

# Increased *EXT1* gene copy number correlates with increased mRNA level predicts short disease-free survival in hepatocellular carcinoma without vascular invasion

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## Abstract

*Exostosin-1 (EXT1)* has been demonstrated to participate in the progression of many cancers. However, it has not been previously described in patients with hepatocellular carcinoma (HCC) without vascular invasion. In this study, we got the accurate data of *EXT1* mRNA Z-score from the CBio data portal of The Cancer Genome Atlas (TCGA), which was used to express the level of *EXT1* gene expression. We analyzed the *EXT1* gene expression between HCC and normal liver tissue and compared the clinical significance of tumor tissue's *EXT1* gene expression of HCC patients without vascular invasion based on data from TCGA database. The association between *EXT1* gene expression and disease-free survival (DFS) was further analyzed. *EXT1* gene copy number was also analyzed in this study. Univariate and multivariate analyses showed that high *EXT1* gene expression group was significantly poorer than that of the low *EXT1* gene expression group ( $P = .004$ ). In addition, *EXT1* gene expression was positively associated with  $\alpha$ -fetoprotein (AFP), which is a well-known marker for HCC. There was a significant positive correlation between *EXT1* copy number and upregulated *EXT1* gene ( $P < .0001$ ). In conclusion, upregulation of *EXT1* could be an important indicator to the short DFS of HCC patients without vascular invasion. *EXT1* gene copy number amplification is one of the mechanisms underlying the upregulation of *EXT1*.

**Abbreviations:** AFP = alpha fetoprotein, AJCC = American Joint Committee on Cancer, ALL = acute lymphoblastic leukemia cancer, ALT = alanine aminotransferase, CI = confidence interval, DFS = disease-free survival, *EXT1* = (*Exostosin-1*), GEO = Gene Expression Omnibus, HBV = Hepatitis B Virus, HCC = hepatocellular carcinoma, HCV = Hepatitis C Virus, HS = heparin sulfate, SMD = Stanford Microarray Database, TCGA = The Cancer Genome Atlas, TNM = tumor node metastasis.

**Keywords:** copy number, disease-free survival, *EXT1*, hepatocellular carcinoma, prognosis

## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common and malignant tumors in the world.<sup>[1]</sup> With the surgical technology being gradually improved and preoperation period of the treatment becoming better, safety of liver resection and survival rate have been raised, but the recurrence rate is still very high.<sup>[2,3]</sup> Although surgical resection offers the best chance of cure of HCC, the prognosis after surgery differs considerably among patients which have hampered both treatment and prognostic prediction. The molecules responsible for early stage HCC,<sup>[4]</sup>

especially in patients with HCC without vascular invasion have not been fully elucidated.<sup>[5]</sup>

*EXT1* is a protein that in humans is encoded by the *EXT1* gene. This gene encodes an endoplasmic reticulum-resident type II transmembrane glycosyltransferase involved in the chain elongation step of heparan sulfate biosynthesis.<sup>[6]</sup> Mutations in this gene cause the type I form of multiple exostoses.<sup>[7]</sup> Furthermore, aberrant *EXT1* expression is significantly associated with prognosis in breast cancer,<sup>[8]</sup> bile duct cancer,<sup>[9]</sup> and acute lymphoblastic leukemia cancer (ALL).<sup>[10]</sup> However, the prognostic value of EXT in patients with HCC without vascular invasion has not been clearly defined.

In the current study, we have taken advantage of Oncomine datasets<sup>[11]</sup> and The Cancer Genome Atlas (TCGA) database by the CBio data porta.<sup>[12]</sup> Then, we performed gene expression of *EXT1* in HCC. We also investigated the clinical significance of *EXT1* expression in HCC cases and assessed whether *EXT1* expression was associated with the prognosis in patients with HCC without vascular invasion. We analyzed genomic copy number variation of *EXT1* gene, which maybe one of the mechanisms underlying the upregulation of *EXT1* which could be related to the prognosis.

## 2. Materials and methods

### 2.1. Ethical approval

This study was approved by the ethics committee of Wuxi No.9 People's Hospital affiliated to Soochow University, P.R. China.

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The authors have no conflicts of interest to disclose.

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All methods were performed in accordance with the relevant guidelines and regulations.

