gel electrophoresis, restriction enzymes *Pvu*II and *Aci*I were used to distinguish between the -765G/C and -1195G/A genotypes, respectively. Results of these assays are illustrated in Figure 1A and B. By sequencing the full-length promoter region of COX-2 of 10 randomly selected subjects, SNPs of -765G/C and -1195G/A were identified, which included -1195AA, -1195GG, -1195GA, -765GG, and -765GC, as shown in Figure 2. However, among the 200 subjects enrolled in this study, the genotype -765CC was not observed. This may be due to the small proportion (about 1%) of this gene type in the Chinese Han population, as previously reported (Li et al., 2009).

-765G>C and -1195G>A polymorphisms and DFS

The genotype distributions of -765G>C and -1195G>A COX-2 gene polymorphisms as well as their association with DFS are summarized in Table 3. Each of the COX-2 polymorphisms in patients was in Hardy-Weinberg equilibrium. Median DFS of all 200 patients was 34 months (95%CI = 21.2-46.8 months). The -1195G>A polymorphism showed a significant association with DFS. Patients with the genotype -1195AG and -1195AA had median DFS of 26 and 47 months, respectively. The overall median DFS of patients with

these two genotypes (-1195AG + AA, altogether 154 patients) was 27 months. On the other hand, the median DFS of patients carrying -1195GG was never reached through the whole 48-month follow-up period. Median DFS in -1195AG carriers was significantly shorter as compared with the -1195GG carriers (P = 0.005, log-rank test). Overall median DFS of patients with -1195AG + AA was also significantly shorter as compared with that of patients carrying the -1195GG gene type (P = 0.008, log-rank test). Survival curves for -1195G>A-associated DFS are shown in Figure 3A.

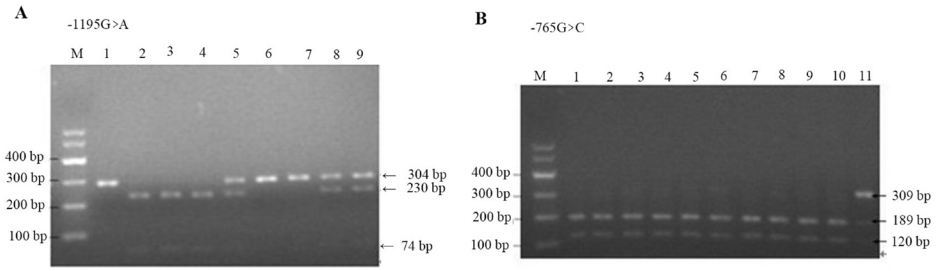

Figure 1. Gel pictures of COX-2 genotypes. **A.** COX-2 -1195G>A polymorphism. *Lane M* = DNA ladder; *lanes 1*, *6*, *7* = AA homozygous (304 bp); *lanes 2*, *3*, *4* = GG homozygous (270 and 34 bp); *lanes 5*, *8*, *9* = GA heterozygous (304, 270, and 34 bp). **B.** COX-2 -765G>C polymorphism. *Lane M* = DNA ladder; *lanes 1* to *10* = GG homozygous (189 and 120 bp); *lane 11* = GC heterozygous (309, 189, and 120 bp).

