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RESEARCH

Overexpression of MMSET is Correlation with Poor Prognosis in Hepatocellular Carcinoma

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Abstract The multiple myeloma SET domain (MMSET) involved in the t(4;14)(p16;q32) chromosomal translocation encodes a histone lysine methyltransferase. High expression of MMSET is common translocation in multiple myeloma (MM) and is associated with the worst prognosis. Recent studies have shown that overexpression of MMSET is significant in other tumor types compared to their normal tissues. However, little is known about its role in hepatocellular carcinoma (HCC). In these study we investigate the expression of MMSET in HCC and to make correlations with clinicopathologic features. Twenty-eight pairs of HCC and adjacent non-tumor tissues, and eight normal liver tissues were collected for MMSET detection by western blotting and real time-PCR analysis. Immunohistochemistry was used to determine the expression of MMSET in HCC and adjacent non-tumor tissues from 103 patients. Overexpression of MMSET was significantly associated with Edmondson stage, vascular invasion. Moreover, Kaplan-Meier curves showed that MMSET upregulated was associated with shorter overall survival and disease-free survival in HCC patient. In conclusion, our study demonstrates for the first time that overexpression of MMSET is an independent prognostic factor and is correlated with poor survival in HCC patients.

Keywords Hepatocellular carcinoma · MMSET · Clinicopathology · Prognosis · Disease-free survival · Overall survival

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Introduction

Hepatocellular carcinoma is one of the most frequent lethal and aggressive tumors and the second leading cause of cancer mortality in China [1]. It is commonly associated with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections and chronic exposure to the aflatoxin B1 [2]. Although treatments for HCC detected in the early stages include surgical resection, liver transplantation, percutaneous ablation, transarterial chemoembolization (TACE) and sorafenib dosing, the 5-year survival rate is still less than 50 % because of the high prevalence of invasion and metastasis [3, 4]. Therefore, it is important to identify novel molecular markers and discover tumorigenicity mechanism for improving the survival of this disease.

The multiple myeloma SET domain (MMSET, also known as WHSC1 or NSD2), locates at chromosome 4p16, encodes a histone lysine methyltransferase which involves in a functionally complex process and either activate or repress transcription [5, 6]. So it involves in numerous diseases and has several complex mechanisms. MMSET first was identified as a candidate gene for Wolf-Hirschhorn syndrome (WHS) which is a malformation syndrome resulting from a partial deletion of chromosomal material of the short arm of chromosome 4 [7]. It also plays important roles in human carcinogenesis. High expression of MMSET is common translocation in multiple myeloma (MM) and is associated with the worst prognosis [8, 9]. Previous study have showen MMSET protein is frequently and highly expressed in neuroblastoma glioma and prostate cancer, contributing to intensely tumorigenicity [10–12]. From Oncomine Cancer Microarray database, MMSET as a tumor-specific gene is correlation with the development of many of the human cancers including esophagus, liver, stomach, colon, anal canal, lung, urinary bladder and so on

[13–15]. Although MMSET has been founded as a tumor associated gene by genome-wide analysis in HCC, little is known about its feature and function [16]. Thus, in our study we used real time PCR, western blotting and immunohistochemistry to evaluate MMSET expression and its correlation with the survival of HCC patients. Consistent with these studies, we suggest that upregulated MMSET expression is associated with poor survival of HCC, and our findings may provide insights that will lead to better diagnosis, prognosis, and therapeutic opportunities for HCC patients.

Materials and Methods

Patients and Tissue Specimens

Hepatocellular carcinoma and adjacent non-tumor tissues were obtained from 28 patients (20 males and 8 females, median age 46.3 years, range 25–72 years) undergoing liver resection between May 2011 and September 2011 at Xiangya Hospital in Central South University. Nine normal liver samples were collected during surgery from patients who underwent resection following acute liver injury. The samples were immediately snap-frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The study was approved by the Ethics Committee of Central South University, and informed consent was obtained from each patient.

For immunohistochemical (IHC) assays, 103 pairs of paraffin-embedded HCC samples and adjacent non-tumor tissues were obtained from patients who underwent curative resection from February 2006 to March 2007 at Xiangya Hospital. None of the patients received chemotherapy or radiotherapy before surgery, or postoperative adjuvant therapy. The follow-up time was 5 years for 96 patients, ranging from 5 months to 60 months.

