Original Article

Copy number variations of *MMP-9* are prognostic biomarkers for hepatocellular carcinoma

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Background: This study aimed to investigate the effect of matrix metalloproteinase-9 (*MMP-9*) copy number variations (CNVs) on hepatocellular carcinoma (HCC) poor prognosis and recurrence.

Methods: A total of 35 patients were collected between January 2016 and December 2018. The copy number and expression level of MMP-9 were measured in 35 HCC tumor tissues and 35 paired adjacent non-tumor tissues using digital polymerase chain reaction (dPCR) and quantitative reverse transcription polymerase chain reaction (RT-qPCR), respectively.

Results: Our results showed that *MMP-9* expression was significantly upregulated in HCC tumor tissues compared to adjacent non-tumor tissues (5.521±9.545 versus 1.000±0.000, P=0.0047). Interestingly, *MMP-9* CNVs only existed in tumor tissues (15/35 versus 0/35, P=0.002). A breakdown analysis by the occurrence of CNVs in tumor tissues had shown that there were significant differences between CNVs group and non-CNVs group in the expression levels of tissue alpha-fetoprotein (AFP) (P=0.015), tumor size (P<0.001), differentiation (P<0.001), microvascular invasion (MVI) (P=0.009), and clinical stage (P<0.001). Receiver operating characteristic (ROC) curves showed that *MMP-9* CNVs and expression were significant predictors of HCC [P<0.0001, area under the curve (AUC) =0.76].

Conclusions: Our results demonstrated that *MMP-9* CNVs were a promising diagnostic biomarker for HCC.

Keywords: Hepatocellular carcinoma (HCC); *MMP-9*; copy number variation (CNV); prognostic indicator

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver. The 5-year survival rate of HCC patients after curative resection was 5–9% from the time of clinical diagnosis (1). In 2012, around 782,500 new patients were reported with liver cancer and 745,500 deaths occurred in the world, and China accounted for about 50% of them (2). With the recent progress in modern medical technologies and the early diagnosis, the resection rate of HCC has been significantly improved. However, a high probability of recurrence after resection has remained a significant challenge in HCC therapy (3). The poor prognosis of HCC is related to high invasion and metastatic capacities of cancer cells (4), which are closely predicted by alpha-fetoprotein (AFP), tumor size, differentiation, clinical stage, and microvascular invasion (MVI) (3).

Matrix metalloproteinase-9 (*MMP-9*) is a matrixin, a class of enzymes that belong to the zinc-metalloproteinases

family. A previous study reported that MMPs played a vital role in cancer invasion and during different stages of cancer progression (5). MMP-9 is highly expressed in HCC, and it participates in angiogenesis by degrading the environmental extracellular matrix and basement membrane (6). Another research showed that *MMP-9* was expressed at a higher level in HCC tissues than in adjacent normal tissues, and *MMP-9* expression was significantly higher in patients with distant metastases or portal vein invasion, indicating that *MMP-9* played a crucial role in the invasion and the metastasis of HCC (7). Several studies were undertaken on *MMP-9* polymorphism of cardio-cerebrovascular disease, and the achieved results confirmed an essential role of *MMP-9* in myocardial infarction (8), temporomandibular disorders (9), intracranial hemorrhage in patients with brain arteriovenous malformation (10), and thoracic aortic dissection (11). However, the role of *MMP-9* polymorphism in HCC is still unknown.

Our genome contains many intermediate size copy number changes, gains, and losses, called copy number variations (CNVs) (12). CNVs can reshape gene structure, modulate gene expression, and contribute to significant phenotypic variation (13). These genomic alterations can range from small insertions or deletions (less than 10 kb) to large ones (over 1 Mb) (14). CNVs are one of the most common genetic variations in the human genome as well as being an important molecular mechanism of pathogenesis in different human diseases such as cancer (15). Digital polymerase chain reaction (dPCR) offers a quantitative method to measure the abundance of a target molecule without requiring a calibration curve, leading to accurate copy number results (16).

In the present study, tumor tissues and adjacent normal tissues were collected from HCC patients in Ningbo Medical Center Lihuili Hospital. This paper aimed to investigate the contribution of *MMP-9* CNVs to HCC prognosis and recurrence.

Methods

Patient and public involvement

The patients were gathered in Ningbo Medical Center Lihuili Hospital between January 2016 and December 2018. All patients were diagnosed by ultrasonography and computed tomography (CT), and histologically diagnosed with HCC. Subjects who had congenital heart disease or other cancers were excluded from the study. The results would be disseminated to each of the participants through the patient's forum.