## 2.2. Public dataset

Bioinformatic analysis was based on data from Oncomine datasets and TCGA database. Oncomine datasets (<https://www.oncomine.org>) is a public cancer microarray platform incorporating 392 independent microarray datasets, totaling >28,880 microarray experiments and spanning 41 cancer types. It unifies a large compendium of other published cancer microarray data, including Gene Expression Omnibus (GEO)<sup>[13]</sup> and Stanford Microarray Database (SMD),<sup>[14]</sup> and uniquely provides differential expression analyses comparing most major types of cancer with their respective normal tissues.

The Cancer Genome Atlas (TCGA) (<https://tcga-data.nci.nih.gov>) database stores genomic and clinical data of a series of common cancers including HCC<sup>[15]</sup>; all data have been made public for analysis. Tissue and tumor specimens were obtained from patients with appropriate consent from institutional review boards under the guidelines of The Cancer Genome Atlas Research Network. Informed consent was obtained from all patients or their guardians, including the use of tissue samples for research purposes. The official TCGA report on HCC was released and the database was analyzed on the CBio data portal (<http://www.cbioportal.org/>). Copy number variance was determined by profiling HCC on Affymetrix SNP 6.0 arrays<sup>[16]</sup> and analysis by GISTIC 2.0.<sup>[17]</sup> Values:  $-2$ =deep deletion;  $-1$ =shallow deletion;  $0$ =neutral/no change (diploid);  $1$ =gain;  $2$ =amplification. Surgical resection of biopsy bio specimens was collected from patients diagnosed with HCC, and had not received prior treatment for their disease (ablation, chemotherapy, or radiotherapy). Cases were staged according to the American Joint Committee on Cancer (AJCC).<sup>[18]</sup>

## 2.3. Patients and analysis of clinical data

From Jan 1<sup>st</sup>, 1995 to Feb 16<sup>th</sup>, 2016, 363 TCGA-HCC cases by whole-exome sequencing and DNA copy number analyses were analyzed, there were 203 patients without vascular invasion (including macroscopic and microscopic vascular invasion) among these patients. The patients without vascular invasion included 134 men cases and 69 women cases with an average age of 60.1 years (from 16–85 years of age). A descriptive table of clinical features, histological features, and molecular features for the 203 cases cohort as well as a patient level summary are shown in tables below. We got the accurate data of *EXT1* mRNA Z-score from the CBio data portal of TCGA-HCC, which was used to express the level of *EXT1* gene expression. We analyzed the clinical significance of tumor tissue's *EXT1* gene expression of HCC patients without vascular invasion. The association with *EXT1* expression and disease-free survival (DFS) was also analyzed. We investigated *EXT1* gene copy number of this study in comparison with its mRNA levels. The heat map data of *EXT1* gene copy number and mRNA levels were collected using the TCGA database by the CBio data portal. *t*-test was used to evaluate the effect of *EXT1* gene copy number on its mRNA levels.

## 2.4. Statistical analysis

The Kaplan–Meier survival analysis (log-rank test) was used to analyze HCC patients' DFS time. The factors with  $P < .05$  from

the Kaplan–Meier analysis were enrolled in the Cox regression hazard model. Log-rank test was applied to compare the survival distribution of 2 groups. Categorical variables were compared using the chi-squared test or Fisher exact test.  $P$  values were 2 tailed. Statistical significance was accepted for  $P$  values  $< .05$ . The data were statistically analyzed with the software SPSS Statistics, version 18.0 (SPSS Inc., Chicago, IL). The tumor tissue's *EXT1* mRNA expression and its copy number variation status in different HCC patients were examined by the GraphPad Prism7 software (GraphPad Software, Inc., La Jolla, CA). Data were presented with mean  $\pm$  SD.