Real Time-PCR

Total RNA was extracted using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Reverse transcription was performed according to the protocol provided with the RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas). Real time-PCR was performed with SYBR GreenPCR Master Mix according to the manufacturer's instructions using the ABI 7900 sequence detection system and accompanying analytical software. Briefly, reverse transcription was performed in a final volume of 20 μL containing 2 μg of total RNA sample, 1 μl Oligo(dT)18 primer, 4 μl 5× reaction buffer, 2 μl dNTP mix, 0.5 μl Ribonuclease Inhibitor, 1 μl RevertAidTM M-MulV Reverse Transcriptase, and 11 μl DEPC-treated water. Real time-PCR contained a total reaction volume of 25 μl containing cDNA, 2× SYBR Green PCR Mastermix, primers and nuclease-free water. The reaction was first denatured at 95 °C for 5 min, then 40 cycles at 94 °C for 20 s, 59 °C for 20 s and followed by 72 °C for 20 s. GAPDH mRNA levels were used for normalization. The primers were for both MMSET type I and II, forward 5′-CGGGATCCATG GAATTTAGCATCA-3′, reverse 5′-CGGGATCCATTTTT GATAGGGGTAGT-3′.

Western Blot Analysis

MMSET protein was extracted using a Total Protein Extraction Kit (ProMab, China). Briefly, all proteins were resolved on 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in running buffer (Bio-Rad, USA), and transferred to nitrocellulose membranes (Pierce, USA). After blocking with 5 % milk for 2 h, the membrane were incubated with primary antibodies against MMSET (Abcam, USA), and β-Actin (Zhongshan, China) at room temperature for 2 h. Then, the membranes were washed with phosphate-buffered saline-Tween20 (PBST) and incubated with secondary antibody goat anti-rabbit IgG/HRP (1:5000) (ProMab) for 1 h at room temperature. Enhanced chemiluminescence detection of the target protein was performed using an ECL chemiluminescence system (Pierce).

Immunohistochemistry

All specimens were fixed with 4 % formaldehyde, dewaxed, embedded, and cut into 3 μm serial sections. The sections were then incubated overnight at 4 °C with anti-MMSET antibody (Abcam). After washing with PBS, sections were incubated with secondary antibodies for 30 min at 37 °C. Then, the sections were washed three times with PBS and treated with DAB for approximately 5 min. Finally, the sections were counterstained with hematoxylin, dehydrated, mounted, and examined by light microscopy. Negative controls included incubation without primary antibody. Immunohistochemical staining was assessed by independent experienced pathologists who were blinded to all clinicopathological features. Five high power fields in each specimen were selected randomly, and nuclear staining was considered to be positive staining for MMSET. More than 500 cells were counted to determine the mean percent. Immunoreactivity in <10 % of cells was considered as negative staining, 10–50 % as moderate positive staining, and >50 % as strong positive staining.

Statistical Analysis

All results were expressed as mean ± SD from at least three separate experiments. MMSET levels in HCC, adjacent

