Protein & Cell

RESEARCH ARTICLE

Combined effects of *p53* and *MDM2* polymorphisms on susceptibility and surgical prognosis in hepatitis B virus-related hepatocellular carcinoma

Yun Yang[1*], Tian Xia[2*], Ning Li[3], Jin Zhang[1], Yuan Yang[1], Wenming Cong[4], Qiang Deng[2], Ke Lan[2✉], Weiping Zhou[1✉]

[1] The Third Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200433, China
[2] Key Laboratory of Molecular Virology and Immunology, Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai 200025, China
[3] Department of Infectious Diseases, Huashan Hospital, Fudan University, Shanghai 200040, China
[4] Department of Pathology, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200433, China
✉ Correspondence: lanke@sibs.ac.cn (K. Lan), ehphwp@126.com (W. Zhou)

Protein & Cell

ABSTRACT

The p53 signaling pathway works as a potent barrier to tumor progression. Two single nucleotide polymorphisms (SNPs) in the gene loci of p53 pathway, *p53* codon 72 Arg72Pro and *MDM2* SNP309 (T > G), have been shown to cause perturbation of p53 function, but the effect of the two SNPs on the risk of hepatocellular carcinoma (HCC) remains inconsistent. This study investigated the influence of combined *p53* Arg72Pro and *MDM2* SNP309 on the risk of developing HCC in patients with chronic hepatitis B virus infection, and evaluated the significance of the two combined SNPs on patient prognosis. In total, 350 HCC patients, 230 non-HCC patients, and 96 healthy controls were genotyped for the *p53* Arg72Pro and *MDM2* SNP309. The combined *p53* Pro/Pro and *MDM2* G/G genotype was significantly associated with HCC risk (*P* = 0.047). Multivariate analysis indicated that combined *p53* Pro/Pro and *MDM2* G/G genotype was an independent factor affecting recurrence and survival (*P* < 0.05). Patients with combined *p53* Pro/Pro and *MDM2* G/G genotypes had a poorer prognosis than other genotypes, *P* < 0.01 for both disease-free survival (DFS) and overall survival (OS). DFS and OS rates also differed significantly between Barcelona Clinic Liver Cancer (BCLC) stage A patients with combined *p53* Pro/Pro and *MDM2* G/G and other genotypes (*P* < 0.05). Thus, the combined *p53* Pro/Pro and *MDM2* G/G genotype is associated with increased risk of developing HCC and is an independent adverse prognostic indicator in early stage HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality (Parkin, 2001). The risk factors associated with HCC include alcohol abuse and aflatoxins adduct (Blonski et al., 2010) but the major risk is chronic viral hepatitis, both B and C (Sherman, 2005; El-Serag and Rudolph, 2007). About 80% of all cases of HCC worldwide are found in Asia and China alone accounts for 55% (Lai and Lau, 2005; El-Serag and Rudolph, 2007). In Asia, infection with the chronic hepatitis B virus (HBV) is the most important etiologic factor of HCC (Yuen et al., 2009). Viral factors (Silini et al., 1996; Kao et al., 2000; Lin and Kao, 2008) and host factors (Clifford et al., 2010; Nault and Zucman-Rossi, 2011) modify the devel-

---

*These authors contributed equally to the work.

opment, progression and clinical outcome of HCC. The risk of HCC is increased in patients with higher levels of HBV replication, determined by tests for hepatits B e antigen (HBeAg) and levels of HBV DNA. Over the last three decades, several mechanisms have been described for the role of HBV in causing HCC including HBV DNA integration in the host genome or transactivation of oncogenes of the host by HBV X protein (HBx) or by another truncated protein derived from the pre-S2/S region of HBV genome (Anzola, 2004; Brechot, 2004; Neuveut et al., 2010). Indirect mechanisms have also been suggested such as HBV causing chronic hepatic injury and hepatocyte regeneration (Anzola, 2004; Brechot, 2004; Neuveut et al., 2010).

p53, the most important tumor suppressor gene, lies at critical joint of the complex signaling network for response to stress, playing a major role in hepatocarcinogenesis, irrespective of the etiology (Buendia, 2000; Edamoto et al., 2003). It is the most common mutated genes of human malignancy. Somatic mutation of p53 is found in 32% of all human liver tumor cases (Petitjean et al., 2007). The integrity of p53 is an especially important barrier for HBV related HCC development. Integration of HBV DNA in human genome is common and the integration is correlated to chromosome instability (Hino et al., 1991; Brechot, 2004). Therefore, it is assumed that chronic HBV infection is correlated to prolongation period of DNA damage, which requires the p53 function for DNA repairing. The p53 integrity is likely an important factor reducing DNA damage caused by HBV integration during tumorigenesis (Puisieux et al., 1995).

Functional inherited single nucleotide polymorphisms (SNPs) exist in the gene loci of the p53 signaling pathway, affecting the cancer risk and clinical outcome (Grochola et al., 2010). The 215 G > C polymorphisms at codon72 of *p53* (Pro72Arg, rs1042522) have been studied in many cancers (Boersma et al., 2006; Han et al., 2008; Horikawa et al., 2008). It has been reported that this polymorphism influences the transcription, senescence and apoptotic function of p53. The functional impact of the Pro72 variant on apoptotic response, which has been replicated in independent investigations (Baptiste et al., 2002; Dumont et al., 2003; Pim and Banks, 2004), is a lowered ability of the Pro72 variant to localize to the mitochondria, and this localization is accompanied by the release of cytochrome *c* into the cytosol (Dumont et al., 2003). However, the Pro72 variant appears to induce a higher level of G1 arrest than the Arg72 variant (Pim and Banks, 2004).

MDM2, a negative regulator of p53, is elevated in many cancers that retain wild type p53. MDM2 binds directly to the N-terminus of p53 and negatively regulates the transcriptional activation function of p53. Further, MDM2 could act as an E3 ubiquitin ligase that targets p53 for ubiquitin modification and targeting it to proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997). Upregulated MDM2, therefore, would lead to more degradation of p53. The *MDM2* SNP T309G polymorphism (rs2279744) localized in the intronic promoter region of *MDM2*, affects the affinity of stimulatory protein (Sp1) with the promoter of *MDM2*, regulating its transcription (Bond et al., 2004). In the cell model, the *MDM2* SNP309G/G genotype shows elevated MDM2 transcription and expression, resulting in attenuation of the p53 pathway and accelerating tumor formation (Bond et al., 2004). It has been reported that the association of *MDM2* SNP309 with various cancers and the correlation between MDM2 expression with poor prognosis (Hirata et al., 2007; Cescon et al., 2009; Toffoli et al., 2009); however, the association of *MDM2* SNP309 with HCC is inconsistent between studies (Dharel et al., 2006; Yoon et al., 2008; Leu et al., 2009).