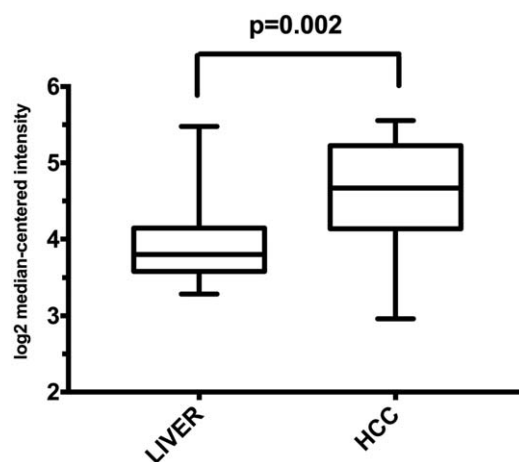
## 3. Results

### 3.1. *EXT1* is upregulated in HCC

To explore the expression and significance of *EXT1* in HCC, a study was performed to compare the gene expression between HCC and normal liver tissue using the Oncomine database. In this study, we chose the *EXT1* expression data in Roessler Liver<sup>[19]</sup> (Hepatocellular Carcinoma vs Normal), including 21 normal liver tissues and 22 HCC tissues. Gene expression profiling was carried out on Affymetrix Gene Chip HG-U133A 2.0 (Affymetrix, Santa Clara, CA) arrays according to the manufacturer's protocol. Then, the gene expression (mRNA) data were plotted. We found that *EXT1* was upregulated in HCC tissues compared with normal liver tissue ( $P = .002$ ) (Fig. 1).

### 3.2. Independent factors influencing prognosis of patients after HCC resection

To estimate the clinical significance of *EXT1* expression in HCC patients without vascular invasion, *EXT1* mRNA expression of the patients who underwent resection of HCC was analyzed. The mRNA Z-score value was used to express the level of *EXT1* gene expression. According to the Z-score value, cases were divided into 2 groups, the high *EXT1* expression group were defined as the Z-score value  $> 2$  while the low *EXT1* expression group were defined as the Z-score value  $< 2$ . The data of 203 HCC patients without vascular invasion was analyzed by Kaplan–Meier analysis. The DFS rates of 1, 2, and 5 years were 74.4%,



**Figure 1.** Expression of *EXT1* mRNA levels in 21 normal liver tissues and 22 HCC tissues. The data were displayed as log<sub>2</sub> median-centered intensity,  $P = .002$ ; *t*-test = 3.118; fold change = 1.548. *EXT1* = (*Exostosin-1*), HCC = hepatocellular carcinoma.

**Table 1****The influencing factors of the DFS in HCC patients without vascular invasion.**

Factors	Kaplan–Meier		Cox regression hazard model	
	Median (mo)	P	HR (95%CI)	P
Age ( $\leq 65 / > 65$ years old)	36.0/33.9	.801		
Gender (male/female)	42.2/21.6	.081		
Race (Asian /Black or African American/ White)	50.0/67.6/19.7	<b>.020</b>	1.156 (0.725–1.843)	.542
History of alcoholism (Yes/No)	42.2/29.7	.233		
HBV infection (none/HBV)	24.0/66.6	<b>.037</b>	1.049 (0.415–2.650)	.920
HCV infection (none/HCV)	42.2/19.2	<b>.024</b>	2.131 (1.161–3.909)	<b>.015</b>
Family history of cancer (Yes/No)	33.9/36.7	.713		
Serum AFP at procurement ( $\leq 20 / > 20$ $\mu\text{g/L}$ )	36.0/25.5	.572		
Cirrhosis (Yes/No)	29.7/33.9	.565		
Child–Pugh classification (A/B)	36.7/24.0	.400		
Surgical margin (R0/R1)	36.7/11.2	<b>.018</b>	2.162 (0.724–6.454)	.167
TNM stage (T1/T2–T4)	60.4/15.0	<b>.000</b>	2.459 (1.427–4.239)	<b>.001</b>
Neoplasm histologic grade (G1/G2/G3/G4)	67.6/33.9/33.1/40.0	.454		
EXT1 expression (high/low)	50.3/18.33	<b>.004</b>	2.223 (1.271–3.887)	<b>.005</b>
EXT1 CNV (increased/not-increased)	33.1/42.0	.645		

CI = confidence interval, CNV = copy number variation, DFS = disease-free survival, *EXT1* = (*Exostosin-1*), HBV = Hepatitis B Virus, HCV = Hepatitis C Virus, HR = hazard ratio, TNM = tumor node metastasis. Bold values indicate significant differences ( $P < .05$ ).

58.7%, and 38.0%, respectively; the median DFS time was 36.0 months. The overall survival rates of 1, 2, and 5 years were 88.4%, 77.0%, and 56.0%, respectively; the median overall survival time was 80.7 months. The results also showed that white people, men, no HBV infection, HCV infection, R1 hepatectomy, TNM stage (T2–T4), high *EXT1* expression were important factors that had influenced the DFS time ( $P < .05$ ). The results of Cox regression hazard model analysis showed that HCV infection, TNM stage (T2–T4), and high *EXT1* expression were independent risk factors influencing DFS ( $P < .05$ ) (Table 1).