Origin of specimens

The HCC tumor tissues (n=35), as well as adjacent non-tumor tissues (the tissues from the edge of tumor tissues larger than 2 cm, n=35), were obtained from patients who underwent surgical resections. The HCC tumor tissues and adjacent non-tumor tissues were both confirmed by pathologic diagnoses. The clinical characteristics including the hepatitis B virus, alpha-fetoprotein (AFP), tumor size, differentiation, microvascular invasion (MVI), and clinical stage are collected for the patients. This study was approved by the Ethics Committee of Ningbo Medical Center Lihuili Hospital (Project Identification Code: DYLL2018028), and informed consent was obtained from all patients before the study.

Genomic DNA isolation and TaqMan® copy number assay

Genomic DNA (gDNA) was isolated from tumor tissues and adjacent-normal tissues using the QIAamp DNA Mini Kit (QIAGEN, Germany). The concentration of the purified gDNA was determined by Infinite M200 PRO (TECAN, Switzerland). The FAM™ dye-labeled TaqMan® Copy Number Reference Assay for *MMP-9* (Cat. No. 4400291) was duplexed with the VIC® dye-labeled TaqMan® Copy Number Reference Assay for *RNase P* (*RPPH1*, Cat. No. 4403326).

MMP-9 copy number assay

The copy number of *MMP-9* was measured in each gDNA sample using the QuantStudio™ 3D Digital PCR System (Life Technologies Corporation, NY, USA). *RPPH1* and *MMP-9* signals were amplified in each PCR. A total volume of 16 μL of PCR was prepared for each sample, containing QuantStudio® 3D Digital PCR Master Mix, TaqMan® Copy Number Assay for *MMP-9*, RNase P Reference Assay, and gDNA sample. The PCR was then loaded into the QuantStudio™ 3D Digital PCR 20K chip, which was loaded onto the Dual Flat Block GeneAmp PCR System 9700. The PCR program was as follows: initial melting at 96 ℃ for 10 minutes followed by 39 cycles at 60 ℃ for 2 minutes, 98 ℃ for 30 seconds, and 2 holds at 60 ℃ for 2 minutes. After PCR amplification, chips were read on the QuantStudio® 3D Digital PCR Instrument. Absolute quantification data were exported from QuantStudio™ 3D

AnalysisSuite™ Software (Life Technologies Corporation, NY, USA). *MMP-9* dPCR was duplexed with *RPPH1* as the baseline control (17). The copy number of *MMP-9* was calculated with the following equation: *MMP-9* copy number = raw *MMP-9* number/(raw *RPPH1* number/2). As the number of copies of *MMP-9* was 2 in the normal population, the *MMP-9* CNV (ΔCN value) in HCC patients was calculated by the following equation: ΔCN value = |measured *MMP-9* copies – 2|.

RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total RNAs were extracted from tumor tissues and adjacent non-tumor tissues using TRIzol reagent (Invitrogen, CA, USA). RNA samples were measured by optical density at 260 nm and reversely transcribed using a PrimeScript RT reagent Kit (Takara, Japan). Quantitative PCR (Q-PCR) was performed in the ViiA 7 Q-PCR System (Applied Biosystems Inc., CA, USA) using SYBR® Green PCR Kit (QIAGEN, Germany). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a controller. The primer sequences were 5'-GCCTGCCACTTCCCCTTCAT-3' for forward primer of *MMP-9*, 5'-CAGAAGCCAAACCGGTCGTC-3' for reverse primer of *MMP-9*, 5'-GGGAAATCGTGCGTGACAT-3' for forward primer of *GAPDH*, 5'-TGTTGCTGTAGCCAAATTCGTT-3' for reverse primer of *GAPDH*. The PCR program was as follows: initial denaturation at 95 ℃ for 2 minutes followed by 45 cycles at 94 ℃ for 10 seconds, 60 ℃ for 10 seconds, and 72 ℃ for 40 seconds. The fold variations in mRNAs were normalized to *GAPDH* and calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The statistical analysis was performed using GraphPad Prism software for Windows, version 5.01 (GraphPad Software Inc., CA, USA). The differences between groups were analyzed by two-tailed t-test. Quantitative data were compared using a one-way analysis of variance (ANOVA) or the Kruskal Wallis test. Spearman's rank correlation coefficient (r) was used to determine a relationship between *MMP9* copy number and differentiation in HCC. Data were analyzed using the Mann-Whitney U test. Receiver operating characteristic (ROC) curves were established to evaluate the diagnostic value for the disease. A two-sided P<0.05 was defined as statistically significant.