Given the crucial importance of *p53* Arg72Pro and *MDM2* SNP309 to the risk of malignancy, and the previously observed biological and clinical effects of *p53* Arg72Pro and *MDM2* SNP309, we evaluated the association between these SNPs with the risk of HBV-related HCC and prognosis in a large cohort of HCC patients with chronic hepatitis B.

RESULTS

Characteristics of HCC patients and HBV carrier controls

The clinicopathologic features of the 580 cases with chronic HBV infection and 96 healthy controls are given in Table 1. Gender and AFP ≥20 ng/mL were significantly different between HCC patients and the other two groups without HCC (HBV carrier controls and healthy controls). Age and Child-Pugh classification were not significantly different between HCC patients and the other two groups without HCC (HBV carrier controls and healthy controls).

Hardy-Weinberg equilibrium (HWE)

The HWE distribution for the genotype frequencies of *p53* Arg72Pro and *MDM2* SNP or *p53* Arg72Pro and *MDM2* SNP 309 was not significantly different in the HCC group, the non-HCC group or in the healthy controls. The genotype distribution in HBV cases and healthy controls was not significantly different and both were consistent with the HWE distribution.

p53 Arg72Pro and *MDM2* SNP309 distributions

We examined the *p53* Arg72Pro and *MDM2* SNP309 distributions for all 676 subjects using the TaqMan SNP genotyping method. The distributions of the *p53* Arg72Pro and *MDM2* SNP309 genotypes with regard to the presence or absence of HCC are given in Tables S1 and S2, respectively, with genotyping data for the 96 healthy subjects for comparison. There were lower Arg/Arg (29.43% vs. 37.39%), and higher Pro/Pro genotypes (20.86% vs. 15.22%) distributed in the HCC group compared to the non-HCC group. In comparison to the Arg/Arg genotype, the crude OR (95% CI) of the presence of HCC for the Pro/Pro genotype was 1.74 (range 1.06–2.85; P = 0.028), and the adjusted OR (95% CI) was 1.88 (range 1.07–3.32; P = 0.029). The frequencies of the

Table 1. Characteristics of HCC patients and controls

	HCC (*n* = 350), *n* (%)	HBV carriers controls (*n* = 230), *n* (%)	Healthy controls (*n* = 96), *n* (%)	*P*-value HCC versus HBV carriers controls	*P*-value HCC versus healthy controls
Gender					
Male	311 (88.86)	141 (61.30)	46 (47.92)	<0.001	<0.001
Female	39 (11.14)	89 (38.70)	50 (52.08)		
Age (years)					
≥50	212 (60.57)	125 (54.35)	52 (54.17)	0.240	0.259
<50	138 (39.43)	105 (45.65)	44 (45.83)		
AFP (ng/mL)					
≥20	217 (62.00)	75 (32.61)	0 (0)	<0.001	<0.001
<20	133 (38.00)	155 (67.39)	96 (100)		
Child-Pugh classification					
A	350 (100)	230 (100)	96 (100)	1.000	1.000
B	0 (0)	0 (0)	0 (0)		
Tumor diameter (cm)[a]	6.78 ± 4.18	-	-	-	-
Tumor encapsulation					
None	138 (39.43)	-	-	-	-
Complete	212 (60.57)	-	-	-	-
Vascular invasion					
Yes	153 (43.71)	-	-	-	-
No	197 (56.29)	-	-	-	-
Satellite nodules					
Yes	57 (16.29)	-	-	-	-
No	293 (83.71)	-	-	-	-
Tumor differentiation					
I/II	62 (17.71)	-	-	-	-
III/IV	288 (82.29)	-	-	-	-
BCLC stage					
A	268 (76.57)	-	-	-	-
B + C	82 (23.43)	-	-	-	-

Tumor diameter are expressed as mean ± SD.
Abbreviations: AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer
Child-Pugh classification: Child-Pugh classification of severity of liver disease according to the degree of ascites, the plasma concentrations of bilirrubin and albumin, the prothrombin time, and the degree of encephalopathy. This score employs five clinical measures of liver disease. Each measure is scored 1–3, with 3 indicating most severe derangement. A total score of 5–6 is considered grade A (well-compensated disease); 7–9 is grade B (significant functional compromise); and 10–15 is grade C (descompensated disease).

MDM2 SNP309 genotype in HBV patients and healthy controls were not significantly different. Multivariate logistic regression analysis showed that, in contrast to the T/T genotype, the crude OR (95% CI) for the G/G genotype was 1.03 (range 0.64–1.64; *P* = 0.920) and the adjusted OR (95% CI) was 1.12 (range 0.65–1.93, *P* = 0.685).

Gene-gene interactions between the *MDM2* and *p53* polymorphisms were also investigated. The combination of the *p53* Pro/Pro and *MDM2* G/G genotypes was significantly correlated with the risk of HCC with a crude OR (95% CI) of 3.27 (1.19–9.00; *P* = 0.022) and an adjusted OR (95% CI) of 3.31 (1.01–10.83; *P* = 0.047). There was no additional effect when the risk of other combined genotypes was analyzed (Table 2).

Combined *p53* Arg72Pro and *MDM2* SNP309 indicates poor prognosis

The median follow-up time for HCC patients was 33.3 months.

Protein & Cell

Table 2. Genotype frequencies of combinations of *MDM2* and *p53* in HBV patients and healthy subjects

		Patients with HBV infection							Healthy subjects (n = 96)[a]
MDM2 SNP 309	*p53* Arg72Pro	Total patients (n = 580)[a]	HCC (n = 350)[a]	Non-HCC (n = 230)[a]	Crude OR (95% CI) HCC versus Non-HCC	P-value	Adjusted OR (95% CI) HCC versus Non-HCC[b]	P-value	
T/T	Arg/Arg	47 (8.10)	25 (7.14)	22 (9.57)	1		1		8 (8.33)
T/T	Arg/Pro	70 (12.07)	43 (12.29)	27 (11.74)	1.40 (0.66–2.96)	0.377	0.90 (0.39–2.05)	0.809	7 (7.29)
T/T	Pro/Pro	31 (5.34)	21 (6.00)	10 (4.35)	1.85 (0.72–4.76)	0.203	0.93 (0.36–2.41)	0.885	4 (4.17)
T/G	Arg/Arg	97 (16.72)	57 (16.29)	40 (17.39)	1.25 (0.62–2.53)	0.527	1.78 (0.77–4.15)	0.181	16 (16.67)
T/G	Arg/Pro	151 (26.03)	93 (26.57)	58 (25.22)	1.41 (0.73–2.73)	0.307	1.31 (0.53–2.41)	0.749	28 (29.17)
T/G	Pro/Pro	44 (7.59)	26 (7.43)	18 (7.83)	1.27 (0.55–2.92)	0.571	1.17 (0.46–2.95)	0.745	6 (6.25)
G/G	Arg/Arg	45 (7.76)	21 (6.00)	24 (10.43)	0.77 (0.34–1.75)	0.532	1.54 (0.53–4.43)	0.427	7 (7.29)
G/G	Arg/Pro	62 (10.69)	38 (10.86)	24 (10.43)	1.39 (0.65–3.00)	0.397	1.99 (0.79–4.98)	0.140	16 (16.67)
G/G	Pro/Pro	33 (5.70)	26 (7.42)	7 (3.04)	3.27 (1.19–9.00)	**0.022**	3.31 (1.01–10.83)	**0.047**	4 (4.17)

[a] Values expressed as *n* (%).
[b] Adjusted for gender, age, and alpha-fetoprotein level.

