Functional polymorphisms in the *NPAS2* gene are associated with overall survival in transcatheter arterial chemoembolization-treated hepatocellular carcinoma patients

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The functional abnormality of circadian regulation genes is involved in the development and progression of hepatocellular carcinoma (HCC). However, the association between functional single nucleotide polymorphisms (SNPs) in circadian gene *NPAS2* and the overall survival of HCC patients treated with transcatheter arterial chemoembolization (TACE) has never been investigated. Six functional SNPs in the *NPAS2* gene were genotyped using the Sequenom iPLEX genotyping system in a cohort of 448 unresectable Chinese patients with HCC treated with TACE. Multivariate Cox proportional hazards model and Kaplan–Meier curves were used for the prognosis analysis. We found that two SNPs, rs1053096 and rs2305160, in the *NPAS2* gene showed significant associations with overall death risk in HCC patients in the recessive model (hazard ratio [HR] = 1.48; 95% confidence interval [CI], 1.13–1.94; $P = 0.004$) and in the dominant model (HR = 1.63; 95% CI, 1.29–2.07; $P < 0.001$), respectively. Moreover, we observed a cumulative effect of these two SNPs on HCC overall survival, indicating a significant trend of increasing death risk with increasing number of unfavorable genotypes (P for trend < 0.001). Compared with the patients without any unfavorable genotypes, the HRs for patients with one and two unfavorable genotypes were 1.41 (95% CI, 1.10–1.82; $P = 0.007$) and 2.09 (95% CI, 1.46–2.97, $P < 0.001$), respectively. The haplotype and diplotype analyses further characterized the association between NPAS2 genotype and survival of HCC patients. Our results for the first time suggest that *NPAS2* gene polymorphisms may serve as an independent prognostic marker for HCC patients treated with TACE.

H epatocellular carcinoma is the sixth most common cancer and the third leading cause of cancer-related deaths worldwide. Almost half of these cases and deaths were estimated to occur in China.[1] According to the data from the National Central Cancer Registry of China, the crude incidence of HCC was 28.71/100 000, and the crude mortality was 26.04/100 000 in 2009.[2] Unfortunately, only a few patients with HCC are diagnosed at an early stage, when the disease is amenable to treatment approaches such as surgical resection, liver transplantation, or local ablation. Most HCC patients, who are diagnosed at advanced stages, had to receive palliative or conservative therapy only.[3] Transcatheter arterial chemoembolization is the current standard of care for patients with intermediate or advanced HCC.[4] It has been shown that TACE significantly improves the OS of unresectable HCC patients.[5] But there is still a lack of effective and specific biomarkers for prediction of TACE treatment responses.

Circadian rhythm is the 24-h cycle in the behavioral, physiological, and biochemical processes.[6] The molecular mechanisms of circadian oscillation are based on a positive /negative transcriptional–translational feedback loop generated by at least nine core circadian genes.[7] Among them, *NPAS2*,

which is located on chromosome 2 at 2q11.2, is one of the most important circadian genes. The *NPAS2* gene encodes a member in the basic helix–loop–helix–PAS class of transcription factors. NPAS2 forms heterodimers with BMAL1 and then activates the expressions of the circadian genes *PER* and *CRY*, which are required for maintaining biological rhythms in many organisms.[8] An animal study showed that the loss of normal function of the *NPAS2* gene may cause defects in several aspects of the circadian system, such as patterns of sleep and behavior.[9] Moreover, evidence has suggested that NPAS2 may play an important role in tumorigenesis by affecting expression of cancer-related genes and could be considered as a novel tumor suppressor.[10] In addition, genetic association studies have shown that a missense SNP (rs2305160) in *NPAS2* gene is associated with the risk of breast cancer,[11] prostate cancer,[12] and non-Hodgkin's lymphoma.[13] More interestingly, potential involvement of circadian genes in cancer progression has recently been reported, indicating that high levels of NPAS2 expression are significantly associated with tumor grade, OS, and disease-free survival in breast cancer patients.[14] Our group has tested six functional SNPs in the *NPAS2* gene in a cohort of 411 resected Chinese colorectal cancer patients, but we did not observe any association between these SNPs and clinical outcomes.[15]

Recently, there has been growing interest in the impact of circadian genes on HCC. For example, one study reported that circadian disruption accelerates hepatocellular carcinogenesis in mice.[16] Another study found that the downregulation of *PER*, *CRY2*, and *TIM* genes causes the disturbance of circadian rhythm in HCC patients, which may benefit the selective survival of cancerous cells and promote carcinogenesis.[17] Of more interest, the overexpression of the *BMAL1* gene and low expression of the *PER1* gene were correlated with liver metastasis in colorectal cancer.[18] Recently, our group reported that a functional polymorphism (rs2640908) in the *PER3* gene is associated with prognosis in HCC treated with TACE.[19] All these data strongly indicated that the functional abnormality of circadian regulation genes is involved in the development and progression of HCC. It has been reported that NPAS2 significantly affects the cell cycle and DNA damage response, which plays an important role in the response of cancer cells to chemotherapeutic reagents.[10] Moreover, the PAS domain of NPAS2 protein mediates the responses of cells to environmental stress, such as ischemia.[20] These data suggest that NPAS2 may play a role in the response of HCC cells to TACE treatment, an important therapeutic regimen for unresectable HCC through both cytotoxicity and ischemic embolism. However, the association between functional SNPs of *NPAS2* and OS of HCC patients treated with TACE has not been reported.