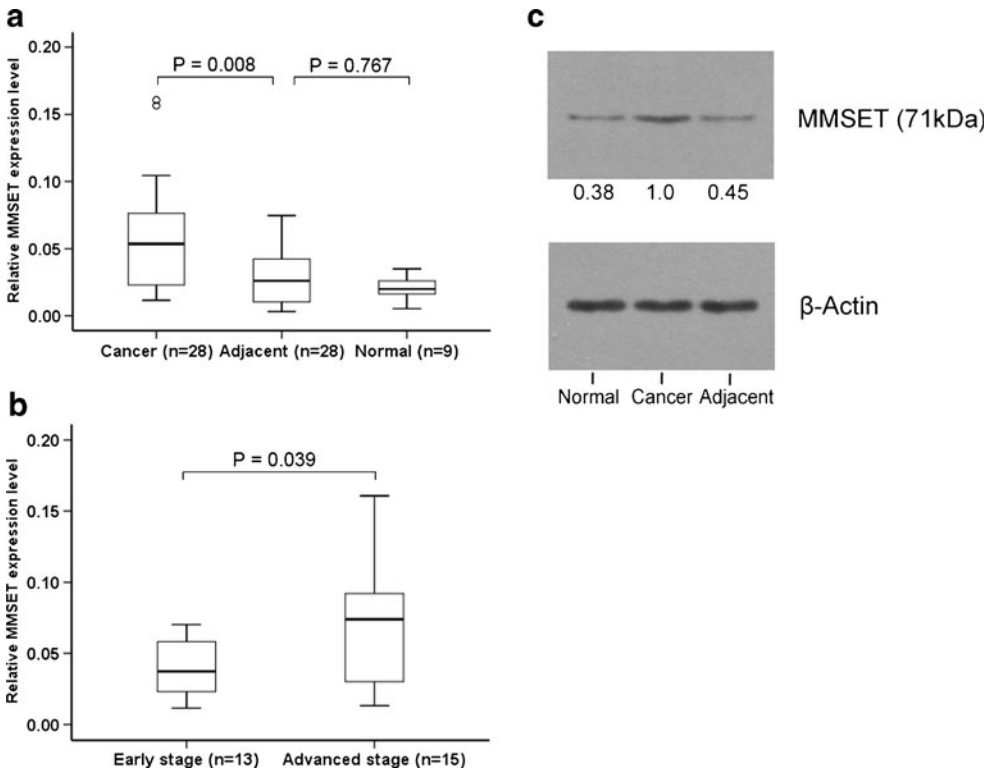

Fig. 1 Comparison of MMSET levels in HCC, adjacent non-tumor tissue and normal tissue. Boxes represent the medians and interquartile ranges of the normalized threshold values. **a**, Relative MMSET expression levels in different tissues: Cancer, 28 paired HCC; Adjacent, adjacent non-tumor tissue samples; and Normal, nine normal liver tissues. **b**, Relative MMSET expression levels in HCC tissues at different stages: early and advanced. **c**, It is a representative western blot analysis of MMSET expression in all specimens

non-tumor tissue and normal tissue was examined by Wilcoxon test. The association between MMSET and clinicopathological features was analyzed using $\chi 2$ test. Survival curves were obtained using Kaplan-Meier curves and log-rank tests. Multivariate prognostic factors were examined using Cox's proportional hazards model. A value of $P<0.05$ was considered statistically significant. Statistical calculations were executed using SPSS software (v.18.0).

Result

The Expression of MMSET in HCC and Adjacent non-Tumor Tissues

MMSET mRNA and protein expression were detected in the 28 pairs of primary HCC and adjacent tissues, and in the nine normal liver tissues. The results in Fig. 1a show that

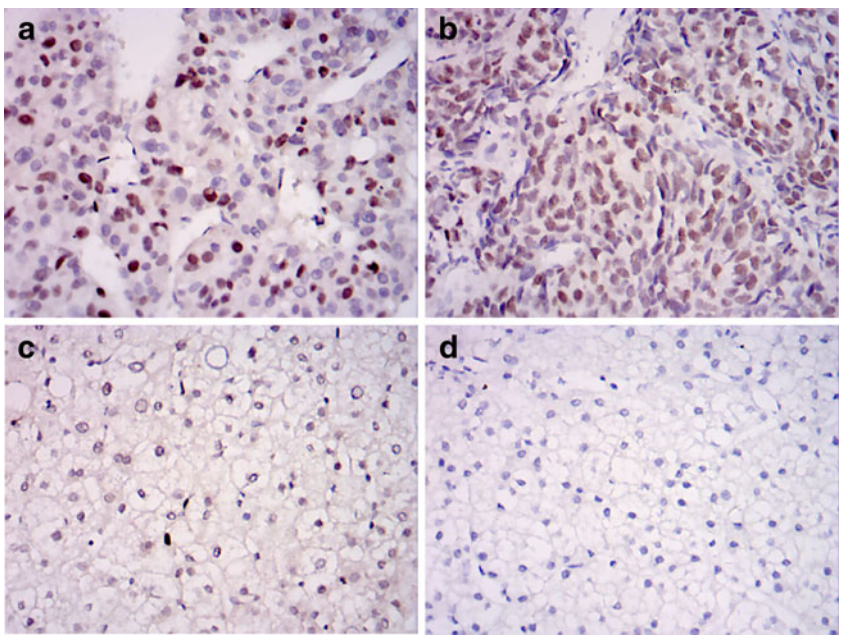

Fig. 2 Immunohistochemical analysis of MMSET in HCC, adjacent non-tumor, and normal liver tissues (original magnification × 400). Representative moderate nuclear staining (**a**) and strong nuclear staining (**b**) of MMSET in HCC tissues. Representative negative staining of MMSET in adjacent non-tumor tissue (**c**) and normal liver tissue (**d**)

