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The Expression of Dynamin 1, 2, and 3 in Human Hepatocellular Carcinoma and Patient Prognosis

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The classical dynamin family consists of dynamin 1, 2, and 3, which have different expression levels in different tissues to regulate cell membrane fission and endocytosis. Recent studies have reported increased expression of dynamins in human cancer, but their expression in hepatocellular carcinoma (HCC) remains to be determined. This study aimed to investigate the expression of dynamin 1, 2, and 3 in tissue sections of human HCC using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry.

Material/Methods: The expression of dynamin 1, 2, and 3 were investigated in 192 cases of HCC and 14 paired samples of HCC and adjacent normal liver tissue by qRT-PCR and immunohistochemistry. The clinical significance of dynamin 1, 2, and 3 were determined by correlating their expression levels with patient clinicopathological factors and survival rates. Independent prognostic factors were determined using the Cox regression hazard model.

Results: In tissue samples from 192 patients with HCC, the expression of dynamin 1, 2, and 3 were upregulated in 41.15%, 29.69%, and 8.33% of cases, respectively. Dynamin 1 had a significantly increased mRNA expression level in HCC compared with adjacent normal liver tissues and was significantly correlated with alpha fetoprotein (AFP) levels, T stage, and TNM stage. Only dynamin 1 expression was correlated with the reduced overall survival (OS), and was identified as an independent prognostic biomarker of human HCC.

Conclusions: Upregulation of dynamin 1 at the protein and mRNA level was an independent prognostic biomarker of reduced OS in patients with HCC.

MeSH Keywords: Biological Markers • Carcinoma, Hepatocellular • Dynamin II • Prognosis

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Background

Worldwide, primary liver cancer is the fifth most common malignancy [1]. Hepatocellular carcinoma (HCC) accounts for more than 90% of all primary liver cancers, with approximately 800,000 new cases annually [2]. The morbidity of HCC is increasing globally, with an increasing incidence from 1.5 to 4.9 per 100,000 individuals in the past 30 years [1]. The main causes of HCC include chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcohol abuse, and metabolic syndrome that is associated with diabetes and obesity [3].

Although there have been recent developments in targeted therapy for patients with HCC, including sorafenib and lenvatinib [4], the improvements in patient prognosis and overall survival (OS) following the use of these new drugs has been modest [5]. Even following radical surgery, the 5-year OS rate for patients with HCC is approximately 30% [6]. Because the prognosis of HCC remains poor, there is a need for new predictive and prognostic biomarkers for HCC.

Dynamin is a 96 kDa GTPase, and the classical dynamin family consists of dynamin 1, 2, and 3, which have different expression levels in different tissues to regulate cell membrane fission, endocytosis, and secretion of vesicles [7,8]. Dynamin consists of a GTPase domain, a middle domain, a GTPase effector domain, and a pleckstrin homology domain, which synergize to anchor membranes and hydrolyze GTP [9]. Dynamins 1 and 3 are mainly expressed in neural tissue, and dynamin 2 has a wide range of tissues [10]. However, recent studies have shown that dynamin 1 was upregulated in non-neuronal cells downstream of cancer-associated signaling pathways [11]. Although previous studies investigated the functions of dynamin in the regulation of endocytosis and secretion, there is increasing study data supporting the role of dynamin in the progression of human cancer, particularly dynamin 2 [12,13]. Ectopic expression of dynamin 1 and 2 have been shown in several types of cancers, including non-small cell lung cancer (NSCLC) and bladder cancer [12,13]. The expression of dynamin 1 and 2 have been shown to promote *in vitro* tumor cell migration in some specific malignant cell lines [14,15].

Therefore, this study aimed to investigate the expression of dynamin 1, 2, and 3 in tissue sections of human HCC using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry. The study also aimed to correlate the tissue expression of dynamin proteins and mRNA with clinicopathological factors and overall survival (OS) rates.

Material and Methods

Patients studied and ethical approval

Between 2009 and 2013, there were 345 patients with hepatocellular carcinoma (HCC) who were managed at the general surgery department of Qilu Hospital of Shandong University and Yidu Central Hospital, who provided the initial study cohort. From this cohort, 192 patients were selected who underwent radical surgery for HCC with clinical follow-up. There were 14 paired patient samples of HCC liver tissue with adjacent normal liver tissue that were collected and stored in liquid nitrogen from 2019.9. Fresh liver tissues were sampled during surgery, without affecting the procedures for tissue diagnosis. Tumor samples were taken from the non-necrotic areas of the tumor, and the normal adjacent liver tissue was sampled at least 1 cm away from the tumor.