Results

Characteristics of the tested participants

The clinical phenotypes of tumors and treatments for HCC patients are listed in *Table 1*. There were 12 patients in clinical stage I, 12 patients in the clinical stage II, 5 patients in clinical stage IIIa, 5 patients in the clinical stage IIIb, 1 patient in clinical stage IIIc. All the patients were treated by surgical resection. As shown in *Table 2*, there were 29 patients with positive hepatitis B virus and 20 patients with positive AFP. Among the participants, 2 patients were well differentiated, 21 patients were moderately differentiated, and 12 patients were poorly differentiated. The MVI test showed that 10 patients were not MVI, 16 patients were M1 (low risk), 9 patients were M2 (high risk). The histopathological pictures of tumor tissues and adjacent non-tumor tissues are shown in *Figure 1*.

MMP-9 CNVs degree (ΔCN value) and mRNA fold change in HCC tumor tissues and adjacent non-tumor tissues

We measured the copy number of *MMP-9* in gDNAs isolated from tumor tissues (n = 35) as well as adjacent non-tumor tissues (n=35) in HCC patients using dPCR. Our results showed that *MMP-9* CNVs (ΔCN value) were significantly higher in tumor tissues (0.589±0.770) than in adjacent normal tissues (0.146±0.112, P=0.002, *Figure 2*). We also found that the fold change of *MMP-9* mRNA was significantly higher in tumor tissues (5.521±9.545) than adjacent non-tumor tissues (1.000±0.000, P=0.0047).

Relationship between MMP-9 CNVs and clinicopathological factors in HCC patients

Our results showed that there were 15 HCC patients with CNVs in tumor tissues (CNV group) and 20 HCC patients without CNVs in tumor tissues (non-CNV group). Therefore, we compared the clinicopathological factors between CNVs group and non-CNVs group. As shown in *Table 3*, significant differences of tissue AFP expression (P=0.015), tumor size (P<0.001), differentiation (P<0.001), MVI (P=0.009), and clinical stage (P<0.001) were found between CNV group and non-CNV group. In contrast, there was no significant differences between CNV group and non-CNV group for the other clinicopathological

Table 1 Clinical grading of tumors and treatments for each patient

Patients	Age	Gender	Grading of tumor	Treatment
Patient 1	43	Male	IIIa	Surgical resection
Patient 2	56	Male	II	Surgical resection
Patient 3	48	Male	II	Surgical resection
Patient 4	45	Male	I	Surgical resection
Patient 5	82	Male	II	Surgical resection
Patient 6	61	Male	I	Surgical resection
Patient 7	54	Male	II	Surgical resection
Patient 8	65	Male	IIIb	Surgical resection
Patient 9	60	Male	I	Surgical resection
Patient 10	67	Male	I	Surgical resection
Patient 11	66	Male	I	Surgical resection
Patient 12	69	Male	I	Surgical resection
Patient 13	60	Male	I	Surgical resection
Patient 14	42	Male	IIIb	Surgical resection
Patient 15	76	Female	II	Surgical resection
Patient 16	69	Female	I	Surgical resection
Patient 17	69	Male	I	Surgical resection
Patient 18	51	Male	IIIc	Surgical resection
Patient 19	61	Male	II	Surgical resection
Patient 20	43	Male	IIIa	Surgical resection
Patient 21	65	Male	IIIb	Surgical resection
Patient 22	43	Male	IIIb	Surgical resection
Patient 23	58	Male	IIIb	Surgical resection
Patient 24	71	Female	II	Surgical resection
Patient 25	57	Male	I	Surgical resection
Patient 26	58	Male	II	Surgical resection
Patient 27	64	Male	IIIa	Surgical resection
Patient 28	47	Male	I	Surgical resection
Patient 29	71	Male	IIIa	Surgical resection
Patient 30	64	Male	IIIa	Surgical resection
Patient 31	64	Male	II	Surgical resection
Patient 32	63	Male	II	Surgical resection
Patient 33	69	Male	II	Surgical resection
Patient 34	55	Male	I	Surgical resection
Patient 35	68	Male	II	Surgical resection