The results indicate that high *EXT1* expression is an important and independent factor influencing the DFS. Therefore, we focused on this factor and performed further analyses.

### 3.3. The clinical significance of *EXT1* expression in HCC patients without vascular invasion

Then the correlations between *EXT1* expression and clinicopathological characteristics of HCC were analyzed. As shown in Table 2, we found that the expression of *EXT1* of HCC patients was positively associated with the corresponding  $\alpha$ -fetoprotein (AFP) level ( $\chi^2 = 6.948$ ,  $P = .010$ ). As AFP is frequently used for

the prognosis of HCC, the correlation indicated that *EXT1* might be a potential biomarker of HCC.

The DFS of the patients in high and low *EXT1* expression group. The results of Kaplan–Meier analysis showed that in the high *EXT1* expression group, the median DFS was 18.3 months. The 1-, 2-, and 5-year DFS rates were 61.0%, 38.6%, and 0.0%, respectively. In the low *EXT1* expression group, the median DFS was 50.3 months. The 1-, 2-, and 5-year DFS rates were 78.0%, 63.5%, and 44.1%, respectively. The analysis showed that there was statistically significant difference in the DFS between the 2 groups ( $P = .004$ ). The DFS of the high *EXT1* expression group was significantly shorter (Fig. 2). The result suggests that *EXT1* gene expression is important to DFS of the HCC patients.

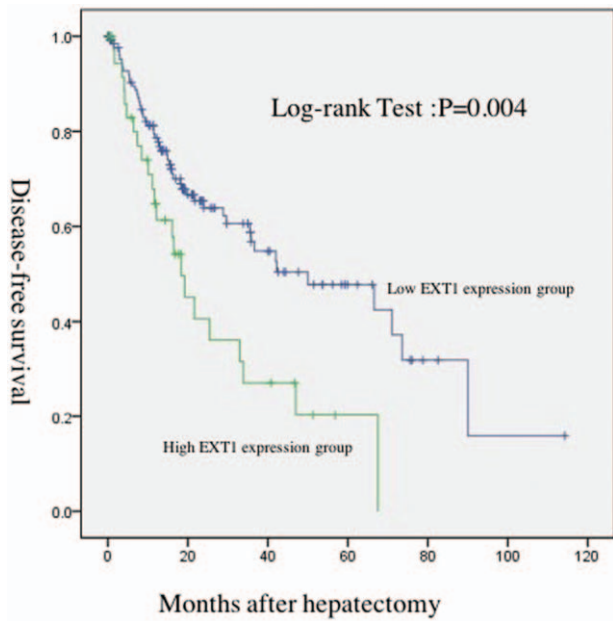
### 3.4. The expression of *EXT1* correlates with the gene copy number in HCC patients without vascular invasion

To verify *EXT1* gene copy number variation and their impact on expression of *EXT1* gene in HCC, we used the CBio data portal to quarry the TCGA dataset. Overall, the average *EXT1* mRNA Z-score level was 0.8341 (range,  $-1.4292$ – $8.6263$ ). Patients were divided into 2 groups: increased *EXT1* gene copy number group (gene amplification), not increased *EXT1* gene copy

**Table 2****Clinical pathological data in the high *EXT1* expression group and the low *EXT1* expression group.**

Factors	High <i>EXT1</i> expression group (n = 43)	Low <i>EXT1</i> expression group (n = 156)	$\chi^2$	P-value
Age ( $\leq 65 / > 65$ years old)	24/19	88/68	0.005	1.000
Gender (male/female)	26/17	104/52	0.572	.473
Race (Asian /Black or African American/ White)	20/2/20	61/9/80	0.669	.716
History of alcoholism (yes/no)	14/23	44/107	1.054	.325
HBV infection (none/HBV)	22/16	99/53	0.688	.453
HCV infection (none/HCV)	29/9	127/25	1.084	.344
Family history of cancer (yes/no)	12/25	54/83	0.603	.567
Serum AFP at procurement ( $\leq 20 / > 20$ $\mu\text{g/L}$ )	13/20	84/46	6.948	<b>.010</b>
Cirrhosis (yes/no)	8/19	39/72	0.293	.588
Child–Pugh classification (A/B)	28/4	110/11	0.333	.519
TNM stage (T1/T2–T4)	27/16	113/42	1.662	.255
Neoplasm histologic grade (G1/G2/G3/G4)	4/18/17/3	29/77/47/3	5.995	.112

CI = confidence interval, DFS = disease-free survival, *EXT1* = (*Exostosin-1*), HBV = Hepatitis B Virus, HCV = Hepatitis C Virus, TNM = tumor node metastasis.