There were no significant differences in disease-free survival (DFS) or overall survival (OS) with respect to the various *p53* genotypes. Similar results were observed for the *MDM2* genotypes (data not shown). The effect of combined *p53* Pro/Pro and *MDM2* G/G on DFS and OS was found to be negatively correlated with 1-, 3- and 5-year DFS rates (53.85%, 30.77%, and 19.24%, respectively for combined *p53* Pro/Pro and *MDM2* G/G genotypes versus 69.44%, 56.79%, and 51.85%, respectively for the other genotypes; *P* = 0.006). The 1-, 3- and 5-year survival rates were lower for combined *p53* Pro/Pro and *MDM2* G/G genotype patients compared to patients with other genotypes (84.62%, 53.85%, and 42.31% versus 90.12%, 74.07%, and 69.13%, respectively, *P* = 0.001, Fig. 1A and 1B).

The clinicopathologic characteristics of the HCC patients with combined *p53* Pro/Pro and *MDM2* G/G genotypes and the other genotypes are given in Table 3. Tumor size was significantly larger in patients with combined *p53* Pro/Pro and *MDM2* G/G genotypes (9.66 ± 4.99 cm) compared to other genotypes (6.55 ± 4.03 cm) (*P* = 0.035). The proportion of complete tumor encapsulation was significantly lower in patients with combined *p53* Pro/Pro and *MDM2* G/G genotypes compared to other genotypes (38.46% versus 62.65%, *P* = 0.015). A descriptive Cox proportional hazard model was used to estimate the independent impact of each variable on DFS and OS. Univariate analysis showed that combined *p53* Pro/Pro and *MDM2* G/G genotypes, AFP expression level, tumor encapsulation, vascular invasion, satellite nodules, tumor differentiation, tumor size and liver cirrhosis were each significantly associated with shorter DFS (Table 4), and combined *p53* Pro/Pro and *MDM2* G/G genotypes, AFP expression level, tumor encapsulation, vascular invasion, satellite nodules, tumor differentiation, and tumor size were associated with shorter OS (Table 4). Finally, multivariate Cox

regression analysis showed that combined *p53* Pro/Pro and *MDM2* G/G genotypes (hazard ratio (HR) = 1.76; *P* = 0.016), AFP expression level (HR = 1.41; *P* = 0.036), vascular invasion (HR = 1.57; *P* = 0.006), satellite nodules (HR = 1.61; *P* = 0.010), tumor differentiation (HR = 2.12; *P* = 0.012), and tumor size (HR = 2.09; *P* < 0.001) were predictive of shorter DFS (Table 4). Combined *p53* Pro/Pro and *MDM2* G/G genotypes (hazard ratio (HR) = 1.86; *P* = 0.017), satellite nodules (HR = 1.78; *P* = 0.009), and tumor size (HR = 1.95; *P* = 0.002) were predictive of shorter OS (Table 4).

Impact of combined *p53* Arg72Pro and *MDM2* SNP309 on prognosis of HCC patients with different Barcelona Clinic Liver Cancer (BCLC) stages

All 350 patients were stratified according to BCLC classification. A total of 268 HCC patients were classified as stage A. Among these patients, 17 were identified as having combined *p53* Pro/Pro and *MDM2* G/G genotypes, and 251 were identified with other genotypes. Patients with both *p53* Pro/Pro and *MDM2* G/G genotypes had a poorer surgical prognosis compared to those with other genotypes (58.82%, 41.18%, and 29.41% versus 79.68%, 66.13%, and 60.96% for 1-, 3-, and 5-year DFS rates, respectively; *P* = 0.042; 82.35%, 52.94%, and 41.18% versus 96.01%, 80.88%, and 74.90% for 1-, 3-, and 5-year survival rates, respectively; *P* = 0.001; Fig. 1C and 1D). All 268 BCLC stage A patients were stratified into two subgroups according to tumor size, using 5 cm as the cutoff value. Patients with tumors > 5 cm in diameter (*n* = 114) had a poorer prognosis than those with tumors ≤ 5 cm in diameter (*n* = 154) (66.67%, 54.39%, and 49.12% versus 87.01%, 72.08%, and 66.23% for 1-, 3-, and 5-year DFS rates, respectively; *P* = 0.001; 92.11%, 69.30%, and 64.91% versus 97.40%, 86.36%, and 78.57% for 1-, 3-, and 5-year survival rates, respectively; *P* = 0.002). Furthermore,

Protein & Cell

the impact of combined *p53* Pro/Pro and *MDM2* G/G on the prognoses of the two subgroups was examined. The prognosis of patients in the > 5 cm subgroup (*n* = 10) with both *p53* Pro/Pro and *MDM2* G/G genotypes was poorer than for patients (*n* = 104) with other genotypes (40.00%, 20.00%, and 10.00% versus 69.23%, 57.69%, and 52.88% in 1-, 3-, and 5-year DFS rates, respectively; *P* = 0.006; 80.00%, 30.00%, and 20.00% versus 93.90%, 73.08%, and 69.23% for 1-, 3-, and 5-year survival rates, respectively; *P* < 0.001, Fig. 2A and 2B). In the ≤5 cm subgroup, however, there were no significant differences in cumulative DFS or OS rates between patients with both *p53* Pro/Pro and *MDM2* G/G genotypes (*n* = 7)

and patients with other genotypes (*n* = 147) (*P* = 0.873 for DFS and *P* = 0.770 for OS, Fig. 2C and 2D). Similar results were also observed in BCLC B and C stage patients (*P* = 0.730 for DFS and *P* = 0.819 for OS, Fig. S1A and S1B).

