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An integrated analysis of SOCS1 down-regulation in HBV infection-related hepatocellular carcinoma

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SUMMARY . Persistent inflammation together with genetic/epigenetic aberrations is strongly associated with chronic Hepatitis B virus (HBV) infection-related hepatocarcinogenesis. Here, we investigated the alterations of the suppressor of cytokine signalling (SOCS) family genes in HBV-related hepatocellular carcinoma (HCC). A total of 116 patients with HCC were enrolled in this study. The methylation statuses of SOCS1-7 and CISH genes were quantitatively measured and clinicopathological significance of SOCS1 methylation was statistically analysed. The gene copy number variation was assayed by aCGH. Luciferase reporter assay and Western blot were used to detect the involvement of SOCS1 in p53 signalling. We found high frequencies of SOCS1 gene hypermethylation in both tumour (56.03%) and adjacent nontumour tissues (54.31%), but tumour tissues exhibited increased methylation intensity (24.01% *vs* 13.11%, $P < 0.0001$), particularly in patients with larger tumour size or cirrhosis background ($P < 0.0001$). In addition, the frequency and intensity of SOCS1 hypermethylation in tumour tissues were both significantly higher than those in nontumour tissues in male gender patients and in patients ≥45 years old ($P = 0.0214$ and $P < 0.0001$, $P = 0.0232$ and $P < 0.0001$, respectively). SOCS1 gene deletion was found in 8 of 25 aCGH assayed tumour specimens, which was associated with lower SOCS1 mRNA expression ($P = 0.0448$). Furthermore, ectopic SOCS1 overexpression could activate the p53 signalling pathway in HCC cell lines. Hypermethylation of SOCS2-7 and CISH genes was seldom found in HCC. Our results suggested that the gene loss and epigenetic silencing of SOCS1 were strongly associated with HBV-related HCC.

Keywords: hepatitis B virus, hepatocellular carcinoma, methylation, p53, suppressor of cytokine signalling 1.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies in the world and the second leading cause of cancer mortality in China [1,2]. In China, over 80% of HCC is attributed to chronic hepatitis B virus (HBV) infection [3]. DNA methylation, which is the first epigenetic alteration identified in cancer [4], is a common phenomenon in various kinds of human cancers including HCC [5]. The silencing of tumour suppressor genes by CpG island (CGI) methylation has been shown to occur very early in cancer development and may contribute to cancer initiation [6]. However, the diversity and tumour stage specificity of epigenetic alterations are less well defined than genetic defects [7].

The suppressor of cytokine signalling (SOCS) family is negative feedback inhibitors of the JAK/STAT cytokine signalling pathway [8]. SOCS1-5 CGI hypermethylation has been reported in primary human HCC [9,10] and mouse HCC models [11]. Our recent aCGH study revealed a frequent loss of SOCS family genes in HCC, especially at the SOCS1 coding region [12]. In HCC, SOCS1 was mainly found to be involved in the JAK/STAT pathway. However, one recent work suggested that SOCS1 was involved in p53 signalling pathway activation in lung fibroblasts [13,14]. The novel association of SOCS1 with p53 has not been addressed in HCC.

Although hypermethylation-mediated SOCS gene silencing has been reported in HCC, the relevant hypermethylation frequency of SOCS1 in HCC tumour tissues varied from

Abbreviations: CGI, CpG island; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SOCS, suppressor of cytokine signalling.

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40.9% to 67%, while it varied from 0% to 41% in the nontumour tissues [9,15–21]. In addition, although the methylation of SOCS6, SOCS7 and CISH was all found to be increased in other kinds of human cancers [22,23], their methylation statuses in HCC have never been addressed. In this study, the methylation status of the whole SOCS family was quantitatively investigated, with particular attention to the change in methylation intensities in primary HCC tissues and their corresponding cirrhotic nontumour liver tissues. Also, the clinicopathological significance of these genes CGI methylation status was statistically analysed. We also demonstrated for the first time the positive regulation by SOCS1 of the p53 signalling pathway in hepatocytes.