All tissue samples were obtained with prior informed consent from the patients. The overall survival (OS) time was calculated from the time of surgery to death or final follow-up. There were no severe complications during the perioperative period, and all patients had a survival time more than two months after surgery. The tumor staging used was according to the 7th edition of the American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) TNM tumor staging system. The study was supervised and approved by the Ethics Committees of Qilu Hospital of Shandong University and Yidu Central Hospital, China.

Tissue microarrays

The tissue microarrays (TMAs) were prepared, as previously reported, using formalin-fixed and paraffin-embedded tissue samples from the patients with HCC [16]. Hematoxylin and eosin (H&E) staining was performed on the routine tissue sections to assist in the identification of the location of the immunohistochemistry staining. Each case had two 1 mm arrays to improve the sampling accuracy.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from fresh tissues using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. The qRT-PCR was performed with the PrimeScript RT reagent kit and SYBR Premix Ex Taq (Applied Biosystems, Waltham, MA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA of Dynamin 1, with GAPDH as the internal control. The primers used in the study included:
Dynamin 1, forward: AGACCATTGTGAAAAAGCAGGT;
Dynamin 1, reverse: CTTCTTGGTGCACTGTCTAACG;
Dynamin 2, forward: GCATGGGCACGCCACATCTG;

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Dynamin 2, reverse: GCTGCTGTCCCTGGAGAAGGAG;
Dynamin 3, forward: AGTTCGCCTTGAGATTGAAGC;
Dynamin 3, reverse: CGTGTGGGGAATAGACTCGTAAA;
GAPDH, forward: GGACCTGACCTGCCGTCTAG;
GAPDH, reverse: GTAGCCCAGGATGCCCTTGA-3'.

Immunohistochemistry

The streptavidin peroxidase complex method was used to perform immunohistochemistry staining, according to previous studies [17,18]. Briefly, tissues were de-paraffinized and rehydrated using graded alcohol and xylene, and then incubated in 3% H_2O_2 to block endogenous peroxidase. Citrate buffer was used for optimal antigen retrieval, and 5% bovine serum albumin (BSA) was used to block nonspecific antibody binding. Primary antibodies were used at a dilution of 1: 200 to dynamin 1 (ab52611; Abcam, Cambridge, MA, USA), dynamin 2 (ab3457; Abcam, Cambridge, MA, USA), and dynamin 3 (PA1-662; Invitrogen, Carlsbad, CA, USA). Secondary antibodies and the streptavidin peroxidase complex were used (Sangon, Shanghai, China) followed by incubation in 3,3'-diaminobenzidine (DAB) solution (Sangon, Shanghai, China) to visualize the localization of the primary antibodies. The slides were counterstained with hematoxylin and mounted in resin.

Each tissue section was evaluated histologically by two pathologists who were unaware of the pathological data. The immunohistochemistry results were semi-quantified using a visual immunohistochemistry score that included the staining intensity and the percentage of immunopositive cells stained. The staining intensity scores were 0, 1, 2, and 3, which represented negative, weak, moderate, and strong immunostaining. The percentage scores of 1, 2, and 3 represented 0–25%, 25–50%, and >50% of positively immunostained cells. The final immunohistochemistry score was obtained by multiplying these two scores [19]. A combined immunohistochemistry score ≥4 was positive, and a score <4 was negative for the expression of dynamin isoforms by immunohistochemistry.

Statistical analysis

All data were analyzed with SPSS version 22.0 software (IBM, Chicago, IL, USA). The correlations between dynamin expression and clinicopathological factors were calculated using the chi-squared (χ^2) test. The overall survival (OS) curves were calculated by the Kaplan-Meier method, and the statistical differences between the subgroups were evaluated with the log-rank test. The Cox proportional hazards regression model identified the independent prognostic parameters. $P<0.05$ was regarded as statistically significant.

Results

Patient demographic and clinical characteristics

This study included a consecutive cohort of 192 patients with hepatocellular carcinoma (HCC), with a mean postoperative follow-up of 38.2 months. The number of male patients was greater than female patients (173 *vs.* 19) (Table 1). Most patients with HCC had chronic hepatitis B virus (HBV) infection (73.96%), while only 6.77% of patients with HCC had hepatitis C virus (HCV) infection, which was consistent with the known strong association between HBV infection and HCC in China. There were 10.94% of patients who had intrahepatic metastasis (multiple tumor nodules), but only 1.04% of patients with HCC had lymphatic metastasis.