DISCUSSION

In this study, we confirmed the *p53* Pro72 genotype is associated with higher risk of HBV-related HCC. Furthermore, we showed the combination of *p53* and *MDM2* polymorphisms is related to the oncogenesis, tumor progression and clinical outcome of HBV-related HCC after hepatic resection.
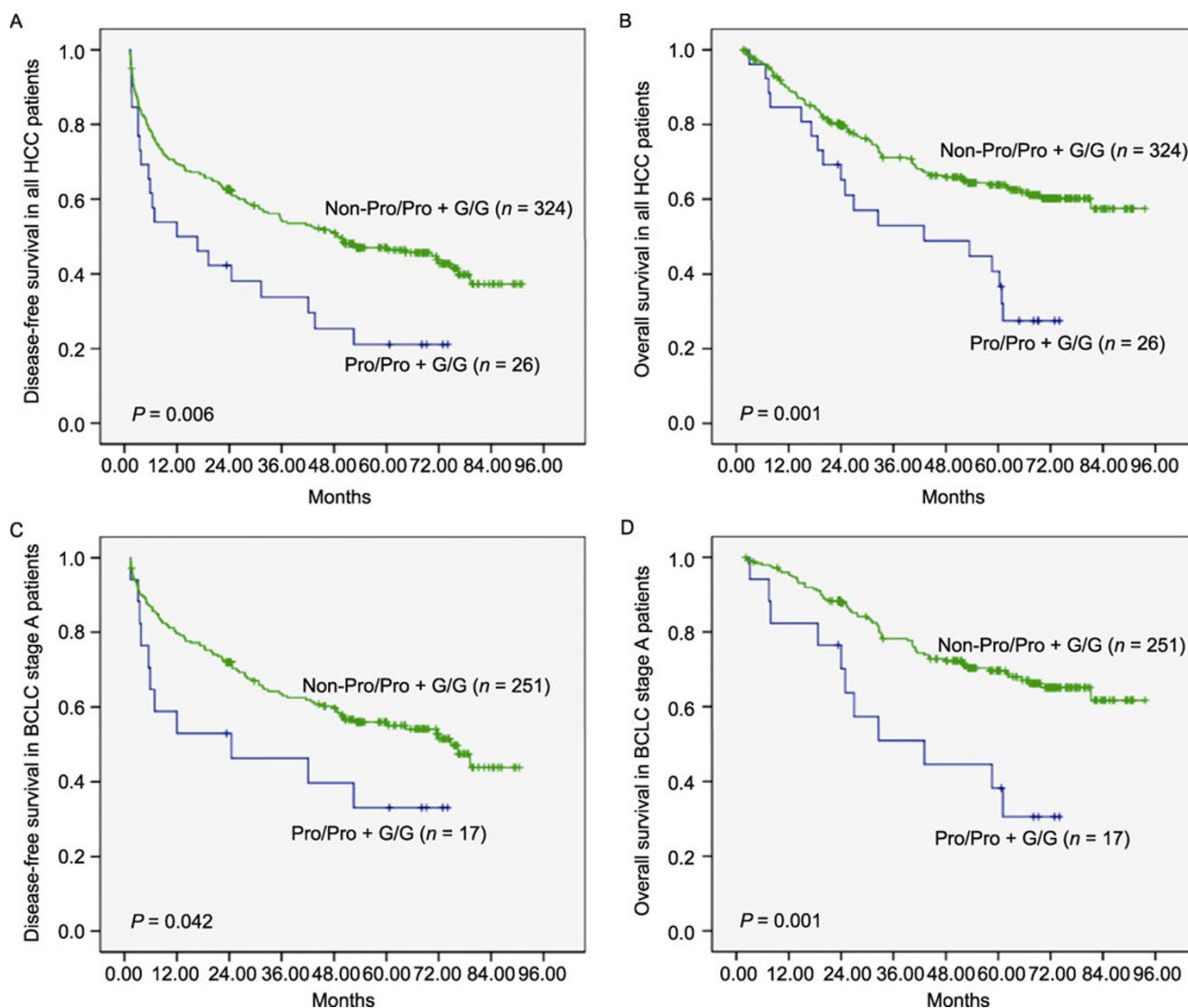
Figure 1. Kaplan-Meier analysis of 350 hepatocellular carcinoma (HCC) patients and survival curves of 268 Barcelona Clinic Liver Cancer (BCLC) stage A patients. (A) Cumulative disease-free survival (DFS) curve of HCC patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes (*P* = 0.006). (B) Cumulative overall survival (OS) curve of HCC patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes (*P* = 0.001). (C) Cumulative DFS curve of BCLC stage A patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes (*P* = 0.042). (D) Cumulative OS curve of BCLC stage A patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes (*P* = 0.001).

Table 3. Clinical characteristics of HCC patients with combined genotypes of *p53* Pro/Pro and *MDM2* G/G and the other genotypes

	HCC patients with combined genotypes of *p53* Pro/Pro + *MDM2* G/G	HCC patients with the other genotypes	*P*-value
	n = 26 (%)	*n* = 324 (%)	
Age[a]	50.57 ± 8.88	51.42 ± 10.71	0.350
Gender			1.000
Male	26 (100%)	285 (87.96%)	
Female	0 (0%)	39 (12.04%)	
HBeAg			0.698
Positive	5 (19.24%)	73 (22.53%)	
Negative	21 (80.76%)	251 (77.47%)	
AFP (ng/mL)			0.735
≥20	17 (65.38%)	200 (61.73%)	
<20	9 (34.62%)	124 (38.27%)	
Liver cirrhosis			0.796
Yes	16 (61.54%)	191 (58.95%)	
No	10 (38.46%)	133 (41.05%)	
Tumor diameter (cm)[a]	9.66 ± 4.99	6.55 ± 4.03	**0.035**
Tumor encapsulation			**0.015**
None	16 (61.54%)	121 (37.35%)	
Complete	10 (38.46%)	203 (62.65%)	
Vascular invasion			0.795
Yes	12 (46.15%)	141 (43.52%)	
No	14 (53.85%)	183 (56.48%)	
Satellite nodules			0.673
Yes	5 (19.23%)	52 (16.05%)	
No	21 (80.77%)	272 (83.95%)	
Tumor differentiation			0.392
I/II	3 (11.54%)	59 (18.21%)	
III/IV	23 (88.46%)	265 (81.79%)	
BCLC stage			0.162
A	17 (65.38%)	251 (77.47%)	
B + C	9 (34.62%)	73 (22.53%)	

[a] Age and Tummor diameter are show as mean±SD, calculated by a two-sample *t*-test
Abbreviations: HBeAg, Hepatitis B e antigen; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer.

We asked whether there was a statistical gene–gene interaction between the *MDM2* and *p53* polymorphisms. Combined effects of *p53* Arg72Pro and *MDM2* SNP309 have been examined in lung cancer, nasopharyngeal carcinoma and non-polyposis colorectal cancer, with different results (Zhang et al., 2006; Talseth et al., 2007; Xiao et al., 2010). In this study, these two polymorphisms produced a combined effect on HCC risk in chronic HBV infection patients. The presence of both the *p53* Pro/Pro and *MDM2* G/G genotypes resulted in a positive synergistic effect on the risk of HCC, with an adjusted OR (95% CI) of 3.31 (1.01–10.83, *P* = 0.047), whereas those who lacked both genotypes did not show a positive combined effect on the risk of HCC. Functional difference of the *p53* polymorphism at codon 72 has been reported and the Arg72 variant induces apoptosis markedly better than the Pro/Pro genotype (Baptiste et al., 2002; Dumont et al., 2003; Cescon et al., 2009). In contrast, the Pro72 form appears to induce a higher level of G1 arrest than the Arg72 form. In addition, the *MDM2* SNP309 G/G genotype results in elevated MDM2 mRNA and protein expression level and subsequently attenuated tumor suppresses function of p53. A multiplicative interaction is to be