MATERIALS AND METHODS

Cell lines and tissue samples

Primary HCC and the adjacent nontumour tissues ($n = 116$) were obtained from patients who underwent routine curative surgery at Henan Tumor Hospital in Zhengzhou, Henan Province of China, from 2008 to 2011. All patients were diagnosed by ultrasonography and computed tomography, and confirmed by liver biopsy. All were serum HBsAg or HBV DNA positive and had not been treated with radiotherapy or chemotherapy prior to surgery. Among them, 105 patients were histologically diagnosed with HCC accompanying cirrhosis. The clinicopathological characteristics of the patients with HCC are shown in Table S1. The study protocol was approved by the institute ethics committee, and informed consent was obtained from all patients and donors before the start of study.

Human HCC cell lines SNU449, Huh-1, Huh-7, PLC/PRF/5, HepG2, SMMC7721, Hep3B and SK-hep-1 were kept in our laboratory and maintained in either DMEM or RPMI 1640 supplemented with 10% foetal bovine serum (GIBCO, Carlsbad, CA, USA).

High-density oligonucleotide aCGH analysis

A genome-wide analysis of copy number changes was performed as described previously [12].

Methylation assay

The quantificational methylated DNA analysis was performed as previously described [24]. The primers used for real-time PCR are shown in Table S2. For determination of a specimen's CGI hypermethylation status, the cut-off value was set at 10% [24].

Gene expression analysis

Real-time quantitative PCR was performed to measure the expression levels of SOCS1 using the Roche lightcycler 480 sequence detection system (Roche, Mannheim, Germany) according to the manufacturer's instructions. The housekeeping gene CTBP was included as control reference to normalize the expression levels, and primers used for detection of SOCS1 and CTBP have been described previously [24,25]. Each experiment was performed in triplicate, and the expression levels of SOCS1 were determined by the comparative Ct method ($2^{-\triangle Ct}$) after normalization to the CTBP.

5-aza-2′-deoxycytidine treatment of cell lines

Cells were seeded in 6-well plates at a concentration of $2–2.5 \times 10^5$ cells per well. After 24 h, cells were treated with DMSO and 2 μM of 5-aza-2′-deoxycytidine for 3 days or 500 nM of trichostatin A for the last 24 h.

Plasmid construction

The SOCS1 expression vector was generated by PCR cloning from cDNA using a pair of primers with BamHI and XhoI sites in their 5′ end, respectively, as follows: forward: 5′-CGCGGATCCATGGTAGCACACAACCAGGT-3′ and reverse: 5′-GACGCTCGAGAATCTGGAAGGGGAAGGAG-3′. The full-length coding sequence was subcloned into pcDNA3.1/V5-his-tag expression vector and was sequenced for verification. The p21-Luc plasmid was described as before [26]. The MDM2-Luc plasmid was a gift from Dr. Jianhua Yang, the Baylor College of Medicine. An MDM2-Luc plasmid was used as a reporter plasmid which contained the wild-type MDM2 promoter upstream of the luciferase gene.

Luciferase reporter assay

Cells were seeded in 12-well plates, and 24 h later, they were cotransfected with 0.3 μg of MDM2-Luc or p21-Luc together with 0.6 μg expression plasmid of either SOCS1 or p53, and 25 ng of pRL-TK for each well as an endogenous control. The luciferase activity in each well was quantified 36 h after transfection using a dual luciferase reporter kit (Promega, Madison, WI, USA), following the manufacturer's protocol.

Western blot

Western blot was conducted as described before [26], using primary antibodies against His-tag, p53, p21 and α-tubulin (MBL, Aichi, Japan) and secondary antibodies conjugated with Cy5.5 (Amersham Pharmacia Biotech, Buckinghamshire, UK). Band signals were visualized using the Odyssey Imager (LI-COR Biosciences, Lincoln, NE, USA).