To evaluate whether functional SNPs of *NPAS2* could be a potential biomarker to predict HCC prognosis, we assessed the effects of six functional SNPs in the *NPAS2* gene on survival in a cohort of 448 unresectable Chinese HCC patients. To the best of our knowledge, this is the first study to investigate the predictive role of SNPs in the *NPAS2* gene for clinical prognosis of unresectable HCC patients treated with TACE.

Materials and Methods

Study group. A total of 578 Han Chinese patients who had unresectable HCC were recruited from an ongoing study at the Department of Pain Management, Tangdu Hospital affiliated to The Fourth Military Medical University in Xi'an and the Eastern Hepatobiliary Surgery Hospital affiliated to the Secondary Military Medical University in Shanghai, China. These patients were recruited between December 2006 and November 2011. As described previously,[19] HCC diagnosis was based on the National Comprehensive Cancer Network clinical practice guidelines in oncology. All cases had no previous history of other cancers or cancer-related treatments. There were no recruitment restrictions on age, gender, and TNM stage. After recruitment, all patients received TACE as the first-line treatment within a week after diagnosis. A combination of oxaliplatin, fluorouracil, and doxorubicin was used for the majority of unresectable HCC patients. In the present study, 130 cases were excluded, including 83 patients with incomplete clinical information or lost during follow-up, 35 patients with distant metastases or Child–Pugh score C, and 12 patients with poor DNA quality. Finally, a total of 448 patients were included in the primary cohort and successfully genotype-assayed. Written informed consent was obtained from each participant before enrolment. Our study was approved by the hospital ethics committees both in Shanghai and Xi'an.

Demographic and clinical data. Demographic variables, including age, sex, ethnicity, residential region, and family history of cancer, were collected by well-trained interviewers. Detailed clinical information was collected through medical chart review or consulting with treating physicians, including the date of diagnosis, history of hepatitis virus infection, tumor number, tumor size, lymph node invasion and/or distant metastases, PVTT, Child–Pugh score, serum AFP, treatment protocol, and response. Clinical staging was determined according to the TNM classification system. The follow-up information was updated at 3-month intervals through onsite interview, direct calling, or medical chart review by a trained clinical specialist. The latest follow-up data in this analysis were obtained in January 2013. For each patient, 5 mL venous blood was collected for genomic DNA extraction using the EZNA Blood DNA Midi Kit (Omega Bio-Tek, Norcross, GA, USA) in the laboratory.

Single nucleotide polymorphism selection and genotyping. Functional SNPs in the *NPAS2* gene were selected using a set of web-based SNP selection tools (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm) according to the previous description.[15] Finally, a total of six SNPs were selected, which include one non-synonymous SNP (rs2305160), one in transcription factor binding sites (rs3811558), two in splice sites (rs1562313 and rs9223), and two in miRNA-binding sites (rs2305158 and rs1053096). Genotyping was carried out using the Sequenom iPLEX genotyping system (Sequenom, San Diego, CA, USA). Laboratory persons who carried out the genotyping were blinded to patient information. The average call rate for the SNP array was 98.4%. Strict quality control measures were implemented during genotyping with over 99.0% concordance with the main genotyping results.

Immunohistochemistry. Tissue samples from another cohort of 243 surgical HCC patients were used for evaluation of NPAS2 expression by immunohistochemistry as previously described[21] with rabbit anti-human NPAS2 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) as primary antibody. The visualization signal was developed with the Histostain Plus kit (Life Technologies, Grand Island, NY, USA). The nuclear NPAS2 immunostaining score was evaluated for all

Table 1. Distribution of patient characteristics and prognosis analysis in Chinese patients with hepatocellular carcinoma treated with transcatheter arterial chemoembolization (TACE) (*n* = 448)