Table 4. Univariate and Multivariate analysis of factors associated with disease-free survival and overall survival of patients with HCC

	Disease-free survival		Overall survival	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Univariate analysis				
Gender (male vs. female)	1.235 (0.784–1.947)	0.363	1.407 (0.819-2.491)	0.217
Age (Year) (≥60 vs. <60)	0.864 (0.593–1.259)	0.446	1.116 (0.724–1.719)	0.621
Genotype (G/G + Pro/Pro vs. Non-G/G + Pro/Pro)	1.986 (1.260–3.131)	0.003	2.207 (1.338–3.640)	0.002
AFP (≥20 vs. <20 ng/mL)	1.784 (1.320–2.411)	<0.001	1.859 (1.273–2.716)	0.001
HBeAg (positive vs. negative)	1.128 (0.799–1.593)	0.492	1.153 (0.756–1.759)	0.508
Tumor encapsulation (no vs. yes)	1.529 (1.145–2.043)	0.004	1.665 (1.170–2.369)	0.005
Vascular invasion (yes vs. no)	2.257 (1.679–3.033)	<0.001	1.689 (1.181–2.415)	0.004
Satellite nodules (yes vs. no)	2.200 (1.552–3.120)	<0.001	2.313 (1.523–3.512)	< 0.001
Tumor differentiation (III + IV vs. I + II)	4.006 (2.049–7.833)	<0.001	2.667 (1.352–5.262)	0.005
Tumor size (>5 vs. ≤5cm)	2.575 (1.863–3.558)	<0.001	2.641 (1.758–3.968)	< 0.001
Liver cirrhosis	1.352 (1.011–1.825)	0.049	1.099 (0.767–1.576)	0.607
Multivariate analysis				
Genotype (G/G + Pro/Pro vs. Non-G/G + Pro/Pro)	1.763 (1.112–2.795)	0.016	1.855 (1.116–3.083)	0.017
AFP (≥20 vs. <20ng/mL)	1.414 (1.023–1.954)	0.036	1.483 (0.994–2.215)	0.054
Tumor encapsulation (no vs. yes)	0.999 (0.737–1.353)	0.993	1.167 (0.810–1.680)	0.408
Vascular invasion (yes vs. no)	1.565 (1.136–2.156)	0.006	1.211 (0.833–1.760)	0.315
Satellite nodules (yes vs. no)	1.609 (1.123–2.306)	0.010	1.778 (1.157–2.734)	0.009
Tumor differentiation (III + IV vs. I + II)	2.122 (1.179–3.819)	0.012	1.684 (0.829–3.421)	0.150
Tumor size (>5 vs. ≤5cm)	2.086 (1.482–2.935)	<0.001	1.953 (1.275–2.989)	0.002
Liver cirrhosis	1.318 (0.958–1.814)	0.090	-	-

Abbreviations: HBeAg, Hepatitis B e antigen; AFP, alpha-fetoprotein.
Hazard ratios (95% CI) and *P* values were calculated using univariate and multivariate Cox proportional hazard regression.

expected if a cell carries both *p53* Pro/Pro and *MDM2 G/G* polymorphisms, which heighten the expression of MDM2 and diminish the function of p53.

In this study, we showed that the combination of *p53* Pro/Pro and *MDM2* G/G genotypes was associated with poorer prognosis in HCC patients after liver resection. Kaplan-Meier analysis of DFS and OS in all HCC subjects showed that both tumor recurrence and survival rates differed substantially between patients with both *p53* Pro/Pro and *MDM2* G/G genotypes and those who lacked both genotypes. Multivariate analysis revealed that having both *p53* Pro/Pro and *MDM2* G/G genotypes was an independent and significant risk factor affecting recurrence and survival after curative resection. In addition, tumor size was significantly larger and the proportion of complete tumor encapsulation was significantly lower in patients with combined *p53* Pro/Pro and *MDM2* G/G genotypes compared to other genotypes. These results suggest that the combined *p53* Pro/Pro and *MDM2* G/G genotypes were associated with malignant characteristics of HCC.

According to the BCLC staging system, stage A is considered the early stage of HCC. However, some stage A patients still have a poor prognosis in clinical practice. Our results suggest that stage A patients with both *p53* Pro/Pro and *MDM2* G/G genotypes could have higher cumulative recurrence rates and lower survival rates than those without both genotypes. Because a solitary tumor > 5.0 cm in diameter might affect a patient's prognosis, we stratified the 268 stage A patients with a single nodule in our study into two subgroups, with a cutoff value of 5 cm (Yang et al., 2009). Having both *p53* Pro/Pro and *MDM2* G/G genotypes also predicted higher cumulative recurrence rates and lower survival rates in 114 stage A patients with solitary tumor size > 5 cm in diameter (Fig. 2A and 2B). Although the stage A patients with a single tumor measuring > 5 cm in diameter suffer from high incidences of recurrence and metastasis after hepatic resection, they are still considered to be candidates for surgery, as noted in previous reports and recommended by the BCLC staging system (Bruix et al., 2001; Yang et al., 2009). We found that the combination of *p53* Pro/Pro and *MDM2* G/G genotypes could also significantly affect recurrence and survival in these patients (Fig. 2A and 2B). Thus, the results of this study indicate that the combined *p53* Pro/Pro and *MDM2* G/G genotypes could identify early stage