Statistical analyses

The differences between groups were analysed by two-tailed Student's *t*-test using GRAPHPAD PRISM software (version

5.0a; GraphPad Software, Inc., San Diego, CA, USA). Chi-square test or Fisher's exact test was used to determine the association between the SOCS1 CGI methylation status and the patients' different clinicopathological features, and the statistical analysis was performed using the Statistical Analysis System (SAS 9.1 TS level 1M3, Cary, NC, USA). In all cases, a *P*-value of less than 0.05 was considered significant.

RESULTS

Chromosomal loss caused a dramatic down-regulation of SOCS1 in HCC

We have previously screened the copy number aberrations among a panel of 25 pairs of HBV-related HCC. We noticed a frequent loss of this family gene in HCC tumour tissues, especially at the SOCS1 coding region which was found deleted in eight patients (Table 1). Further, real-time qPCR showed that low mRNA expression (1/2 folds or less) was detected in five of eight (62.5%) tumour tissues with SOCS1 gene loss. In the other 17 cases without chromosomal aberration at this region, five of them (29.41%) showed lower SOCS1 mRNA level when compared with their corresponding nontumour tissues. Moreover, the general expression level of SOCS1 in tumour tissues harbouring the deleted SOCS1 gene was significantly lower than that in the adjacent nontumour tissues ($P = 0.0448$; Fig. 1a). Above all, these observations indicated that chromosomal loss of the SOCS1 gene might be one important reason for its down-regulation in HCC. Meanwhile, other mechanism(s) except chromosome loss may also contribute to SOCS1 lower expression in HCC.

Table 1 The chromosomal aberration rates of suppressor of cytokine signalling (SOCS) genes

Gene	Number of deletion	Number of amplification
SOCS1	32% (8/25)	4% (1/25)
SOCS2	0	12% (3/25)
SOCS3	0	8% (2/25)
SOCS4	16% (4/25)	0
SOCS5	4% (1/25)	8% (2/25)
SOCS6	4% (1/25)	8% (2/25)
SOCS7	0	8% (2/25)
CISH	0	0

Methylation status of the SOCS family genes in HCC cell lines and tissues

Using the method established [24], we detected a strong hypermethylation of SOCS1 in 6/8 HCC cell lines. SOCS3, SOCS4, and SOCS5 also showed hypermethylation but in a lesser frequency (1/8–4/8). No methylation of SOCS2/6/7 and CISH was found in any of the eight HCC cell lines tested (Table S3). Therefore, the CGI methylation statuses