Variable	Total, *n* (%) (*n* = 448)	Deaths, *n* (%) (*n* = 339)	Adjusted HR† (95% CI)	*P*-value‡
Age, years				
≤53	222 (49.6)	168 (49.6)	Ref.	
>53	226 (50.4)	171 (50.4)	1.13 (0.89–1.42)	0.310
Sex				
Female	57 (12.7)	40 (11.8)	Ref.	
Male	391 (87.3)	299 (88.2)	1.27 (0.90–1.77)	0.170
HBV infection				
Negative	65 (14.5)	48 (14.2)	Ref.	
Positive	383 (85.5)	291 (85.8)	0.84 (0.61–1.17)	0.300
PVTT				
No	314 (70.1)	221 (65.2)	Ref.	
Yes	134 (29.9)	118 (34.8)	1.92 (1.49–2.46)	**<0.001**
Child–Pugh score				
A	431 (96.2)	324 (95.6)	Ref.	
B	17 (3.8)	15 (4.4)	1.64 (0.97–2.75)	0.060
TNM stage				
I + II	221 (49.3)	142 (41.9)	Ref.	
III + IV	227 (50.7)	197 (58.1)	1.56 (1.23–1.98)	**<0.001**
Serum AFP, μg/L				
<400	231 (51.6)	157 (46.3)	Ref.	
≥400	217 (48.4)	182 (53.7)	1.51 (1.20–1.89)	**<0.001**
Treatment				
TACE	366 (81.7)	299 (88.2)	Ref.	
TACE + other	82 (18.3)	40 (11.8)	0.42 (0.30–0.59)	**<0.001**

†Adjusted by age, sex, hepatitis B virus (HBV) infection, portal vein tumor thrombus (PVTT), tumor stage, serum α-fetoprotein (AFP), and treatment, where appropriate. ‡Significant *P*-values (<0.05) are in bold. CI, confidence interval; HR, hazard ratio; Ref., reference; TACE, transcatheter arterial chemoembolization.

Table 2. Association between single nucleotide polymorphisms (SNPs) in the *NPAS2* gene and clinical outcome in Chinese patients with hepatocellular carcinoma treated with transcatheter arterial chemoembolization (*n* = 448)

SNP	Genotype and best-fitting model	Death/total 339/448	Death HR† (95%CI)	*P*-value‡
rs9223	WW	200/270	Ref.	
	WV	122/156	1.03 (0.82–1.30)	0.790
	VV	17/21	1.03 (0.62–1.70)	0.910
	Dominant		1.03 (0.83–1.28)	0.780
rs1053096	WW	105/143	Ref.	
	WV	165/220	1.08 (0.85–1.39)	0.520
	VV	68/82	**1.55 (1.14–2.12)**	**0.005**
	Recessive		**1.48 (1.13–1.94)**	**0.004**
rs1562313	WW	198/260	Ref.	
	WV	115/157	0.87 (0.69–1.09)	0.230
	VV	25/30	0.90 (0.59–1.37)	0.620
	Dominant		0.87 (0.7–1.09)	0.220
rs2305158	WW	226/298	Ref.	
	WV	94/128	1.12 (0.88–1.43)	0.360
	VV	19/22	**1.63 (1.01–2.63)**	**0.040**
	Dominant		1.18 (0.94–1.49)	0.150
rs2305160	WW	215/294	Ref.	
	WV	107/133	**1.61 (1.26–2.05)**	**<0.001**
	VV	7/9	**2.15 (1.00–4.64)**	**0.050**
	Dominant		**1.63 (1.29–2.07)**	**<0.001**
rs3811558	WW	98/132	Ref.	
	WV	163/208	1.05 (0.82–1.35)	0.710
	VV	71/101	1.07 (0.78–1.46)	0.670
	Dominant		1.06 (0.83–1.34)	0.660

†Adjusted for age, sex, hepatitis B virus infection, portal vein tumor thrombus, tumor stage, serum α-fetoprotein, and treatment. ‡Significant *P*-values (<0.05) are in bold. CI, confidence interval; HR, hazard ratio; Ref., reference; VV, homozygous variant; WV, heterozygous variant; WW, homozygous wild-type.

samples under double-blinded conditions as previously described.[21]

Statistical analysis. All statistical analyses were carried out using the SPSS 16.0 statistical package (SPSS, Chicago, IL, USA). The survival time was defined as the period from the date of first treatment to the date of death or last follow-up. The three genetic models (additive, dominant, and recessive) were applied to assess the association of single SNPs with clinical outcome of HCC patients, and the best-fitting model was defined as that with the smallest *P*-value for the association analysis. Hazard ratios and 95% CIs were estimated by multivariate Cox proportional hazards model, with adjustment for age, sex, HBV infection, PVTT, TNM stage, serum AFP, and treatment, where appropriate. Overall survival was analyzed by plotting Kaplan–Meier curves and the survival probability distributions were compared using the log–rank test. The Mann–Whitney *U*-test was used to compare the difference in NPAS2 immunostaining score between different genotypes. All statistical tests were two-sided and $P \leq 0.05$ was considered to be statistically significant.