Protein & Cell
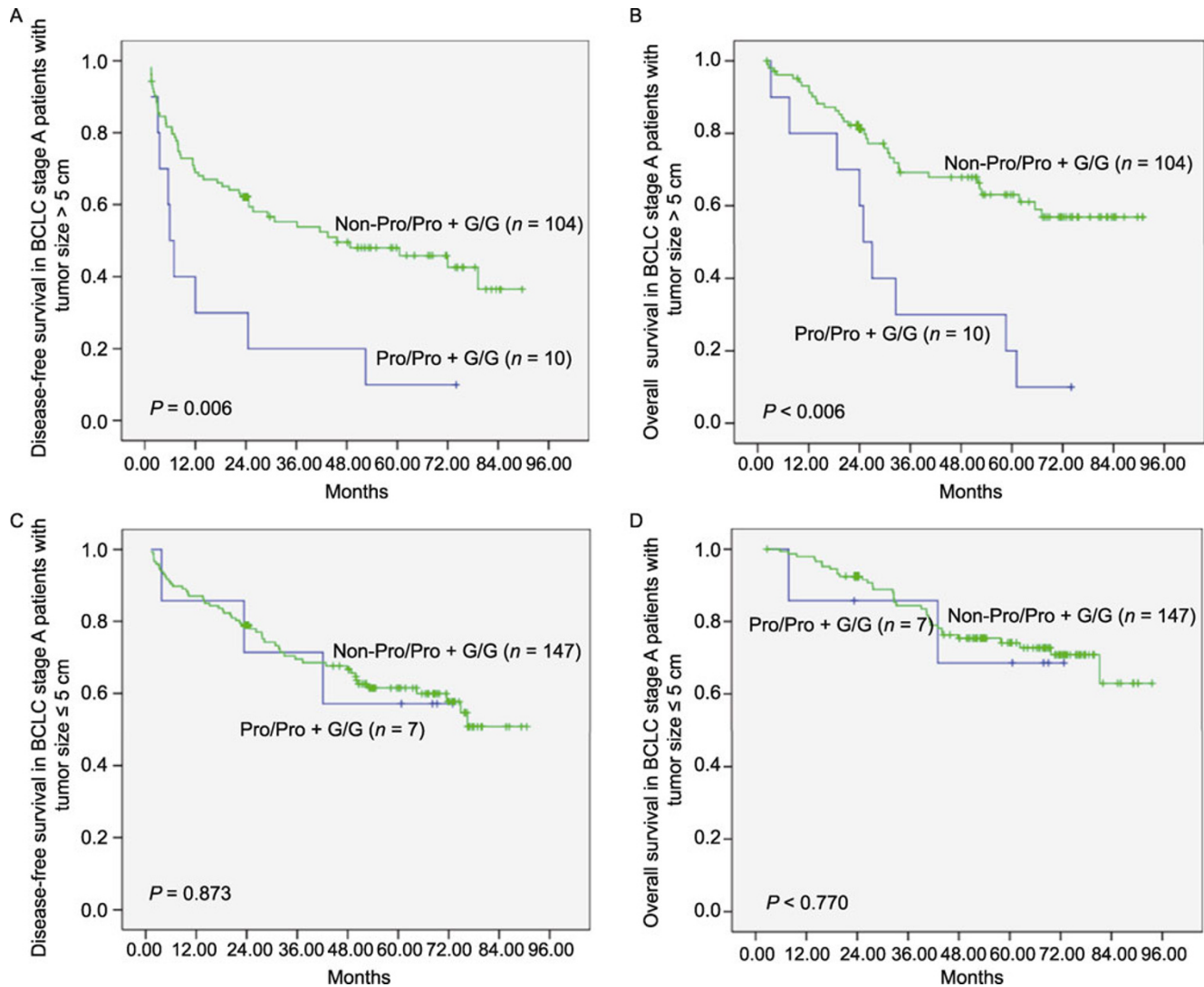
Figure 2. Survival curves of BCLC stage A HCC patients with combined p53 Pro/Pro and MDM2 G/G genotypes and different tumor diameters. (A) Cumulative disease-free survival (DFS) curve of BCLC stage A HCC patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes with tumor diameter > 5 cm ($P = 0.006$). (B) Cumulative overall survival (OS) curve of BCLC stage A HCC patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes with tumor diameter > 5 cm ($P < 0.001$). (C) Cumulative DFS curve of BCLC stage A HCC patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes with tumor diameter ≤ 5 cm ($P = 0.873$). (D) Cumulative OS curve of BCLC stage A HCC patients with combined p53 Pro/Pro and MDM2 G/G and other genotypes with tumor diameter ≤ 5 cm ($P = 0.770$).

HCC patients with a poorer prognosis, especially for BCLC stage A patients with tumors larger than 5 cm. However, having combined *p53* Pro/Pro and *MDM2* G/G genotypes did not substantially affect the prognosis of BCLC stage A patients with solitary tumors ≤ 5 cm in diameter after liver resection.

In the present study, the impact of the combination of *p53* Pro/Pro and *MDM2* G/G genotypes on the prognosis of BCLC stage B+C patients was not significant. This may have been because of the limited number of patients. Additionally, stage B and stage C patients have large multinodular tumors,

gross portal vein tumor thrombosis and other invasive features of HCC (Bruix et al., 2001), and a large proportion of these patients died of tumor recurrence within 2 years (88.89% for combination genotypes of *p53* Pro/Pro and *MDM2* G/G and 63.34% for the other genotypes). The impact of the combined *p53* Pro/Pro and *MDM2* G/G genotypes on the outcomes of these patients could thus not be determined.

In summary, we showed that the combination of *p53* Pro/Pro and *MDM2* G/G genotypes not only resulted in a positive synergistic effect on the risk of HCC, but also substantially affected tumor recurrence and survival in a large

cohort of HCC patients. This combined genotype might thus serve as a risk marker of prognosis after liver resection. The results of this work have both diagnostic and prognostic value for HBV-related HCC patients and will help us to understand the relationship between the p53 signaling pathway and HCC.

PATIENTS AND METHODS

Patients

A total of 580 Chinese patients with chronic HBV infection were enrolled in this study. All patients were stratified into two groups according to the presence (n = 350) or the absence (n = 230) of HCC. A total of 360 HCC patients underwent hepatectomies by five independent surgical teams at the Eastern Hepatobiliary Surgery Hospital in Shanghai from December 2003 to June 2009. The preoperative diagnosis of HCC was based on the diagnostic criteria for HCC used by the European Association for the Study of the Liver (EASL) (Bruix et al., 2001). The postoperative diagnosis of HCC was confirmed pathologically after liver resection. Of the 360 HCC patients, 350 met the following inclusion criteria and thus underwent SNP analysis: preoperative World Health Organization performance status of 0–1; Child-Pugh class A; no distant metastasis; no chemotherapy or radiotherapy before surgery; curative resection; and resected lesions identified as HCC on pathological examination. Five patients had early metastatic disease and/or tumor recurrence within 1 month after resection, three patients received preoperative hepatic arterial chemoembolization, and two patients died from liver failure within 30 days postoperatively. These patients were excluded from this study. A retrospective study was carried out on the remaining 350 HCC patients

Curative resection was defined as complete macroscopic and microscopic removal of the tumor. The maximal diameter of the tumor was taken as the tumor size. The resection volume and surgical procedures were designed according to tumor size, location, and liver functional reserves. The clinical staging of tumors was determined according to the BCLC staging systems (Bruix et al., 2001). The histological grade of tumor differentiation was assigned by the Edmondson Steiner grading system (Edmondson and Steiner, 1954).

Patients with chronic HBV infection without HCC (non-HCC) were recruited from the Huashan Hospital. All Chronic HBV patients without HCC had a chest X-ray, ultrasonography (USG), and contrast computed tomography (CT) or magnetic resonance imaging (MRI) examination. Laboratory blood tests included hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), hepatitis C virus (HCV) anti body, serum alpha-fetoprotein (AFP), serum albumin, serum total bilirubin, alanine aminotransferase (ALT) and prothrombin time. All chronic HBV patients without HCC were evaluated as HCC-free either radiologically or clinically. All patients were positive for the HBsAg and negative for the HCV antibody detection. To obtain an estimate of the genetic distribution of p53 Arg72Pro and MDM2 SNP309 in the general Chinese population, we obtained blood samples from 96 healthy individuals who visited the Eastern Hepatobiliary Surgery Hospital, with no history of liver disease, negative for both HBsAg and HCV antibody. Written informed consent was obtained from all subjects. There were six patients aged under 18 at the time of enrollment in this study, and their written informed consent

was obtained from their guardian. The study was conducted in accordance to the Declaration of Helsinki and Good Clinical Practice guidelines and the protocol was approved by the Institutional Review Board of Eastern Hepatobiliary Surgery Institute, Shanghai, China.