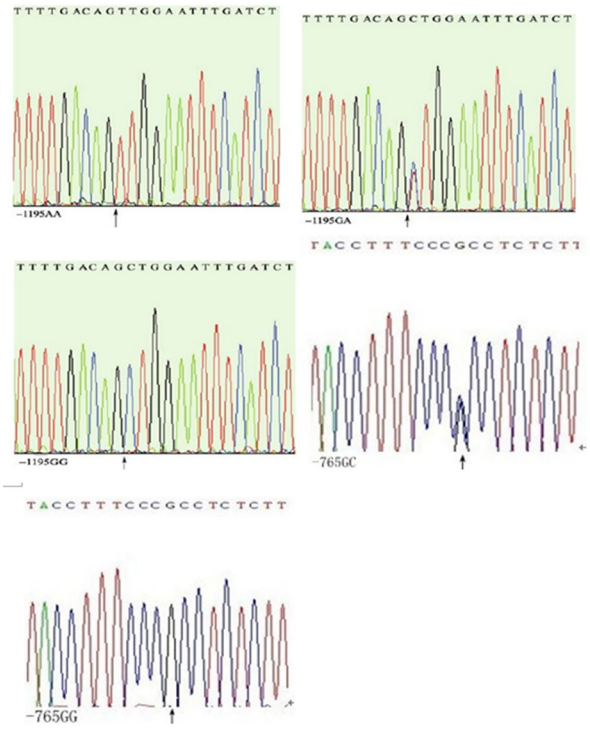

Figure 2. Sequence analysis of the COX-2 promoter region revealing two SNPs. Base changes at the nucleotide positions are indicated by arrows.

Table 3. Associations between COX-2 genotypes and disease-free survival.

Genotype	Gene frequency (N, %)	Median DFS (m)	P
-1195G>A			
GG	46 (22.50)	-	1.000
AG	109 (54.50)	26	0.005
AA	45 (23.0)	43	0.083
AG+AA	154 (77.5)	27	0.008
-765G>C			
GG	183 (91.50)	32	1.000
GC	17 (8.50)	43	0.518
CC	-	-	-
GC+CC	17 (8.50)	43	0.518

P value calculated by log-rank method.

On the other hand, the -765G>C polymorphism showed no significant association with DFS. Patients with the genotypes -765GG and -765GC had median DFS of 32 and 43 months, respectively. The overall median DFS of patients with -765GC and -765CC (-765GC + CC, altogether 17 patients, equaling to -765GC carriers alone as -765CC was never observed in this study) was also 43 months. Median DFS in -765GG and -765GC carriers were significantly different (P = 0.518, log-rank test). Survival curves for DFS grouped by the -765G>C polymorphism are shown in Figure 3B.

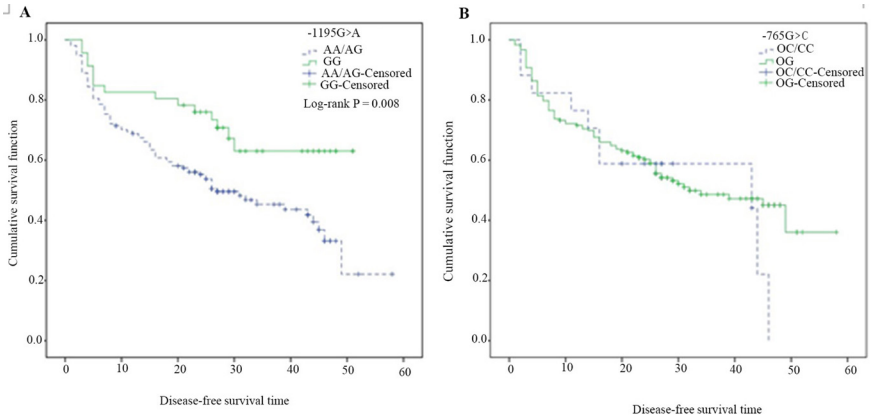

Figure 3. Kaplan-Meier curves of estimated disease-free survival calculated by log-rank test for the entire cohort. **A.** P = 0.008 for patients with -1195G>A polymorphism; **B.** P = 0.518 for patients with the -765G>C polymorphism.

Univariate and multivariate Cox's regression analysis of -765G>C and -1195G>A polymorphisms

Based on our previous knowledge of multiple factors that could affect patient survival, univariate and multivariate Cox regression analysis were employed to identify important factors associated with DFS in patients (Table 4).

In the multivariate Cox proportional hazard model, after adjustment for clinicopathological factors (age, gender, smoking or drinking status, family history of cancer, HBV infection, Child-Pugh classification, blood serum AFP level before surgery, histological grade, clinical stage, portal vein tumor thrombus, and tumor size/number), blood AFP level

and -1195G>A polymorphism were found to be of prognostic significance (P = 0.006 for AFP level, and P = 0.034 for the -1195G>A polymorphism). However, the prognostic value of the -765G>C polymorphism was not statistically significant (P = 0.796).