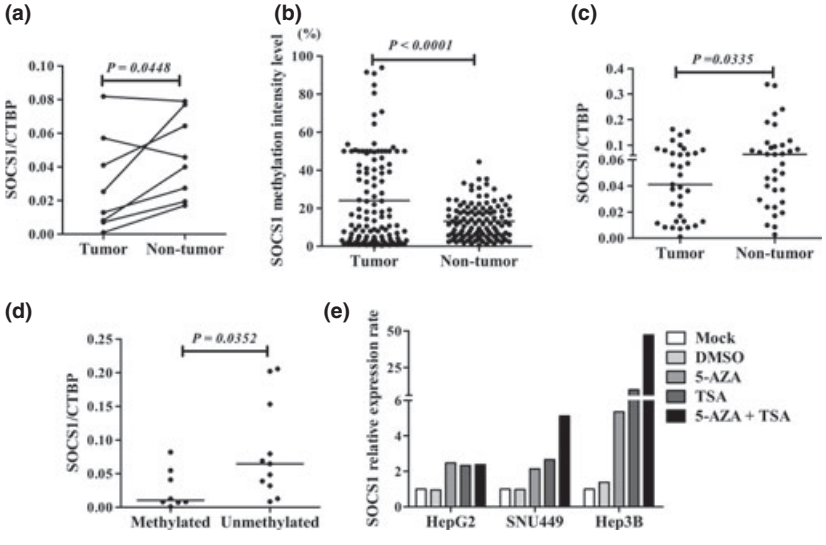

Fig. 1 Both CpG island (CGI) hypermethylation and chromosomal loss can downregulate SOCS1 mRNA expression. **(a)** SOCS1 mRNA expression level in chromosomal deleted samples. **(b)** The methylation intensity of SOCS1 CGI in hepatocellular carcinoma (HCC) tissues ($n = 116$) and adjacent nontumour tissues detected by quantitative methylation–specific PCR. **(c)** SOCS1 mRNA expression level in 35 pairs of HCC tumour and adjacent nontumour tissues. **(d)** SOCS1 mRNA expression level in hypermethylated HCC tissues (intensity ≥ 40%) and unmethylated tissues (intensity < 3%). **(e)** SOCS1 relative expression level in cell lines HepG2, SNU449, Hep3B, after treatment with 5-aza-2′-deoxycytidine for 3 days and/or trichostatin A for the last 24 h. The two-tailed paired or unpaired t-test was used to analyse the difference of expression levels in different groups.

of SOCS1, SOCS3, SOCS4 and SOCS5 were quantitatively analysed in a pilot study of 20 patients with HCC. SOCS1 also showed a relative higher hypermethylation in HCC tumour tissues. In contrast, the hypermethylation of SOCS3 and SOCS5 was found only in one and two cases, and SOCS4 in none of the tumour tissues, respectively. Because of the lower frequency in this exploring cohort, no further tests of SOCS3, four and five genes were performed. We also sequenced the coding region of the SOCS1 gene in several HCC cell lines and found no mutation (data not shown).

The methylation status of SOCS1 was detected in a total of 116 pairs of patients with HBV-related HCC. Almost the same high frequency of SOCS1 hypermethylation was found in tumour (56.03%, 65/116) and the adjacent nontumour (54.31%, 63/116) tissues. However, the hypermethylation intensity in tumour tissues was significantly higher than that in the adjacent nontumour tissues (24.01% *vs* 13.11%, $P < 0.0001$; Fig. 1b). Meanwhile, in 28.45% (33/116) of tumour-derived specimens, the SOCS1 methylation intensity was over 40%, as opposed to only one in the nontumour group. Moreover, pairwise comparison revealed that further stepwise increase in SOCS1 hypermethylation in intensity (≥10%) in tumour tissues was seen in 39.66% (46/116) of tumour tissues, as compared to their adjacent nontumour tissues from the same patients with HCC.

Correlation between CGI methylation and mRNA expression of SOCS1 in HCC tissues and cell lines

Real-time quantitative PCR was used to detect the SOCS1 mRNA expression level in 35 of the 116 pairs of tissues. As expected, the level of SOCS1 mRNA in tumour tissues was statistically lower than that in nontumour tissues ($P = 0.0335$), in a manner inversely related to the methylation intensity of SOCS1 (Figs 1c,d). To further confirm that CGI hypermethylation modulation suppressed SOCS1 gene expression, HCC cell lines HepG2, SNU449 and Hep3B with hypermethylated SOCS1 were treated with the DNA demethylation reagent 5-aza-2′-deoxycytidine, with trichostatin A or both. An obvious increase in SOCS1 expression was detected (Fig. 1e). These results indicated that epigenetic modulation of the SOCS1 gene might be another reason for its low mRNA expression level in HBV infection-related HCC.

Association between SOCS1 hypermethylation status and clinicopathological variables

Associations between the hypermethylation of SOCS1 and patient clinicopathological variables were examined. The number of patients for the association study ranged from 98 to 116, because of missing clinical data of some patients (Table S1). A statistically significant association was found between SOCS1 CGI methylation and male gender ($P = 0.0214$) or older age ($P = 0.0232$) in this cohort (Table 2). Consistently, a significant increase in SOCS1 hypermethylation intensity in tumour tissues was only found among male patients (tumour 27.05% *vs* nontumour 13.38%, $P < 0.0001$, Fig. 2a) or patients older than 45 years old (tumour 26.35% *vs* nontumour 13.21%, $P < 0.0001$, Fig. 2b). Moreover, among the 63 patients with SOCS1 hypermethylation already present in nontumour tissues (with hypermethylation intensity level ≥10%, the threshold established to judge the methylation intensity difference), a further increase in SOCS1 CGI hypermethylation intensity in tumour tissues was more frequently seen in male gender patients(21/44) than that in the female patients (3/19) ($P = 0.0166$).