MMSET mRNA levels in the HCC sections were higher than in the adjacent tissues (0.0597±0.0394 vs. 0.0303±0.0229, $P<0.05$). However, no significant differences were found between adjacent and normal tissues (0.0303±0.0229 vs. 0.0221±0.0093, $P>0.05$). We also found that miR-301a had different expression levels in the HCC tissues at different tumor stages (the HCC tissues were categorized using the Edmondson grades). As shown in Fig. 1b, MMSET mRNA expression in advanced-stage HCCs ($n=15$) at stages III and IV was significantly higher than in early-stage HCCs ($n=13$) I and II tumors ($P<0.05$). Western blot analysis showed that the MMSET protein expression in HCC was significantly higher than that in adjacent or normal liver tissue (Fig. 1c; $P<0.05$).

Correlation of MMSET Expression with Clinicopathological Characteristics in HCC

One hundred three pairs of paraffin-embedded HCC and adjacent tissue were analyzed by IHC staining. The positive expression rate of MMSET protein in HCC tissues (57/103, 55.3 %) was significantly higher than that in the adjacent non-tumor tissue (19/103, 18.4 %, $P<0.05$). As shown in Fig. 2, MMSET positive staining was predominantly observed in the nucleus, but rarely occurred in the cytoplasm. From IHC analysis, the clinicopathological data of the patients are summarized in Table 1. There were significant differences in MMSET expression associated with Edmondson stage and vascular invasion ($P<0.05$). There were no significant differences in gender, age, tumor size, tumor number, AFP, HBV infection, or capsule invasion.

Table 1 Correlation between MMSET expression and clinicopathologic features of HCC patients

Parameters	Case	Gax expression (n)		χ^2	P
		Positive	Negative		
Tissues					
Cancer	103	57	46	30.108	**0.000**
Adjacent	103	19	84		
Gender					
Male	71	40	31	0.092	0.761
Female	32	17	15		
Age					
≥45	77	41	36	0.541	0.462
<45	26	16	10		
Tumor size					
≥5 cm	58	36	22	2.432	0.119
<5 cm	45	21	24		
Tumor number					
Single	69	42	27	2.586	0.108
Multiple	34	15	19		
AFP					
≥20 ng/ml	62	32	30	0.875	0.349
<20 ng/ml	41	25	16		
HBV infection					
Positive	83	48	35	1.074	0.300
Negative	20	9	11		
Edmondson stage					
I–II	40	17	23	4.362	**0.037**
III–IV	63	40	23		
Capsule invasion					
Yes	47	30	17	2.521	0.112
No	56	27	29		
Vascular invasion					
Yes	31	24	7	6.378	**0.012**
No	72	33	39		

Bold values represent P values are considered to be statistically significant at <0.05

Prognostic Significance of MMSET Expression in HCC Patients

At the end of clinical follow-up, survival information was available in 96 of 103 cases. Patients were divided into a MMSET-positive group ($n=54$) and a MMSET-negative group ($n=42$). Kaplan-Meier survival analysis demonstrated that patients with positive MMSET expression had a shorter overall survival than MMSET-negative patients (median survival, 30.93 months vs 39.59 months; $P=0.014$; Fig. 3). Analysis of disease-free survival also obtained a similar result (median survival, 20.89 months vs 28.52 months; $P=0.037$). As shown in Table 2, univariate analysis showed that overall survival and disease-free survival were correlated with Edmondson stage, capsule invasion, vascular invasion and MMSET expression. Furthermore, Table 3 shows that multivariate analysis using the Cox proportional hazards model indicated that MMSET expression, Edmondson stage and vascular invasion may serve as independent prognostic factors for overall survival. Moreover MMSET expression, Edmondson stage, capsule invasion and vascular invasion are considered as independent prognostic factors for disease-free survival.