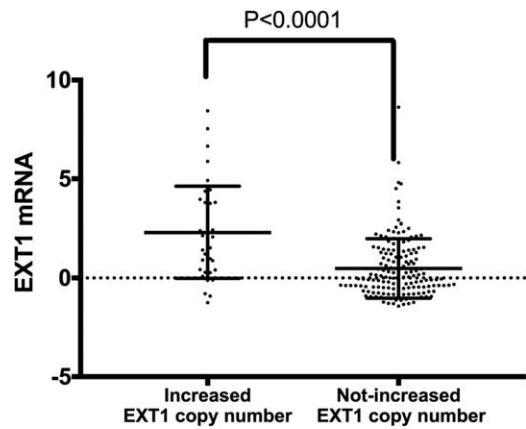


**Figure 2.** Comparison of DFS between high *EXT1* expression group (green line) and low *EXT1* expression group (blue line). Some patients' *EXT1* gene expression data or survival were missing,  $P = .004$ . DFS = disease-free survival, *EXT1* = (*Exostosin-1*).

number group (including gene gain, deep deletion, shallow deletion, diploid). Increased *EXT1* gene copy number group was found in 19.4% patients, *EXT1* mRNA Z-score level was  $2.300 \pm 0.3765$ ,  $n = 38$ . While in the group of not increased *EXT1* gene copy number, *EXT1* Z-score level was  $0.4833 \pm 0.1196$ ,  $n = 158$ . As shown in Figs. 3 and 4, a positive correlation was found between *EXT1* gene copy number and *EXT1* mRNA ( $P < .0001$ ).

#### 4. Discussion

Years ago, techniques such as ultrasonography, computed tomography, and magnetic resonance imaging were not available. HCC was almost always diagnosed when the cancer produced symptoms. Years later, it became feasible to diagnose HCC at earlier stages, before symptoms developed.<sup>[20]</sup> Very early and early-stage HCC patients without both macrovascular and microvascular invasion can benefit from potentially curative treatments, such as surgical resection.<sup>[21]</sup> But long-term survival of early-stage HCC patients is still very low,<sup>[22]</sup> studies now aim

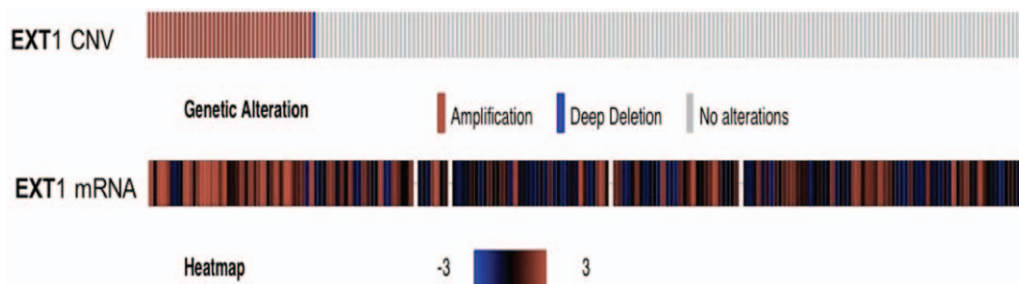


**Figure 4.** Increased *EXT1* gene copy number correlates with increased mRNA levels in HCC patients without vascular invasion,  $P < .0001$ . *EXT1* = (*Exostosin-1*), HCC = hepatocellular carcinoma.

to identify molecular markers for risk and determination of prognosis and treatment<sup>[23]</sup>.

Now *EXT1* gene has been used as an approach to identification of molecular biomarkers for cancer occurrence, classification, and prognosis. But until now little has been known about the role and molecular mechanism of *EXT1* in HCC. In this study, we found that *EXT1* gene expression was upregulated in some cases. These data suggested that *EXT1* gene might participate in the tumorigenesis and metastasis of HCC. Moreover, *EXT1* gene mRNA expression was significantly correlated with serum AFP. AFP has been a useful biomarker for HCC,<sup>[1]</sup> the significant positive correlation between AFP and *EXT1* gene strongly suggested that *EXT1* might be another potential biomarker of HCC.