It has been suggested that 51 years old is the average age of menopause, in those older than the average age of menopause the female-specific hormones like oestrogen are at their lowest levels [27–29]. To investigate whether the gender difference of SOCS1 hypermethylation was oestrogen hormone related, we used the 51 years old threshold to divide the 37 female patients with HCC into two

Table 2 SOCS1 CpG island (CGI) hypermethylation and clinicopathological correlations in hepatocellular carcinoma (HCC)

Feature	Hypermethylated ($n = 65$)	Unmethylated ($n = 51$)	*P*-value
Gender			
Male	50	29	**0.0214**
Female	15	22	
Age			
≥45	55	34	**0.0232**
<45	10	17	
Cirrhosis			
Yes	62	43	0.0997
No	3	7	
TNM stage			
I–II	29	24	0.453
III–IV	28	17	
Portal vein tumour thrombosis			
Present	15	12	0.981
Absent	48	38	
Tumour size			
≥5 cm	50	33	0.0786
<5 cm	11	17	
Tumour encapsulation			
Complete	50	42	0.486
Uncomplete	9	5	

The number of patients analysed ranged from 98 to 116; the number of patients was not identical in all features because of clinical data absence. *P*-values were calculated by chi-square test or Fisher's exact test. *P*-value of <0.05 (two-sided) was considered as significant and written in bold text.

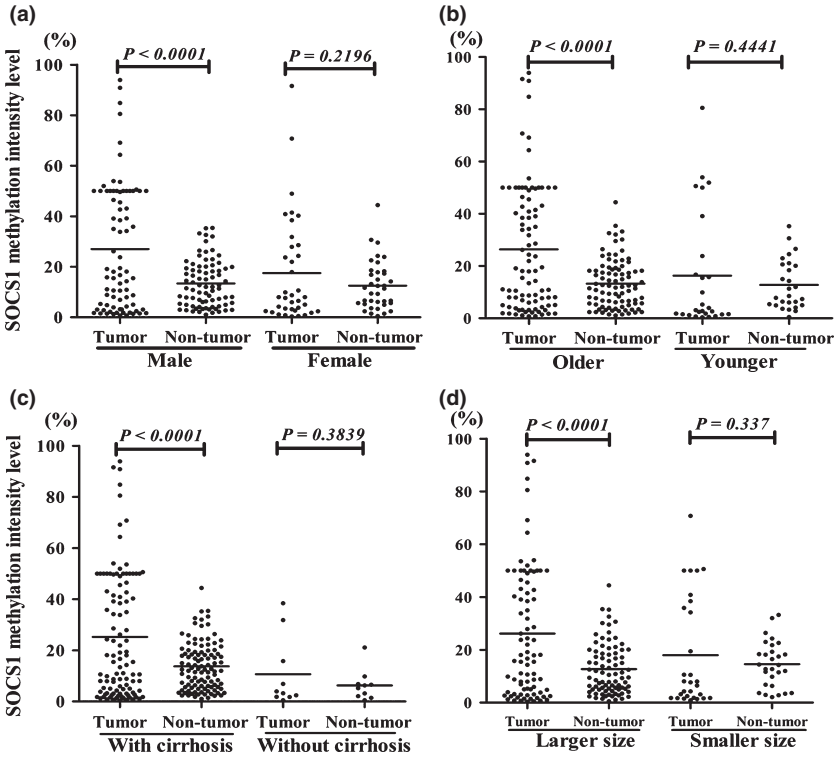

Fig. 2 The association of SOCS1 CpG islandCGI hypermethylation intensities and clinical variables in clinical samples. Data were expressed as the mean hypermethylation intensity for each group, and the *P*-value was computed by paired t-test. **(a)** SOCS1 hypermethylation intensities in different gender patient groups. **(b)** SOCS1 hypermethylation intensities in different age patient groups (older: ≥45 years old; younger: <45 years old). **(c)** SOCS1 hypermethylation intensities in patient groups with or without cirrhosis. **(d)** SOCS1 hypermethylation intensities in patient groups with larger (≥5 cm) or smaller size (<5 cm).