Table 2 Clinic characteristics in 35 HCC patients

Characteristics	No. of cases
Age (years)	60.11±10.10
Gender	
Male	32
Female	3
Hepatitis B virus	
Positive	29
Negative	6
AFP	
Positive	20
Negative	15
Tumor size, cm	
≥5	15
<5	20
Differentiation	
Well	2
Moderate	21
Poor	12
MVI	
M0	10
M1	16
M2	9
Clinical stage	
I	12
II	12
IIIa	5
IIIb	5
IIIc	1

AFP, alpha fetoprotein; MVI, microvascular invasion. M0, no MVI; M1 (low-risk), MVI of <5 and at ≤1 cm away from the adjacent liver tissues; M2 (high-risk), MVI of >5 or at >1 cm away from the adjacent liver tissues.

factors, including age (P=0.659), gender (P=0.446), hepatitis B virus (P=0.351) and *MMP-9* mRNA expression level (P=0.430).

Additionally, our ROC analysis for prediction potential of *MMP-9* CNVs and expression levels for HCC showed that *MMP-9* CNVs and *MMP-9* expression were significantly associated with HCC [CNVs: P=0.001, area under curve (AUC) =0.74; *MMP-9* expression: P=0.047, AUC =0.64, *Figure 3*). Moreover, the combined ROC showed a more enhanced diagnostic ability of *MMP-9* CNVs and expression for HCC (P<0.0001, AUC =0.76, *Figure 3*).

Discussion

Our study indicated that the copy number and transcription level of MMP-9 was significantly higher in tumor tissues than in adjacent normal tissues. Also, *MMP-9* CNVs were shown to be associated with several clinicopathological factors such as AFP expression, tumor size, differentiation, MVI, and clinical stage. Our ROC analysis showed that *MMP-9* CNVs and expression were significant predictors of HCC risk.

The gene copy number can be used as an indicator of major diseases (18). For example, CNV of zinc finger matrine type 4 has the potential as a diagnostic indicator of hematological malignancies (19). A circulating tumor DNA derived CNV detection might be feasible for colorectal cancer (20). Single nucleotide variation of *MMP-9* has been studied in breast cancer (21) and gallbladder cancer (22). In this study, we found a higher copy number of *MMP-9* in HCC tumor tissues than their adjacent non-tumor tissues.

MMP-9 high expression was found to be correlated to the increase in copy number of *MMP-9* in patients with colorectal cancer (18) and brain glioma (23). High expression of MMP-9 is strongly associated with HCC invasion and metastasis (6,24). In this study, the achieved result showed that MMP-9 expression level was higher in tumor tissues than that in adjacent normal tissues, and *MMP-9* CNVs appeared only in tumor tissues. The previous study suggested that increased expression levels of the MMP-9 gene were associated with its copy number gains (18). However, our study was unable to find a link between *MMP-9* CNV and mRNA expression in tumor tissues, and both *MMP-9* copy number gain (16/35) and *MMP-9* copy number loss (19/35) were found in our HCC tumor tissues.

MVI is the most relevant risk factor for tumor recurrence in HCC (25). In HCC patients, higher MVI grades and worse differentiation were found to be closely related to the unfavorable prognosis of HCC (3,26). A previous study found that 9p24.2-p21.1 recurrent loss and 8q11.21-q24.3 increase were correlation with the high tumor grade and MVI in HCC patients (27). Here, we found that *MMP-9* copy number gains were closely related to tumor size, clinical stage, differential differentiation, and high