Discussion

Hepatocellular carcinoma is one of the most lethal and aggressive neoplasms. With the development of genomic and basic research, increasing numbers of cell molecular biomarkers have been considered for development and application as specific targeted therapeutic agents for HCC [1–3]. In this study, the expression levels of MMSET mRNA and protein in the HCC tissues were significantly

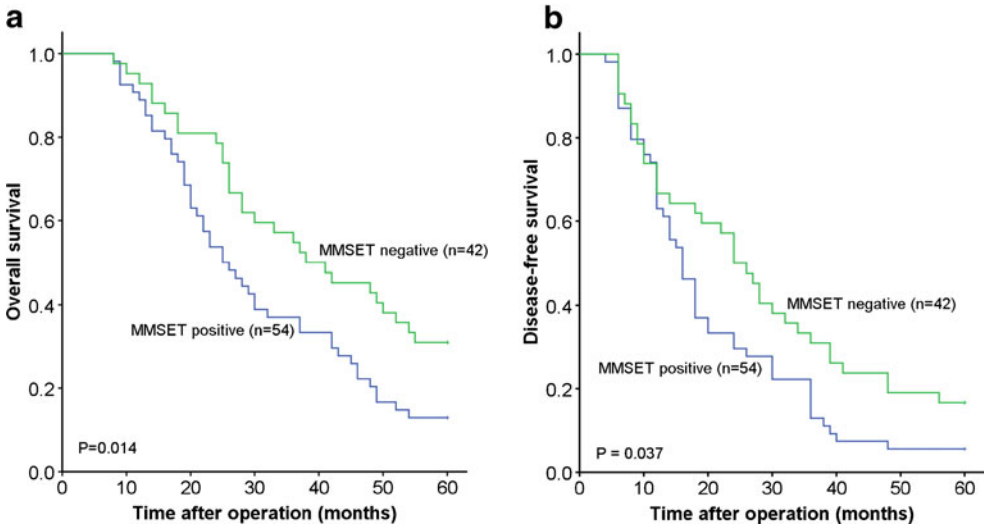

Fig. 3 Kaplan-Meier survival curves of 96 HCC patients based on MMSET expression. **a**, HCC patients with MMSET-positive expression showed significantly shorter overall survival. **b**, HCC patients with MMSET-positive expression showed significantly shorter disease-free survival

higher than in the adjacent tissue and normal liver tissue ($P<0.05$). IHC analysis of a large set of specimens revealed that MMSET protein was positively expressed in 55.3 % (57/103) HCC tissues, compared to 18.4 % (19/103) in adjacent non-tumor liver tissues ($P<0.05$). We correlated MMSET protein expression with respect to various clinicopathological factors in 103 HCC patients. Our results showed that overexpression of MMSET was significantly associated with Edmondson stage and vascular invasion ($P<0.05$). From univariate and multivariate analysis we obtained sufficient evidence to correlate overexpression of MMSET with poor survival in HCC patients, suggesting it might be an important pathogenetic factor in HCC patients.

Histone lysine methylation is a functionally complex process as, depending on sequence-specific lysine methylation sites in histones and on the methylation state of the group of the target lysine, it can either activate or repress transcription [5]. The multiple myeloma SET domain (MMSET), also known as NSD2 or WHSC1, located at chromosome 4p16 encodes a histone lysine methyltransferase [6, 8]. It undergoes alternative splicing resulting in different protein isoforms including MMSET type I, MMSET type II, and RE-IIBP [17, 18]. Previous studies have revealed that MMSET mRNA is expressed in highly proliferating embryonic tissues whereas in adult tissues it is primarily expressed in thymus and testis [8, 19]. On the other hand, MMEST have been shown to be mutated in several diseases. Wolf–Hirschhorn syndrome (WHS) is a malformation syndrome associated with a hemizygous deletion of the distal short arm of chromosome 4 (4p16.3). MMSET, mapping to the 165 kb WHS critical region, as a good candidate gene to be responsible for many of the phenotypic features of WHS [7]. MMSET is identified as a immunoglobulin promoter involved in the t(4;14)(p16; q32) translocation. It have been found in 15–20 % of multiple myeloma and associated with the worst prognosis [8, 9, 20]. From microarrays MMSET protein is frequently and highly expressed in neuroblastoma (MMSET positive in 75 % of neuroblastomas) and associated with negative prognostic factors, and metastatic disease [10]. Li et al. revealed the expression of MMSET increased with ascending glioma grade, and it

Table 2 Univariate survival analysis of overall and disease-free survival in 96 patients with HCC