Results

Distribution of patients' characteristics and prognosis analysis. Patients' characteristics and clinical data are summarized in

Table 1. The patients included 391 men (87.3%) and 57 women (12.7%) with a median age of 53 years (range, 20–80 years); 383 (85.5%) patients were HBsAg positive. The minority (29.9%) of patients had PVTT and almost all patients (96.2%) had a Child–Pugh score A. The percentage of patients with TNM stage I and II was 49.3%. Two hundred and seventeen (48.4%) patients had significantly increased serum AFP (≥400 μg/L). Among all patients, 366 (81.7%) received only TACE treatment whereas the remaining 82 (18.3%) received TACE and other non-surgical treatments, such as radio frequency ablation, percutaneous ethanol injection, gamma knife radiosurgery, and/or immunoradiotherapy. During the median follow-up of 12.1 months (range, 2.0–70.1 months), a total of 339 (75.7%) patients died, and 8.9% patients were lost during follow-up. The median survival time for all patients was 11.1 months. Furthermore, multivariate analysis indicated significant poor OS in HCC patients with PVTT (HR = 1.92; 95% CI, 1.49–2.46), advanced TNM stage III and IV (HR = 1.56; 95% CI, 1.23–1.98), and higher serum AFP (HR = 1.51; 95% CI, 1.20–1.89). But patients who received TACE and other non-surgical treatments showed a significantly decreased risk of death (HR = 0.42; 95% CI, 0.30–0.59) compared with those treated by TACE alone. There was no association between

HCC outcome and age ($P = 0.31$), sex ($P = 0.17$), HBV infection status ($P = 0.30$), or Child–Pugh score ($P = 0.06$).

Association between SNPs and clinical outcome in HCC patients. We assessed the association between each individual SNP and clinical outcome using the multivariate Cox proportional hazard model with adjustment for age, sex, HBV infection, PVTT, TNM stage, serum AFP, and treatment under dominant, additive, and recessive models (Tables 2, S1). We found that rs1053096 was significantly associated with HCC OS (Table 2). Compared to the WW and WV of rs1053096, the VV conferred a significant increased risk of death (HR = 1.48; 95% CI, 1.13–1.94; $P = 0.004$) in HCC patients. In addition, compared to the WW of rs2305160, patients carrying at least one variant allele (WV + VV) had a significant increased risk of death (HR = 1.63; 95% CI, 1.29–2.07; $P < 0.001$) (Table 2). Kaplan–Meier curve analysis showed that there was no significant difference in OS among patients with different genotypes in each SNP except rs2305160 (Fig. S1). Further analysis showed that patients with VV genotype in rs1053096 had better OS than those with WW or WV genotype (Fig. 1a), whereas patients with WW genotype in rs2305160 had worse OS than those with WW or WV geno-

type (Fig. 1b). To further evaluate the predictive effect on HCC outcome of SNPs in the *NPAS2* gene, we carried out a stratified analysis (Table 3). The death risk conferred by rs1053096 remained significant in patients who were more than 53 years old (HR = 1.64; 95% CI, 1.11–2.41, $P = 0.01$), in male patients (HR = 1.44; 95% CI, 1.08–1.92, $P = 0.01$), in patients who were HBsAg positive (HR = 1.46; 95% CI, 1.10–1.94, $P = 0.01$), in patients without PVTT (HR = 1.45; 95% CI, 1.04–2.01, $P = 0.03$), in patients with TNM stage I and II disease (HR = 1.53; 95% CI, 1.01–2.32, $P = 0.05$), and in patients treated by TACE alone (HR = 1.56; 95% CI, 1.17–2.08, $P = 0.003$). Similar results were observed with rs2305160. In particular, P-values were <0.001 in male patients (HR = 1.69; 95% CI, 1.31–2.17), in HBsAg-positive patients (HR = 1.57; 95% CI, 1.22–2.03), those with stage III and IV disease (HR = 1.94; 95% CI, 1.41–2.67), in patients with increased serum AFP (HR = 1.76; 95% CI, 1.28–2.42), and in patients treated with TACE alone (HR = 1.76; 95% CI, 1.37–2.26).