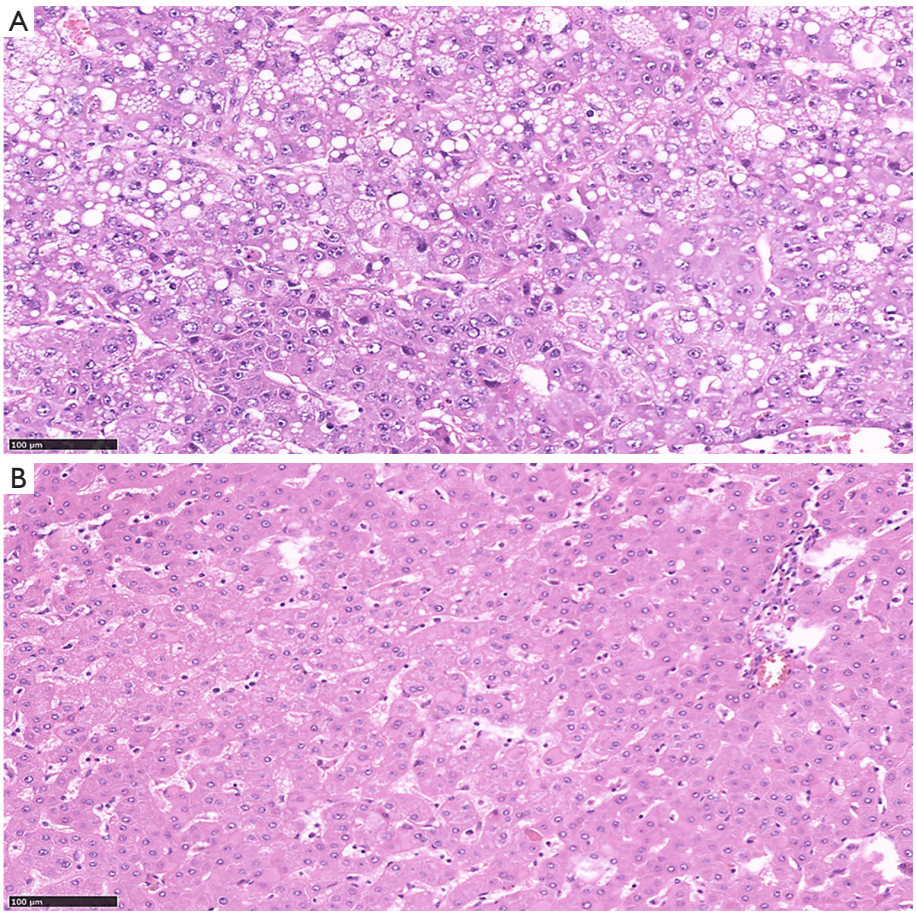

Figure 1 Hematoxylin and eosin (H&E) staining of tumor and adjacent-normal tissue. (A) HE staining of tumor tissue, magnification ×200. (B) HE staining of adjacent-normal tissue, magnification ×200. Scale bar: 100 μm.

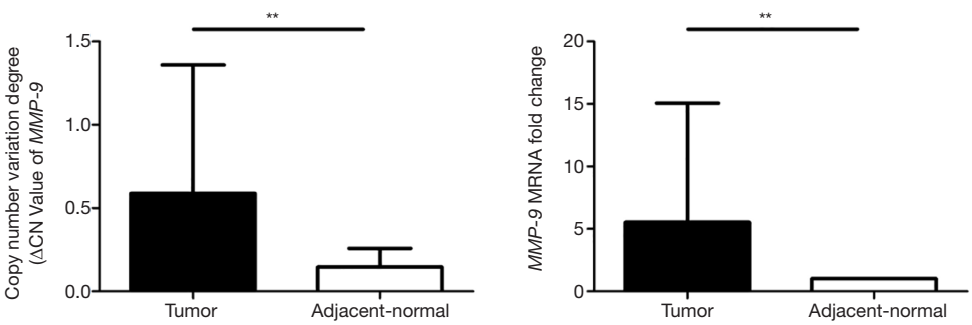

Figure 2 The *MMP-9* CNVs degree and relative expression in HCC tumor tissues and adjacent-normal tissues[#]. [#], *MMP-9* CNVs degree (ΔCN value) in HCC tumor tissues (mean ± SD: 0.589±0.770) compared with adjacent-normal tissues (0.146±0.112, n=35, **P=0.002). The relative expression of *MMP-9* was significant different between the tumor tissues (5.521±9.545) and adjacent-normal tissues (1.000±0.000, **P=0.0047). The available differences between groups were analyzed by paired samples *t*-tests.