Follow-up and treatment for tumor recurrences

Follow-up ended in June 2011; the median follow-up duration was 33.3 months (range, 1.4–93.7 months). Patients were assessed every 2–3 months within the first 2 years, and then every 3–6 months thereafter. All follow-up examinations were performed by physicians who were unaware of the study. All patients were followed-up for postoperative recurrence with regular assessments using serum AFP, chest x-ray and abdominal USG. A CT or MRI examination was performed every 3 months after surgery. The diagnostic criteria for HCC recurrence were the same as the preoperative criteria. If recurrence was confirmed, recurrent lesions were managed aggressively using a multimodal approach including re-resection, transarterial chemoembolization, percutaneous radiofrequency ablation and percutaneous ethanol injection. The treatment was decided according to the pattern of recurrence, liver functional reserve and general condition of the patient at the time of recurrence.

Genome DNA extraction and allelic discrimination

Genomic DNA was extracted from 200 μL of whole blood using the TIANamp Blood DNA Kit (TIANGEN BIOTECH CO.). The p53 Arg72Pro (rs1042522) and MDM2 SNP309 (rs2279744) SNPs were determined using TaqMan® SNP Genotyping Assays (Applied Biosystems). The primers and probes for genotyping were designed with Primer Express v3.0 software (Applied Biosystems), and manufactured by Applied Biosystems. For p53 Arg72Pro, the amplification primers were: 5′-CCAGATGAAGCTCCCAGAATGC-3′, 5′-GCCGC CGGTGTAGGA-3′. The G allele probe was: 5′-VIC-CTCCCCCCG TG GCC-NFQ-3′. The C allele probe was: 5′-FAM-TCCCCGCGTG GCC-NFQ-3′. For MDM2 SNP309, the amplification primers were: 5′-CGGGAGTTCAGGGTAAAGGT-3′, 5′-ACAGGCACCTGCGATCATC-3′. The G allele probe was: 5′-VIC-CCGCTGCGGCGCG- NFQ-3′. The T allele probe was: 5′-FAM-CCGCTTCGGCGCG- NFQ-3′.

Polymerase chain reaction (PCR) amplification and allelic discrimination were done according to the manufacturer's instructions. In brief, a 5-μL reaction mix was set up for each well of 384-well plates with 20 ng of genomic DNA template. The amplify PCR protocol was: denaturation at 95°C for 10 min, then followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. Endpoint reads and allelic discrimination were done with the 7900HT Fast Real-Time PCR System (Applied Biosystems). Laboratory personnel were blinded to clinical outcome and validation was done with a random 5% of samples.

Statistical analysis

DFS and OS were the primary endpoints. DFS was taken from the date of pathologic diagnosis to the date of earliest recurrence, or censored at last known follow-up. OS was defined as the interval between the date of pathologic diagnosis and death. Outcome data were collected from clinical records and the hospital cancer registries. The last date for censoring purposes was June 2011. For individuals

<div style="writing-mode: vertical">Protein & Cell</div>

Protein & Cell

lost to follow-up before that date, censoring occurred at the last date they were evaluated radiologically or clinically without recurrence for DFS, and at the last date known to be alive for OS.

The Hardy-Weinberg equilibrium (HWE) was evaluated using Statistical Analysis System Genetics 9.13 (SAS Institute, Cary, NC). The characteristics of HCC and non-HCC patients were compared using Student's two-sample t-tests for continuous variables and χ^2 tests for categorical variables. The χ^2 test was used to compare genotype frequencies between patients and controls. The association between HCC and genotype was estimated based on the odds ratio (OR) and 95% confidence interval (CI) using a multivariate logistic regression model. DFS and OS curves were analyzed using the Kaplan-Meier method. Differences between curves were assessed using the log-rank test. Cox proportional hazard regression models were used to determine the independent factors for recurrence and survival, based on the variables selected by univariate analysis. Statistical analysis was performed using SPSS 13.0 (SPSS Inc, Chicago IL). Statistically significant differences were set at $P \leq 0.05$ for a two tailed test.

ACKNOWLEDGEMENTS

ABBREVIATIONS

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; CT, computed tomography; DFS, disease-free survival; HBeAg, hepatits B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; HWE, Hardy-Weinberg equilibrium; MRI, magnetic resonance imaging; OR, odds ratio; OS, overall survival; SNP, single nucleotide polymorphism; USG, ultrasonography

REFERENCES

Anzola, M. (2004). Hepatocellular carcinoma: role of hepatitis B and hepatitis C viruses proteins in hepatocarcinogenesis. J Viral Hepat 11, 383–393.

Baptiste, N., Friedlander, P., Chen, X.B., and Prives, C. (2002). The proline-rich domain of p53 is required for cooperation with anti-neoplastic agents to promote apoptosis of tumor cells. Oncogene 21, 9–21.

Blonski, W., Kotlyar, D.S., and Forde, K.A. (2010). Non-viral causes of hepatocellular carcinoma. World J Gastroenterol 16, 3603–3615.

Boersma, B.J., Howe, T.M., Goodman, J.E., Yfantis, H.G., Lee, D.H., Chanock, S.J., and Ambs, S. (2006). Association of breast cancer outcome with status of p53 and MDM2 SNP309. J Natl Cancer Inst 98, 911–919.

Bond, G.L., Hu, W., Bond, E.E., Robins, H., Lutzker, S.G., Arva, N.C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., et al. (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell 119, 591–602.

Brechot, C. (2004). Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. Gastroenterology 127, S56–61.

Bruix, J., Sherman, M., Llovet, J.M., Beaugrand, M., Lencioni, R., Burroughs, A.K., Christensen, E., Pagliaro, L., Colombo, M., Rodes, J., et al. (2001). Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. J Hepat 35, 421–430.

Buendia, M.A. (2000). Genetics of hepatocellular carcinoma. Semin Cancer Biol 10, 185–200.

Cescon, D.W., Bradbury, P.A., Asomaning, K., Hopkins, J., Zhai, R., Zhou, W., Wang, Z., Kulke, M., Su, L., Ma, C., et al. (2009). p53 Arg72Pro and MDM2 T309G polymorphisms, histology, and esophageal cancer prognosis. Clin Cancer Res 15, 3103–3109.

Clifford, R.J., Zhang, J., Meerzaman, D.M., Lyu, M.-S., Hu, Y., Cultraro, C.M., Finney, R.P., Kelley, J.M., Efroni, S., Greenblum, S.I., et al. (2010). Genetic variations at loci involved in the immune response are risk factors for hepatocellular carcinoma. Hepatology 52, 2034–2043.