Cumulative effect of unfavorable genotypes on HCC OS. To further assess the cumulative effect of genetic variants on HCC OS, we carried out a combination analysis by including
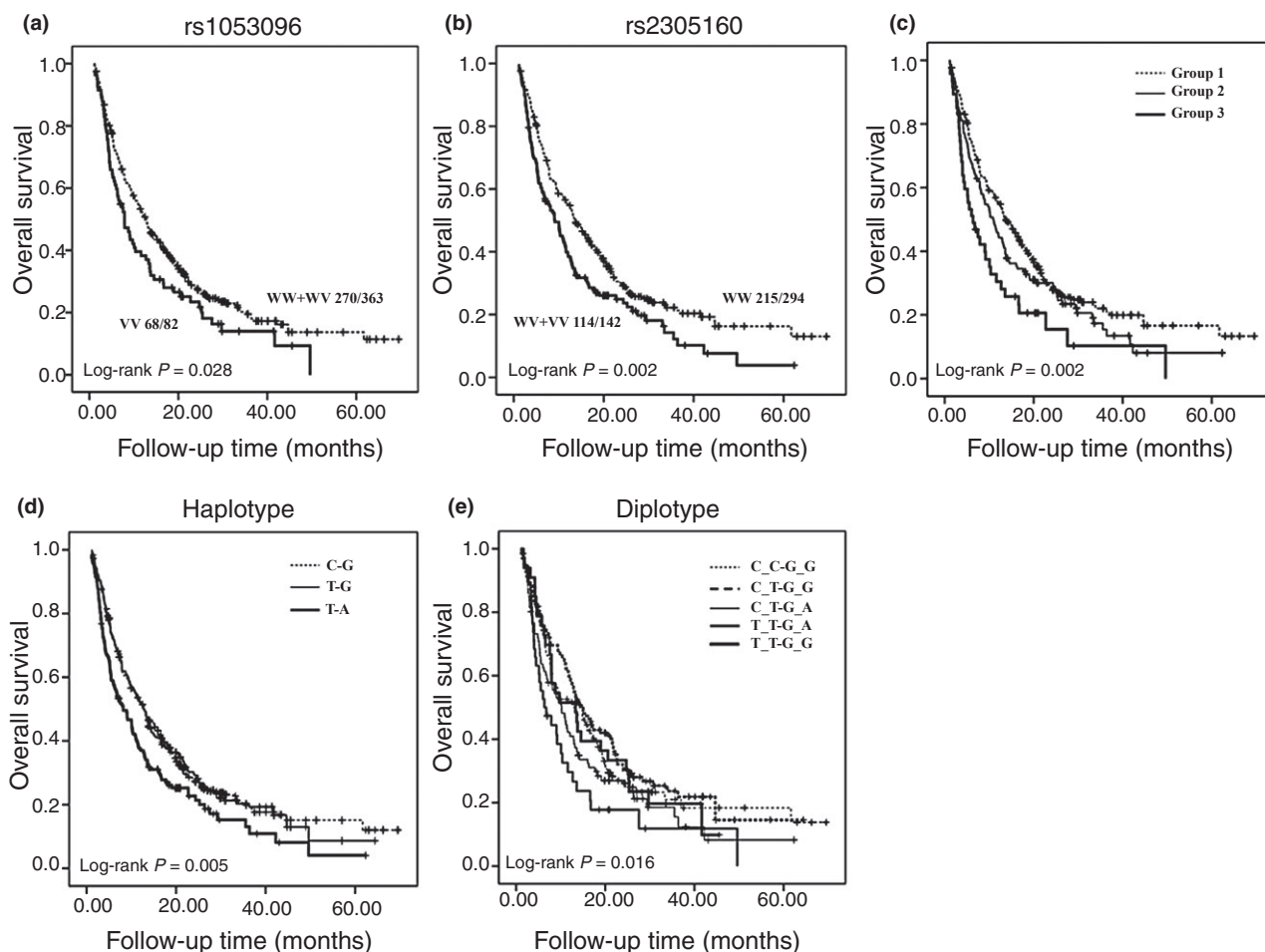
Fig. 1. Kaplan–Meier plots of overall survival in Chinese patients with hepatocellular carcinoma treated with transcatheter arterial chemoembolization. Overall survival analysis of patients with single nucleotide polymorphisms (SNPs) rs1053096 (a) and rs2305160 (b) in the *NPAS2* gene. VV, homozygous variant; WV, heterozygous variant; WW, homozygous wild-type. (c) Cumulative effect of unfavorable genotypes. Group 1, no unfavorable genotypes; Group 2, one unfavorable genotype; Group 3, two unfavorable genotypes. (d) Haplotype analysis of combined SNPs rs1053096 and rs2305160. (e) Diplotype analysis of combined SNPs rs1053096 and rs2305160. The differences between indicated groups in each analysis were compared by the log–rank test.

Table 3. Associations between single nucleotide polymorphisms rs1053096 and rs2305160 in the *NPAS2* gene and clinical outcome in Chinese patients with hepatocellular carcinoma treated with transcatheter arterial chemoembolization (TACE) (n = 448), stratified by host characteristics