Expression of dynamin isoforms in HCC

The expression of the three dynamin isoforms was detected by immunohistochemistry in the 192 HCC tissue specimens. Dynamin 1 and 3 were expressed in both the tumor cell cytoplasm and cell membrane, and dynamin 2 was mainly expressed in the cell membrane (Figure 1A–1C). Dynamin 1, was previously considered to be expressed in the nervous system and tumor tissue [11]. In this study, dynamin 1 had the highest expression in HCC among all the isoforms of dynamin, and was present in 41.15% of all HCCs. The upregulation of dynamin 2 and 3 was observed in 29.69% and 8.33% cases, respectively (Table 1). The mRNA levels of dynamin 1–3 were investigated in 14 tissue samples of HCC with paired adjacent normal liver tissues. The mRNA levels were standardized with adjacent liver tissues of dynamin 1 as 1.0. In this study, dynamin 2 had the highest mRNA expression levels in normal liver tissue, and dynamin 1 had the highest mRNA expression levels in HCC. Dynamin 1 mRNA was significantly upregulated in HCC compared with normal liver tissue (Figure 1D).

The clinicopathological significance of dynamin

The clinicopathological significances of the expression of dynamin isoforms in the liver tissues were evaluated with the chi-squared (χ^2) test to analyze their association with clinicopathological factors. The demographic factors included data on gender and age. The clinicopathological factors included data on the tumor size and tumor number, and the histopathological findings of vascular invasion, histological grade, tumor T stage, lymphatic metastasis, TNM stage. Clinical data included the presence of HBV and HCV infection and serum levels of alpha fetoprotein (AFP) (Table 2). Dynamin 1 expression was significantly associated with AFP, T stage, and TNM stage ($P=0.001$, 0.003, and 0.002, respectively). The overexpression of dynamin 1 was associated with high levels of AFP. Also, patients with high dynamin 1 expression showed an advanced T stage,

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Table 1. Basic characters of patients with HCC.

Characters	Number	Percentage
Sex		
Female	19	9.90%
Male	173	90.10%
Age		
<60	148	77.08%
≥60	44	22.92%
Tumor size (cm)		
≤5	98	51.04%
>5	94	48.96%
Tumor number		
Single	171	89.06%
Multiple	21	10.94%
Histopathological grade		
I+II	123	64.06%
III	69	35.94%
Vascular invasion		
Negative	127	66.15%
Positive	65	33.85%
HBsAg		
Negative	50	26.04%
Positive	142	73.96%
HCV		
Negative	179	93.23%
Positive	13	6.77%
AFP		
<500 ng/ml	109	56.77%
≥500 ng/ml	83	43.23%
T stage		
I	72	37.50%
II	67	34.90%
III	49	25.52%
IV	4	2.08%
Lymph invasion		
Negative	190	98.96%
Positive	2	1.04%
TNM stage		
I	72	37.5%
II	68	35.4%
III	50	26.0%
IV	2	1.0%
Dynamin 1		
Low	113	58.85%
High	79	41.15%
Dynamin 2		
Low	135	70.31%
High	57	29.69%
Dynamin 3		
Low	176	91.67%
High	16	8.33%

HBsAg – hepatitis B surface antigen; HCV – hepatitis C virus; AFP – alpha fetoprotein.

indicating that dynamin 1 may promote tumor progression. There were no significant factors that were significantly associated with the expression of dynamin 2 and 3.

The prognostic roles of dynamin 1, 2, and 3 determined by univariate analysis

The prognostic significances of dynamin 1, 2, and 3, and other clinicopathological factors were initially analyzed using univariate analysis with the log-rank test (Table 3). The overall survival (OS) curves of low and high dynamin expression were identified using the Kaplan-Meier method. In the dynamin family, dynamin 1 was the only isoforms affecting the prognosis of patients with HCC. Patients with high expression of dynamin 1 had a worse prognosis than those with low expression of dynamin 1, with a 5-year OS rate of 58.4% compared with 34.0% (Figure 2A). However, dynamin 2 and 3 had no significant influence on the OS in this study (Figure 2A, 2B).