The HR of patients with AFP ≤ 400 ng/mL (with AFP > 400 ng/mL as reference) was 0.495 (95%CI = 0.298-0.820). The HR of patients with -1195GA + GG (with -1195GG as reference) was 2.214 (95%CI = 1.062-4.614), while the HR of patients with -765GC+CC (not statistically significant, with -765GG as reference) was 1.122 (95%CI = 0.468-2.690).

These results suggested that AFP ≤ 400 ng/mL prior to surgery was an independent influence factor of HCC prognosis, and is indicative of lower risk of disease progression and better clinical outcomes. On the other hand, -1195GA and -1195AA genotypes were an indicative of higher risk of disease progression and poor clinical outcomes when compared with those carrying the -1195GG genotype. Lastly, the -765G>C polymorphism was not found to be an independent influence factor of HCC prognosis in our study.

Table 4. Univariate and multivariate Cox regression analyses for DFS.

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	P	HR (95%CI)	P
Age (≤50 *vs* >50 years)	1.274 (0.848-1.914)	0.234	0.963 (0.525-1.766)	0.902
Gender (Male *vs* Female)	1.195 (0.638-2.238)	0.579	2.059 (0.657-6.448)	0.215
Smoking status (No *vs* Yes)	0.679 (0.458-1.009)	0.055	0.684 (0.372-1.260)	0.223
Drinking status (No *vs* Yes)	0.744 (0.497-1.112)	0.149	1.082 (0.558-1.989)	0.800
HBsAg (- *vs* +)	0.969 (0.559-1.678)	0.909	1.363 (0.650-2.857)	0.412
Family history of cancer (No *vs* Yes)	1.219 (0.564-2.636)	0.615	1.270 (0.432-3.734)	0.664
Child-Pugh classification (A *vs* B)	2.438 (0.340-17.507)	0.376	NC	NC
Histological grade (highly moderately *vs* poorly differentiated)	0.638 (0.413-1.129)	0.137	0.699 (0.408-1.198)	0.192
AFP before surgery (ng/mL) (≤400 *vs* >400 ng/mL)	0.612 (0.410-0.913)	0.016	0.495 (0.298-0.820)	0.006
Tumor size (≤5 *vs* >5 cm)	0.661 (0.436-1.003)	0.052	0.863 (0.472-1.479)	0.583
Portal vein tumor thrombus (No *vs* Yes)	0.612 (0.367-1.023)	0.061	0.603 (0.265-1.372)	0.228
Number of tumors (Single *vs* Multiple)	0.701 (0.442-1.112)	0.131	0.810 (0.428-1.553)	0.517
COX-2 -765G>C	1.237 (0.643-2.380)	0.524	1.122 (0.468-2.690)	0.796
(GC *vs* GG)	-	-	NC	NC
(CC *vs* GG)			NC	NC
COX-2 -1195G>A	1.800 (0.933-3.472)	-	2.214 (1.062-4.614)	0.034
(AA *vs* GG)	2.161 (1.228-3.802)	0.080	NC	NC
(AG *vs* GG)		0.008	NC	NC

NC = not calculated.

DISCUSSION

HCC is one of the most prevalent malignancies in the world, and is the third most common cause of cancer-related deaths (Forner et al., 2012). Although new means of diagnosis and treatments have improved the survival of HCC, it is still rarely cured (Sun et al., 2015). Identification of biologic markers that are capable of predicting the progression of HCC and disease prognosis after surgical treatments may facilitate our understanding and management of this disease.