Variable	Genotype	rs1053096			Genotype	rs2305160		
		Death/total	HR† (95% CI)	P-value‡		Death/total	HR† (95% CI)	P-value‡
Age, years								
≤53	WW+WV	133/179	Ref.		WW	111/154	Ref.	
	VV	35/42	1.32 (0.91–1.93)	0.150	WV+VV	54/64	**1.70 (1.21–2.40)**	**0.002**
>53	WW+WV	137/184	Ref.		WW	104/140	Ref.	
	VV	33/40	**1.64 (1.11–2.41)**	**0.010**	WV+VV	60/78	**1.51 (1.08–2.10)**	**0.020**
Sex								
Female	WW+WV	32/48	Ref.		WW	28/39	Ref.	
	VV	8/9	1.75 (0.78–3.90)	0.170	WV+VV	11/15	1.47 (0.66–3.30)	0.350
Male	WW+WV	238/315	Ref.		WW	187/255	Ref.	
	VV	60/73	**1.44 (1.08–1.92)**	**0.010**	WV+VV	103/127	**1.69 (1.31–2.17)**	**<0.001**
HBV infection								
Negative	WW+WV	41/56	Ref.		WW	34/47	Ref.	
	VV	7/8	1.39 (0.58–3.30)	0.460	WV+VV	13/17	**2.46 (1.18–5.12)**	**0.020**
Positive	WW+WV	229/307	Ref.		WW	181/247	Ref.	
	VV	61/74	**1.46 (1.10–1.94)**	**0.010**	WV+VV	101/125	**1.57 (1.22–2.03)**	**<0.001**
PVTT								
No	WW+WV	173/254	Ref.		WW	140/207	Ref.	
	VV	47/57	**1.45 (1.04–2.01)**	**0.030**	WV+VV	75/99	**1.59 (1.19–2.12)**	**0.002**
Yes	WW+WV	97/109	Ref.		WW	75/87	Ref.	
	VV	21/25	1.39 (0.85–2.28)	0.180	WV+VV	39/43	**1.52 (1.00–2.31)**	**0.050**
TNM stage								
I + II	WW+WV	112/180	Ref.		WW	84/138	Ref.	
	VV	29/38	**1.53 (1.01–2.32)**	**0.050**	WV+VV	53/76	1.23 (0.86–1.75)	0.250
III + IV	WW+WV	158/183	Ref.		WW	131/156	Ref.	
	VV	39/44	1.35 (0.94–1.93)	0.100	WV+VV	61/66	**1.94 (1.41–2.67)**	**<0.001**
Serum AFP, µg/L								
<400	WW+WV	128/195	Ref.		WW	105/158	Ref.	
	VV	29/35	1.40 (0.93–2.12)	0.110	WV+VV	46/65	**1.46 (1.01–2.10)**	**0.040**
≥400	WW+WV	142/168	Ref.		WW	110/136	Ref.	
	VV	39/47	1.42 (0.99–2.05)	0.060	WV+VV	68/77	**1.76 (1.28–2.42)**	**<0.001**
Treatment								
TACE	WW+WV	239/298	Ref.		WW	184/234	Ref.	
	VV	59/66	**1.56 (1.17–2.08)**	**0.003**	WV+VV	107/123	**1.76 (1.37–2.26)**	**<0.001**
TACE + other	WW+WV	31/65	Ref.		WW	31/60	Ref.	
	VV	9/16	0.85 (0.39–1.83)	0.670	WV+VV	7/19	0.68 (0.29–1.59)	0.380

†Adjusted by age, sex, hepatitis B virus (HBV) infection, portal vein tumor thrombus (PVTT), tumor stage, serum α-fetoprotein (AFP), and treatment, where appropriate. ‡Significant P-values (<0.05) are in bold. CI, confidence interval; HR, hazard ratio; Ref., reference; VV, homozygous variant; WV, heterozygous variant; WW, homozygous wild-type.

the two SNPs with a significant association in the single SNP analysis. The unfavorable genotypes were defined as VV for rs1053096 and WV + VV for rs2305160. When we combined these unfavorable genotypes, there was a significant trend of increasing death risk with increasing number of unfavorable genotypes. Compared with the reference (group 1 without any unfavorable genotypes), the HRs for patients with one (group 2) and two (group 3) unfavorable genotypes were 1.41 (95% CI, 1.10–1.82; P = 0.007) and 2.09 (95% CI, 1.46–2.97, P < 0.001), respectively. A significant dose–response trend was also observed (P < 0.001) (Table 4). The Kaplan–Meier analysis showed that there was a significant trend for decreased OS with the increasing number of unfavorable genotypes (log–rank, P = 0.002; Fig. 1c).