Table 3 Relationship of *MMP-9* CNVs in tumor tissues with clinicopathological factors of HCC patients*

Characteristics	CNVs group, n=15	Non-CNVs group, n=20	P value
Age (mean ± SD)	61±8.960	59.45±11.057	0.659
Gender (n, %)			0.446
Male	14 (93.3)	18 (90.0)	
Female	1 (6.7)	2 (10.0)	
Hepatitis B virus (n, %)			0.351
Positive	12 (80.0)	17 (85.0)	
Negative	3 (20.0)	3 (15.0)	
AFP (n, %)			0.015
Positive	10 (66.7)	10 (50.0)	
Negative	5 (33.3)	10 (50.0)	
Tumor size, cm (n, %)			<0.001
≥5	9 (60.0)	6 (30.0)	
<5	6 (40.0)	14 (70.0)	
Differentiation (n, %)			<0.001
Well	0 (0)	2 (10.0)	
Moderate	8 (53.3)	13 (65.0)	
Poor	7 (46.7)	5 (25.0)	
MVI (n, %)			0.009
M0	2 (13.3)	8 (40.0)	
M1	8 (53.3)	8 (40.0)	
M2	5 (33.3)	4 (20.0)	
Clinical stage (n, %)			<0.001
I	4 (26.7)	8 (40.0)	
II	5 (33.3)	7 (35.0)	
IIIa	2 (13.3)	3 (15.0)	
IIIb	3 (20.0)	2 (10.0)	
IIIc	1 (6.7)	0 (0)	
MMP-9 mRNA expression	4.76±2.494	4.13±2.18	0.430

AFP, alpha fetoprotein; MVI, microvascular invasion. M0, no MVI; M1 (low-risk), MVI of <5 and at ≤1 cm away from the adjacent liver tissues; M2 (high-risk), MVI of >5 or at >1 cm away from the adjacent liver tissues. *, The P value is calculated according to the percentage. Data were analyzed using one-way analysis of variance (ANOVA), the Kruskal Wallis test or Mann-Whitney U test. The cut-off concentrations used to distinguish positive and negative results were 20 μg/L for AFP and 0.9 S/CO for hepatitis B virus.

MVI grading in HCC patients.

Serum AFP is the most widely used serological marker for the diagnosis of HCC (28). AFP was found to induce *MMP-9* expression by activating protein kinases and transcription factors (29). Our study found that AFP status in the *MMP-9* CNV group was significantly higher than that in non-CNV group. Therefore, we speculate that there is a vital role of AFP in *MMP-9* CNV and its expression in HCC patients. Future work is needed to verify our hypothesis.

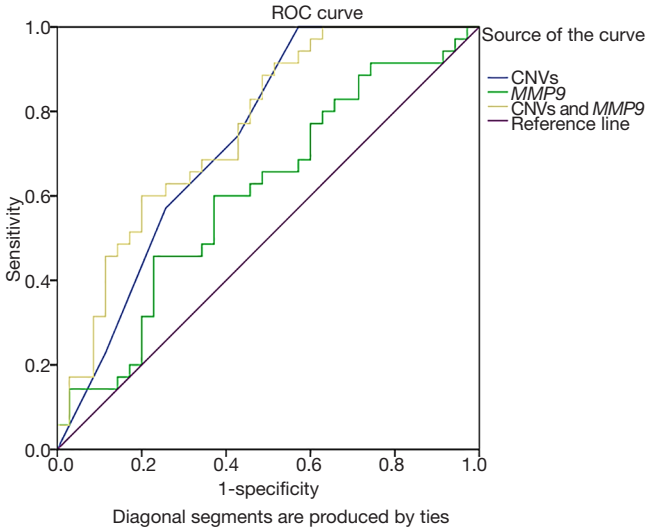

Figure 3 ROC curves analysis for the *MMP-9* CNVs and expression in HCC patients. ROC, receiver operating characteristic; AUC, area under curve; CNVs, copy number variations; HCC, hepatocellular carcinoma. *MMP9* CNVs, P=0.001, AUC =0.74; *MMP9* expression, P=0.047, AUC =0.64; *MMP9* CNVs and expression, P<0.0001, AUC =0.76.

Strengths and limitations of this study

(I) *MMP-9* CNVs appeared only in tumor tissues.
(II) *MMP-9* CNVs were significantly correlated with AFP, tumor size, differentiation, MVI and clinical stage in the HCC patients.
(III) ROC curves showed that the CNVs and expression levels of *MMP-9* were significant predictors of HCC.

Conclusions

This study showed that copy number gains and higher expression of *MMP-9* existed in HCC tumor tissues. *MMP-9* CNVs were associated with a series of clinical indicators, including AFP status, tumor size, differentiation, MVI, and clinical stage. Our findings indicate that *MMP-9* CNVs have potential diagnostic value for HCC screening and prognosis.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.11.52). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Ethics Committee of Ningbo Medical Center Lihuili Hospital (Project Identification Code: DYLL2018028), and informed consent was obtained from all patients before the study.

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