Carcinogenesis of HCC is a complex process associated with various risk factors. Some of the confirmed risk factors include exposure of aflatoxin B, chronic infection with hepatitis B virus or hepatitis C virus, excessive consumption of alcohol and tobacco, iron overload, and diabetes (Forner et al., 2012). In the last few years, numerous studies have pointed out that SNPs of certain cancer-related genes may be associated with the initiation and progression of HCC, either independently or in cooperation with identified risk factors (Jin et al., 2011; Nahon

et al., 2012). The -765G>C (rs20417) and -1195G>A (rs689466) polymorphisms in the COX-2 gene are among the most extensively studied SNPs. As an inflammation-associated enzyme, COX-2 plays important roles in cell apoptosis, proliferation, differentiation, and angiogenesis, and is thus involved in the pathogenesis of solid tumors (Coskunpinar et al., 2011; Gangwar et al., 2011; Talar-Wojnarowska et al., 2011). Recently, a number of studies have focused on the influence of COX-2 SNPs on risk of various cancers including HCC, but very few have investigated their role in the progression of these diseases (Festa-Vasconcellos et al., 2012).

In this study, we aimed to investigate the role of COX-2 -765G>C and -1195G>A polymorphisms on HCC progression. We also addressed the prognostic value of these two COX-2 polymorphisms in HCC. To the best of our knowledge, this is the first study demonstrating the association between -765G>C and -1195G>A polymorphisms in the COX-2 gene and post-operative DFS in Chinese Han patients with HCC. In the present study, we found that the -1195G>A polymorphism was significantly associated with DFS. Median DFS of patients with the -1195AG genotype and the overall median DFS of patients with the -1195GA + GG genotypes were both significantly shorter as compared with that of -1195GG carriers. However, no such difference was observed between the genotypes -765GG and -765GC. We then performed univariate and multivariate Cox's regression analysis, and found that the prognostic significance of blood serum AFP level and the -1195G>A polymorphism were present following adjustments for clinicopathological factors ($P = 0.006$ for AFP level, and $P = 0.034$ for -1195G>A polymorphism). However, the prognostic value of the -765G>C polymorphism was not statistically significant ($P = 0.796$). Our results suggested that patients with AFP ≤ 400 ng/mL and carrying the -1195GG genotype may experience lower risk of disease progression and better outcome after standard surgical treatment.

So far, published data regarding the clinical significance of COX-2 -765G>C and -1195G>A polymorphisms have been controversial (Talar-Wojnarowska et al., 2011) The -765C allele has been reported to be associated with either increased or decreased risk for various cancers by different studies (Zhao et al., 2009; Coskunpinar et al., 2011; Gangwar et al., 2011). The -1195G>A polymorphism was also considered to have opposite effects on tumorigenesis in different types of cancers, as reported by different research groups (Kristinsson et al., 2009; Pereira et al., 2010). In addition to the limited number of studies investigating the influence of these two COX-2 polymorphisms on cancer prognosis, the results of these few studies available are controversial. In a study conducted by Bi et al. (2010), the COX-2 -1195G/A polymorphism has been shown to be a potential predictive marker for survival in patients with advanced non-small cell lung cancer treated with chemoradiotherapy or radiotherapy alone. Another study by Lurje et al. (2008) also indicated that polymorphisms in COX-2 (-765G>C and others) may be a useful molecular marker for predicting clinical outcomes in patients with metastatic colorectal cancer treated with cetuximab. However, a study in 2012 claimed that in the Caucasian population, COX gene polymorphisms were not associated with prognosis of gastric adenocarcinoma (García-González et al., 2012). In our study, only the -1195G>A polymorphism was found to be a potential predictive marker for post-operative DFS in HCC patients.

As with all clinical outcome studies, our study and other studies we discussed previously are limited by sample size and the enrolled population. A relatively small sample size would limit the power to detect small differences in HR values, especially in these low-frequency homozygous variant polymorphisms. We set the α value to be 0.05 in this study, which resulted in 80% power to detect HRs >1.4. It is possible that we missed minor statistical

differences, which could be detected by further research with larger sample sizes. Additionally, results may vary among different populations, leading to controversial results. All patients included in our study (and in many other studies too) received standard surgical or medical treatment. Therefore, assessing genotype combinations associated with clinical outcome in an untreated control group is ethically not possible (Lurje et al., 2008). Nevertheless, this study still provided information and evidence for future research regarding the association between COX gene polymorphisms and prognosis of cancer patients.

Conflicts of interest

The authors declare no conflict of interest.

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