Haplotype and diplotype of *NPAS2* gene and HCC OS. We carried out haplotype and diplotype analyses to further characterize the impact of *NPAS2* genotypes on the OS of HCC patients. There were four haplotypes in terms of rs1053096 and rs2305160 (CG, 56.0%; TG, 26.7%; TA, 16.4%; and CA, 0.9%) and seven diplotypes (CC-GG, 30.5%; CT-GG, 29.3%; CT-GA, 19.9%; TT-GA, 8.8%; TT-GG, 7.6%; TT-AA, 2.1%; and CC-GA, 1.8%). The frequency of haplotypes and diplotypes <5% were combined as another group. When compared to the CG haplotype, there was a 1.59-fold increase of death risk for the TA haplotype (95% CI, 1.28–1.97; P < 0.001) (Table 5). Kaplan–Meier curves indicated that significant difference of OS existed among the three haplotypes (log–rank, P = 0.005; Fig. 1d). Patients with the CT-GA diplotype had a significantly increased risk of death (HR = 1.50; 95% CI, 1.08–2.08; P = 0.01), and those with TT-GA had a 1.94-fold increased risk of death (95% CI, 1.29–2.92; P = 0.001), compared with those carrying the CC-GG diplotype (Table 5). The P-value was also significant by Kaplan–Meier analysis (log–rank, P = 0.016; Fig. 1e). In addition, we also carried out haplotype and diplotype analyses for six SNPs in the

Table 4. Cumulative effect of unfavorable genotypes on overall survival in Chinese patients with hepatocellular carcinoma (n = 448)

Group†	Death/total	HR‡ (95% CI)	P-value§
Group 1 (0)	188/259	Ref.	
Group 2 (1)	101/127	**1.41 (1.10–1.82)**	**0.007**
Group 3 (2)	39/47	**2.09 (1.46–2.97)**	**<0.001**
P for trend			**<0.001**

†Patients were grouped by the number of unfavorable genotypes (0–2) in single nucleotide polymorphisms rs1053096 (VV) and rs2305160 (WV+VV) in the NPAS2 gene. ‡Hazard ratios (HRs) were adjusted by age, sex, hepatitis B virus infection, portal vein tumor thrombus, tumor stage, serum α-fetoprotein, and treatment. §Significant P-values (<0.05) are in bold. CI, confidence interval; Ref. reference.

Table 5. Haplotype and diplotype analysis of rs1053096 and rs2305160 single nucleotide polymorphisms in the NPAS2 gene in Chinese patients with hepatocellular carcinoma (n = 448)

Group	Frequency, %	Death, n (%)	HR† (95% CI)	P-value‡
Haplotype				
C-G	56.0	359 (54.7)	Ref.	
T-G	26.7	177 (27.0)	1.04 (0.87–1.24)	0.680
T-A	16.4	114 (17.4)	**1.59 (1.28–1.97)**	**<0.001**
Other groups	0.9	6 (0.9)	0.81 (0.36–1.83)	0.610
Diplotype				
C_C-G_G	30.5	97 (29.6)	Ref.	
C_T-G_G	29.3	91 (27.7)	0.88 (0.66–1.18)	0.390
C_T-G_A	19.9	68 (20.7)	**1.50 (1.08–2.08)**	**0.010**
T_T-G_A	8.8	32 (9.8)	**1.94 (1.29–2.92)**	**0.001**
T_T-G_G	7.6	27 (8.2)	1.16 (0.75–1.80)	0.490
Other groups	3.9	13 (4.0)	1.22 (0.67–2.21)	0.510

†Adjusted by age, sex, hepatitis B virus infection, portal vein tumor thrombus, tumor stage, serum α-fetoprotein, and treatment. ‡Significant P-values (<0.05) are in bold. CI, confidence interval; HR, hazard ratio; Ref., reference.

NPAS2 gene, but no significant result was observed (data not shown).

Association between genotypes and expression of NPAS2. To assess the potential impact of genotypes of SNPs on the expression of NPAS2, we examined the genotype and expression of NPAS2 in another 243 HCC cases. As shown in Table S2, we found that patients who had the VV genotype of rs1053096 showed a significantly higher expression level of NPAS2 when compared with those who had the WW and WV genotypes (P = 0.03). In addition, no significant difference of NPAS2 expression was found between patients carrying different genotypes of other SNPs.

Discussion

In the present study, we evaluated the effects of six functional SNPs in NPAS2 on the prognosis of a cohort of 448 unresectable Chinese patients with HCC treated with TACE. The most important finding was that two SNPs (rs1053096 and rs2305160) are significantly associated with HCC OS. Additionally, we identified an accumulative death risk with increasing number of unfavorable genotypes. Furthermore, we observed that several haplotypes and diplotypes were significantly associated with HCC death risk, indicating the potential interactions between these SNPs on the OS of HCC patients. To the best of our knowledge, this is the first study to report

that NPAS2 gene polymorphisms may serve as an independent prognostic marker for HCC patients treated with TACE.