Features	Overall survival			Disease-free survival		
	HR	95 % CI	P	HR	95 % CI	P
Gender	1.080	0.662–1.762	0.759	1.023	0.648–1.615	0.922
Age	1.215	0.734–2.011	0.448	1.204	0.750–1.934	0.442
Tumor size	0.915	0.581–1.441	0.703	0.949	0.619–1.454	0.810
Tumor number	1.342	0.826–2.180	0.235	1.084	0.679–1.729	0.736
AFP	0.855	0.532–1.375	0.519	0.843	0.539–1.319	0.455
HBV infection	1.049	0.596–1.846	0.869	0.861	0.500–1.485	0.592
Edmondson stage	2.067	1.273–3.356	**0.003**	1.759	1.126–2.746	**0.013**
Capsule invasion	1.964	1.246–3.097	**0.004**	2.316	1.498–3.581	**<0.001**
Vascular invasion	3.583	2.179–5.894	**<0.001**	2.811	1.744–4.529	**<0.001**
MMSET expression	0.565	0.355–0.901	**0.017**	0.642	0.415–0.993	**0.047**

Bold values represent P values are considered to be statistically significant at <0.05

Table 3 Multivariate survival analysis of overall and disease-free survival in 96 patients with HCC

Features	Overall survival			Disease-free survival		
	HR	95 % CI	*P*	HR	95 % CI	*P*
Edmondson stage	2.062	1.236–3.439	**0.006**	1.805	1.129–2.886	**0.014**
Capsule invasion	1.395	0.842–2.311	0.196	1.826	1.141–2.921	**0.012**
Vascular invasion	3.753	2.195–6.417	**<0.001**	2.928	1.767–4.853	**<0.001**
MMSET expression	0.511	0.316–0.828	**0.006**	0.575	0.363–0.910	**0.018**

Bold values represent *P* values are considered to be statistically significant at <0.05

appeared to be directly involved in the proliferative capacity of glioblastoma multiforme cells in vitro [11]. Kang et al. discovered that MMSET was involved in androgen receptor mediated transcription, implicating MMSET in the promotion of prostate carcinogenesis [12]. Searching for uncharacterized proteins with SET domains, Kim et al. identified several different transcripts of the MMSET gene, which was involved in certain types of leukemia and may exert a crucial impact on the regulation of leukemia pathogenesis [19]. Moreover, a search in the Oncomine Cancer Microarray database showed the MMSET expression was elevated in a variety of human cancer types(esophagus, liver, stomach, colon, anal canal, lung, urinary bladder, female genitals, and skin) in comparison to normal counterparts and most benign tumors [13–15].

Although MMSET has been found in several diseases for many years, its function and mechanism has not been completely pinpoint. MMSET complexing with HDAC1 and HDAC2 influences gene expression and potentially acts as a pathogenic agent in multiple myeloma [21]. Simultaneous dysregulation of FGFR3/MMSET and CCND1 into two different IGH alleles but lacking concurrent activation of both proto-oncogenes requires a complete cytogenetic and molecular genetic analysis [8, 22]. Overexpression of MMSET in myeloma cells leads to aberrantly high global levels of H3K36 dimethylation, accompanied by a decrease in levels of H3K27 methylation [23]. Lauring et al. found MMSET played a significant role in multiple myeloma and contributes to cellular adhesion, clonogenic growth, and tumorigenicity [17]. Previous study indicated that MMSET promoted oncogenic transformation in t(4;14) MM patients by transcriptional activation of ID-1 expression [24]. Toyokawa et al. concluded that MMSET carcinogenic effects were mediated by interaction with β-catenin and effected on the WNT pathway [25]. MMSET, playing a restorative role within the genome, regulates histone H4K20 methylation and p53-binding protein 1 (53BP1) accumulation at DNA damage to repair breaks and maintain genetic stability [26]. So we suppose that due to MMSET impelling cancer cells to repair themselves, chemotherapy treatment couldn't well interrupt cancers DNA. However, MMSET associated with tumorigenicity and pathogenesis need to explore.

In conclusion, we have demonstrated that MMSET overexpression is positively correlated with some core clinicopathologic parameters of HCC and the poor prognosis of patients. This information may help to develop novel therapeutic approaches for HCC and prolong patient survival.

Conflict of Interest None

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