Table 3 SOCS1 CpG island (CGI) hypermethylation and the average age of menopause correlation in different gender patients

		Hyperthylated	Unmethylated	*P*
Male	>51	26	13	0.482
(*n* = 79)	≤51	23	16	
Female	>51	11	9	0.052
(*n* = 37)	≤51	4	13	

groups. Although no statistically significant correlation was detected, a higher frequency of SOCS1 methylation was observed in the older group ($P = 0.052$). In contrast, in the 79 male patients, no similar age-related trend was found (Table 3). Above all, the occurrence of SOCS1 hypermethylation was more likely to happen in male patients and female patients above the average age of menopause, which indicated that SOCS1 hypermethylation might attribute more significantly to HCC development among the male patients, at least with chronic HBV infection.

We also noticed an association of SOCS1 hypermethylation frequency with tumour size ($P = 0.0786$) and liver cirrhosis background ($P = 0.0997$) in this cohort of patients (Table 2). Noticeably, when the average intensity of SOCS1 hypermethylation between tumour tissues and nontumour tissues in the patients with cirrhosis was compared, a significant difference was found (tumour 25.27% *vs* nontumour 13.76%, $P < 0.0001$). In contrast, no significant difference was observed in patients without cirrhosis (10.66% *vs* 6.3%, $P = 0.3839$; Fig. 2c). In addition, patients with larger tumour size (≥5 cm) showed higher SOCS1 hypermethylation in tumour tissues compared with the corresponding adjacent nontumour liver tissues (tumour 26.16% *vs* nontumour 12.69%, $P < 0.0001$; Fig. 2d). These observations implicated that the increase in SOCS1 CGI hypermethylation intensity was associated with tumour size and a cirrhotic background.

SOCS1 was involved in the p53 signal pathway in HCC

To investigate whether SOCS1 was involved in the p53 signal pathway in cells of hepatocyte origin, the HepG2 cell line harbouring wild-type p53 was transiently transfected

with either SOCS1 or a control vector. We found that ectopic SOCS1 expression increased endogenous p53 and p21 protein levels (Fig. 3a). Such SOCS1 induced p53 signalling pathway activation was further confirmed by MDM2 or p21 promoter–luciferase reporter assays in HepG2 cells with wild-type p53, but not in PLC/PRF5 and Huh7 cell lines in which p53 was mutant (Fig. 3b,c). Above all, these revealed the ability of SOCS1 to positively regulate the p53 signalling pathway.

DISCUSSION

The association of SOCS1 down-regulation with CGI hypermethylation in HCC remains uncertain. Some previous investigations showed that SOCS1 CGI hypermethylation was significantly different in frequency between tumour and nontumour tissues [16,18], while others reported that there was already a high frequency of hypermethylation in nontumour tissues [21,30]. In this study, using the improved method for quantitative DNA methylation assay [24], we confirmed that at least in chronic HBV infection-related HCC, SOCS1 CGI hypermethylation already occurred with a high frequency in nontumour tissues. Most importantly, our experiments demonstrated that the intensity of SOCS1 CGI hypermethylation in tumour tissues was significantly higher than that in precancerous cirrhotic tissues. This finding suggested that the CGI methylation-mediated SOCS1 suppression could be an early event during HBV infection-related HCC and may play a role at different stages during the multistep process of HBV infection-related HCC development.

Similar to our previous report on p16INK4a [24], SOCS1 hypermethylation was more likely to happen in male gender patients. Concordantly, in those with hypermethylation in adjacent nontumour tissues, increased hypermethylation in corresponding tumour tissues was also more likely to happen in male patients. The gender difference of SOCS1 hypermethylation may implicate another explanation of male susceptibility to HCC development. This suggestion has been supported by the further observation in female gender patients: the frequency of SOCS1 methylation was significantly higher in patients older than the average age of menopause. It is reasonable to assume that the gender difference of SOCS1 methylation might be due to female-specific hormones like oestrogen. In breast cancer, DNA methylation of multiple oestrogen receptor downstream targets was preferentially observed in ER-negative breast tumours [31]. In addition, oestrogen was found to be able to attenuate tumour progression in HCC by reducing tumour cell invasion, arresting cell cycle progression and promoting apoptosis [32]. Therefore, whether oestrogen can affect the methylation status of SOCS1 in HCC would be an interesting topic to study. Additionally, we found that SOCS1 CGI hypermethylation was more likely to happen in patients aged 45 years or older, and with significantly increased intensity, when compared with the younger age group. This observation also indicated that SOCS1 hypermethylation might contribute to the occurrence of HCC particularly in older individuals.