Previous reports have suggested that NPAS2 may directly regulate other genes in the circadian regulatory system.[22] For example, the heterodimer of transactivators NPAS2 with BMAL1 regulate the transcription of PER1, PER2, CRY1, and CRY2.[23] Decreased expressions of PER1, PER2, PER3, and CRY2 in HCCs were observed.[17] It was reported in both in vitro and in vivo studies that overexpression of PER1 and PER2 inhibited cancer cell growth,[24] and induced apoptosis.[25] Yi et al.[26] reported that overexpression of NPAS2 increased PER1 and PER2 expression and consequently inhibited the growth of cancer cells. This could be one molecular explanation for the observed association between NPAS2 expression and OS among breast cancer patients.[14] A genome-wide ChIP-on-chip study identified 16 transcriptional targets of NPAS2, and nine of these genes were involved in cancer development.[26] Furthermore, Hoffman et al.[10] reported that NPAS2 is also involved in DNA damage response and cell-cycle regulation, and therefore plays an important role in tumorigenesis by affecting the expression of cancer-related genes, and could be considered a novel tumor suppressor. All these data strongly support the idea that NPAS2 is involved in the development and progression of human cancers, including HCC.

The functional prediction indicates that several microRNA binding sites exist near the sequence of rs1053096, which is located in the 3′-UTR region of the NPAS2 gene, suggesting the potential regulation on NPAS2 expression by rs1053096. Previous evidence showed that SNP sequences in 3′-UTRs of many other genes were strongly involved in the expression regulation of mRNA either by providing mutated binding sites for proteins and microRNAs to alter mRNA stability, or by forming hairpin loop structures to stabilize and thus slow down mRNA degradation. In this study, we found that the genotype of rs1053096 was significantly associated with the expression level of NPAS2. Therefore, the SNP rs1053096 in the NPAS2 gene could possibly affect the expression of NPAS2 and ultimately regulate its biological functions in cancer cells. In our study, for the first time, we found that SNP rs1053096 was associated with the prognosis of HCC patients, indicating that variants in this SNP site may affect the functions of NPAS2 in HCC cells. However, the underlying mechanisms need to be studied further. Moreover, the prognostic value of rs1053096 in other types of cancer warrant detailed investigation.

Another SNP, rs2305160, which is a non-synonymous SNP in the NPAS2 gene, has previously been reported to be a susceptibility biomarker for human cancers in earlier case–control studies.[11–13] The current results showed that the homozygous variant of rs2305160 is significantly associated with poor OS in HCC patients. It may have several potential impacts on NPAS2 functions. First, an amino acid change caused by this polymorphism may alter the NPAS2 protein structure. Second, Zhu et al.[11] has predicted that rs2305160 is located in the PAS sensory domain, therefore, it may affect protein sensory capacity directly or interfere with NPAS2/BMAL1 heterodimerization, which is essential for normal sensory function. Further functional assay is still needed to verify our hypothesis.

In our study, the mortality rate in unresectable HCC patients after TACE treatment is similar to previous reports in Chinese patients.[27,28] The TACE technique combines transarterial embolization with regional chemotherapy. The major advantage of this treatment is the high concentration of chemo-agents in tumor tissue, which bring toxicity to cancer cells. TACE

shows great application to HCC patients by restricting the location of chemo-agents to HCC tissue, therefore improving the treatment response. The final clinical outcome is usually determined by the balance between tumor progression and treatment response. On the basis of these reports, we put forward the hypothesis that rs1053096 and rs2305160 of the *NPAS2* gene may influence the treatment response of TACE through regulating cell proliferation, apoptosis, and DNA damage response by controlling transcription and affecting the protein sensory capacity of *NPAS2*. Further validations of this hypothesis using independent populations, high-density mapping, and functional characterizations are warranted.

Our study has one specific strength. The patient group was mainly enrolled from Xi'an and adjacent areas. This region is highly attractive for carrying out population-based research because of the demographic stability and low mobility rate. The homogenous patient characteristics and treatments, as well as the extremely low rate of patient loss during follow-up, greatly reduced the confounding effects of the heterogeneous therapeutic methods in most cancer clinical outcome studies. Our study also has limitations. Our sample size may not be large enough to detect minimal associations and interactions, and the generalizability of our findings identified from this Chinese patient group needs to be further validated in other ethnic populations.

In conclusion, our study reported the first epidemiological evidence supporting a role for rs1053096 and rs2305160 in the prognosis prediction of Chinese HCC patients treated with TACE. If validated, these two SNPs may potentially be developed as a simple non-invasive biomarker to help clinicians identify specific patients who will benefit from TACE.

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

AFP	α-fetoprotein
CI	confidence interval
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HR	hazard ratio
OS	overall survival
PVTT	portal vein tumor thrombus
SNP	single nucleotide polymorphism
TACE	transcatheter arterial chemoembolization
VV	homozygous variant
WV	heterozygous variant
WW	homozygous wild-type

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Kaplan–Meier survival curves of patients with hepatocellular carcinoma by single nucleotide polymorphisms in the *NPAS2* gene. VV, homozygous variant; WV, heterozygous variant; WW, homozygous wild-type.

Table S1. Association of rs1053096 and rs2305160 with clinical outcome in patients with hepatocellular carcinoma.

Table S2. NPAS2 scores by subgroups with different single nucleotide polymorphism genotypes.