Dharel, N., Kato, N., Muroyama, R., Moriyama, M., Shao, R.X., Kawabe, T., and Omata, M. (2006). MDM2 promoter SNP309 is associated with the risk of hepatocellular carcinoma in patients with chronic hepatitis C. Clin Cancer Res 12, 4867–4871.

Dumont, P., Leu, J.I.J., Della Pietra, A.C., George, D.L., and Murphy, M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat Genet 33, 357–365.

Edamoto, Y., Hara, A., Biernat, W., Terracciano, L., Cathomas, G., Riehle, H.M., Matsuda, M., Fujii, H., Scoazec, J.Y., and Ohgaki, H. (2003). Alterations of RB1, p53 and Wnt pathways in hepatocellular carcinomas associated with hepatitis C, hepatitis B and alcoholic liver cirrhosis. Int J Cancer Suppl 106, 334–341.

Edmondson, H.A., and Steiner, P.E. (1954). Primary Carcinoma of the Liver - a Study of 100 Cases among 48,900 Necropsies. Cancer 7, 462–503.

El-Serag, H.B., and Rudolph, K.L. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 132, 2557–2576.

Grochola, L.F., Zeron-Medina, J., Meriaux, S., and Bond, G.L. (2010). Single-nucleotide polymorphisms in the p53 signaling pathway. Cold Spring Harb Perspect Biol 2, a001032.

Han, J.Y., Lee, G.K., Jang, D.H., Lee, S.Y., and Lee, J.S. (2008). Association of p53 codon 72 polymorphism and MDM2 SNP309 with clinical outcome of advanced nonsmall cell lung cancer. Cancer 113, 799–807.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature 387, 296–299.

Hino, O., Tabata, S., and Hotta, Y. (1991). Evidence for increased in vitro recombination with insertion of human hepatitis B virus DNA. Proc Natl Acad Sci U S A 88, 9248–9252.

Hirata, H., Hinoda, Y., Kikuno, N., Kawamoto, K., Suehiro, Y., Tanaka, Y., and Dahiya, R. (2007). MDM2 SNP309 polymorphism as risk factor for susceptibility and poor prognosis in renal cell carcinoma. Clin Cancer Res 13, 4123–4129.

Horikawa, Y., Nadaoka, J., Saito, M., Kumazawa, T., Inoue, T.,

Yuasa, T., Tsuchiya, N., Nishiyama, H., Ogawa, O., and Habuchi, T. (2008). Clinical implications of the MDM2 SNP309 and p53 Arg72Pro polymorphisms in transitional cell carcinoma of the bladder. Oncol Rep 20, 49–55.

Kao, J.H., Chen, P.J., Lai, M.Y., and Chen, D.S. (2000). Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 118, 554–559.

Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. Nature 387, 299–303.

Lai, E.C., and Lau, W.Y. (2005). The continuing challenge of hepatic cancer in Asia. Surgeon 3, 210–215.

Leu, J.D., Lin, I.F., Sun, Y.F., Chen, S.M., Liu, C.C., and Lee, Y.J. (2009). Association between MDM2-SNP309 and hepatocellular carcinoma in Taiwanese population. World J Gastroenterol 15, 5592–5597.

Lin, C.L., and Kao, J.H. (2008). Hepatitis B viral factors and clinical outcomes of chronic hepatitis B. J Biomed Sci 15, 137–145.

Nault, J.C., and Zucman-Rossi, J. (2011). Genetics of hepatobiliary carcinogenesis. Semin Liver Dis 31, 173–187.

Neuveut, C., Wei, Y., and Buendia, M.A. (2010). Mechanisms of HBV-related hepatocarcinogenesis. J Hepat 52, 594–604.

Parkin, D.M. (2001). Global cancer statistics in the year 2000. Lancet Oncol 2, 533–543.

Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P., and Olivier, M. (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Human Mutat 28, 622–629.

Pim, D., and Banks, L. (2004). p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. Int J Cancer 108, 196–199.

Puisieux, A., Ji, J., Guillot, C., Legros, Y., Soussi, T., Isselbacher, K., and Ozturk, M. (1995). p53-mediated cellular response to DNA damage in cells with replicative hepatitis B virus. Proc Natl Acad Sci U S A 92, 1342–1346.

Sherman, M. (2005). Hepatocellular carcinoma: epidemiology, risk factors, and screening. Semin Liver Dis 25, 143–154.

Silini, E., Bottelli, R., Asti, M., Bruno, S., Candusso, M.E., Brambilla, S., Bono, F., Iamoni, G., Tinelli, C., Mondelli, M.U., et al. (1996). Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: a case-control study. Gastroenterology 111, 199–205.

Talseth, B.A., Meldrum, C., Suchy, J., Kurzawski, G., Lubinski, J., and Scott, R.J. (2007). MDM2 SNP309 T > G alone or in combination with the TP53 R72P polymorphism does not appear to influence disease expression and age of diagnosis of colorectal cancer in HNPCC patients. Int J Cancer 120, 563–565.

Toffoli, G., Biason, P., Russo, A., De Mattia, E., Cecchin, E., Hattinger, C.M., Pasello, M., Alberghini, M., Ferrari, C., Scotlandi, K., et al. (2009). Effect of TP53 Arg72Pro and MDM2 SNP309 polymorphisms on the risk of high-grade osteosarcoma development and survival. Clin Cancer Res 15, 3550–3556.

Xiao, M., Zhang, L., Zhu, X., Huang, J., Jiang, H., Hu, S., and Liu, Y. (2010). Genetic polymorphisms of MDM2 and TP53 genes are associated with risk of nasopharyngeal carcinoma in a Chinese population. BMC cancer 10, 147.

Yang, L.Y., Fang, F., Ou, D.P., Wu, W., Zeng, Z.J., and Wu, F. (2009). Solitary large hepatocellular carcinoma a specific subtype of hepatocellular carcinoma with good outcome after hepatic resection. Ann Surg 249, 118–123.

Yoon, Y.J., Chang, H.Y., Ahn, S.H., Kim, J.K., Park, Y.K., Kang, D.R., Park, J.Y., Myoung, S.M., Kim do, Y., Chon, C.Y., et al. (2008). MDM2 and p53 polymorphisms are associated with the development of hepatocellular carcinoma in patients with chronic hepatitis B virus infection. Carcinogenesis 29, 1192–1196.

Yuen, M.F., Hou, J.L., Chutaputti, A., and Asia Pacific Working Party on Prevention of Hepatocellular, C. (2009). Hepatocellular carcinoma in the Asia pacific region. J Gastroenterol Hepat 24, 346–353.

Zhang, X., Miao, X., Guo, Y., Tan, W., Zhou, Y., Sun, T., Wang, Y., and Lin, D. (2006). Genetic polymorphisms in cell cycle regulatory genes MDM2 and TP53 are associated with susceptibility to lung cancer. Human Mutat 27, 110–117.

Protein & Cell