Except aberrant CGI methylation, here in this study, we also found a high frequency of loss of heterozygosity (LOH) at the SOCS1 gene in HCC, which also contributed to the lower SOCS1 mRNA expression in HBV infection-related HCC. Additionally, we observed an up-regulation of SOCS1 expression in SNU449 and Hep3B cells after trichostatin A treatment, which indicated that histone deacetylation might also contribute to the SOCS1 down-regulation as reported in myeloproliferative neoplasms [33]. Combined with the finding that SOCS1 was targeted by miRNA-155 which was upregulated in HCC [34], we can summarize that SOCS1 down-regulation in HCC was mainly due to chromosomal loss, methylation, deacetylation and microRNA regulation.

SOCS1 was recently suggested to be involved in p53 signalling activation [13,14]. Here, we demonstrated for the first time the presence of the functional SOCS1/p53 axis in HCC, which linked the inflammatory response with DNA damage in liver cancer. The common loss of function

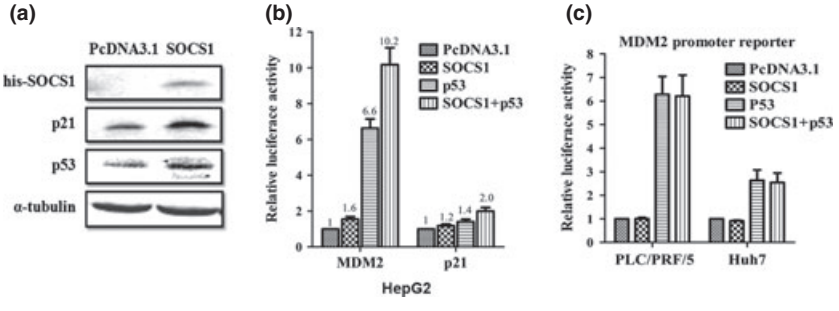

Fig. 3 SOCS1 was involved in p53 signal pathway in hepatocellular carcinoma (HCC). **(a)** Western blot analysis of p53 and p21 proteins in HepG2 cells transfected with pcDNA3.1 or SOCS1. **(b)** Luciferase assay in HepG2 cells transiently transfected with MDM2 or p21 promoter–luciferase plasmid, together with p53 or SOCS1 expression plasmids. The data represent the mean and standard deviation of three independent experiments. **(c)** Luciferase assay in PLC/PRF5 and Huh7 cells transiently cotransfected MDM2 promoter–luciferase plasmid with p53 or SOCS1 expression plasmids.

mutation of p53 in HCC has been demonstrated by us and other groups [12,35,36]. Analysed together with the p53 status, we found that up to 80% of HCC specimens had either SOCS1 hypermethylation or p53 mutation, implicating a majority of patients with HBV infection-related HCC in an SOCS1/p53 axis aberration.

In conclusion, our findings suggest that the aberrant SOCS1 expression through epigenetic silencing or deletion contributes to HBV-related HCC tumourigenesis. Based on our discovery here, it is reasonable to suggest that the methylation modulation-mediated SOCS1 expression silencing is not only an early event in cirrhosis but also plays a role in tumourigenesis originating from cirrhosis. However, further studies are still needed to provide an explanation for this mechanism.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1: Clinicopathological parameters of 116 patients with HCC.

Table S2: summary of primers sequences for methylation assay and the size of the PCR products.

Table S3: Methylation status of the SOCS family in HCC cell lines.