We used the Oncomine data (Roessler Liver) to explore the possibility, we found that *EXT1* was upregulated in HCC tissues compared with normal liver tissue. In fact, *EXT1* was localized in the endoplasmic reticulum of fibroblast cell lines that exhibits glycosyltransferases activities involved in the chain elongation step of heparin sulfate (HS) biosynthesis. Expression of *EXT1* may promote carcinogenesis via its glycosyltransferases activities to synthesize HS.<sup>[24]</sup> The increase in HS has been reported in many cancer types such as in HCC,<sup>[25]</sup> pancreatic cancer,<sup>[26]</sup> breast cancer.<sup>[27]</sup> Its role in tumor progression towards an aggressive phenotype, particularly invasiveness and preparation of metastatic niche, has been proposed. Consistent with these



**Figure 3.** An expression heat map of frequent copy number amplification of *EXT1* gene and mRNA in HCC patients. Some patients' *EXT1* gene expression or copy number data were missing. *EXT1* = (*Exostosin-1*), HCC = hepatocellular carcinoma.

results, we believed that the upregulation of *EXT1* is a consequence of the development of HCC.

Furthermore, we also showed the evidence for the correlation between *EXT1* gene copy number and its expression level. *EXT1* was always associated with diseases including hereditary multiple exostoses, an autosomal dominant bone disorder of its mutation<sup>[28,29]</sup> or, more recently, its expression levels.<sup>[30]</sup> The copy number of *EXT1* gene or the correlation between *EXT1* gene copy number and its expression level has not been previously examined. In this study, we found a positive correlation between *EXT1* gene copy number and *EXT1* mRNA ( $P < .0001$ ). One possible mechanism is gene amplification.<sup>[31]</sup> Gene amplification can be defined as any duplication of a region of DNA that contains a gene. Gene amplification include retro transposition event, ectopic recombination, polyploidy, aneuploidy, and replication slippage. From the perspective of molecular genetics, gene copy amplification is one of many ways in which a gene can be overexpressed. If gene copy amplification occurred in the same reading frame in the coding portion of a gene, it may lead to a long chain of the amino acid, possibly creating increase of protein expression and if this occurred in the non-coding part of the gene, it may affect gene expression and regulation too.<sup>[32]</sup> For example, gene copy amplification and overexpression of *c-myc*, could be frequently observed in many cancers.<sup>[33]</sup>

But there were still some cases of upregulated *EXT1* without increased gene copy number. There are some other mechanisms may contribute to *EXT1* over-expression. Variants located in one or both *EXT1* promoters might influence *EXT1* expression. We searched the relevant documents, and found the *EXT1* promoter was identified within a region of 123 bp and the promoter single nucleotide polymorphisms (SNP) rs3401643 which was lost with the presence of the C-allele resulting in a 56% increase in *EXT1* promoter activity, that could explain part of the *EXT1* over-expression.<sup>[34]</sup> Certain microRNA may also modulate *EXT1* expression. A recent report described there is a negative correlation between *miR-1303* and *EXT1* expression in pediatric astrocytomas, which strongly suggested the regulation of *EXT1* by this miRNA. *EXT1* is also regulated by *let-7g-3p*. This miRNA had a similar expression pattern to that of *miR-1303*, which suggested a synergic action of these miRNAs over the regulation of *EXT1* expression.<sup>[35]</sup> It is certain that some other mechanisms can affect the *EXT1* mRNA as well as its gene copy number. These mechanisms can explain for some outlier value (cases). We recognize it is a limitation of not having a larger series of patients to explore whether *EXT1* gene copy number as well as upregulated mRNA associated with HCC patients' survival. In vitro or in vivo assays will also be done in our further study and will further illuminate factors affecting the expression of *EXT1* and the prognosis of HCC. Animal models such as nude mice would also be selected to further demonstrate this discovery in this study.

Taken together, this study has identified upregulation of *EXT1* could be an important indicator to the short DFS of HCC patients without vascular invasion. Our data also implied that *EXT1* could be a useful target in patients with HCC without vascular invasion. *EXT1* gene copy number amplification may be one of the mechanisms underlying the upregulation of *EXT1*.

### Author contributions

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**Supervision:** Shurong Fan.

**Validation:** Sheng Dong, Lijun Lu, Shurong Fan.

**Visualization:** Sheng Dong, Lijun Lu, Shurong Fan.

**Writing – original draft:** Sheng Dong, Lijun Lu, Shurong Fan.

**Writing – review & editing:** Sheng Dong, Shurong Fan.

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