In the clinicopathological factors, patients with a large liver tumor size (P<0.001) and positive vascular invasion (P=0.001) had a poorer prognosis (Figure 3A, 3B). Advanced T stage (P<0.001) and TNM stage (P<0.001) were significant prognostic factors for patients with HCC (Figure 3C, 3D). Male patients

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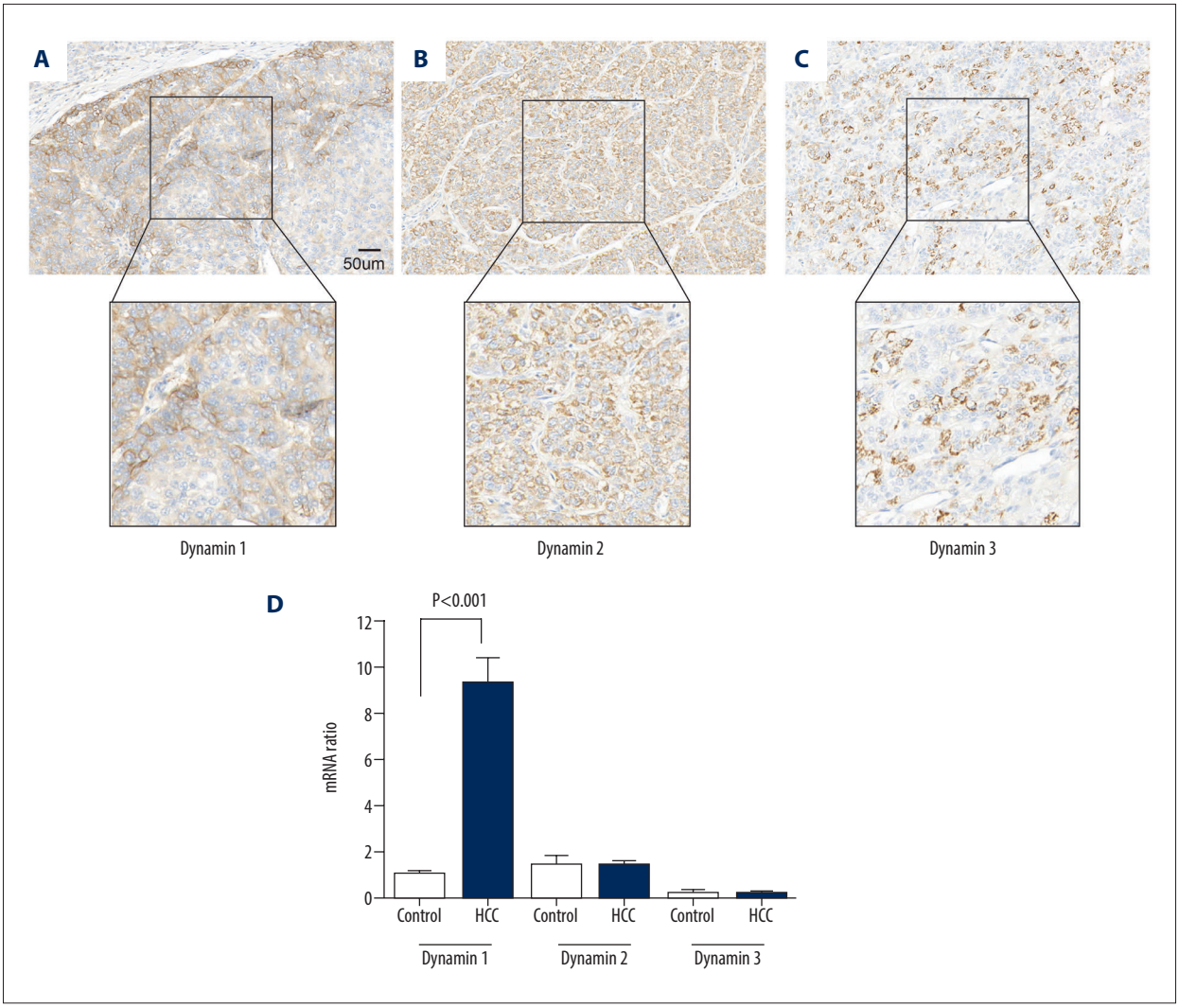

Figure 1. The expression of dynamin 1, 2, and 3 in tissue sections of hepatocellular carcinoma (HCC) and adjacent normal liver tissues using immunohistochemistry and quantitative real-time polymerase chain reaction (qRT-PCR). (**A–C**) The expression of dynamin 1, 2, and 3 detected with immunohistochemistry. Representative photomicrographs of the HCC tissue show positive immunostaining for dynamin. (**D**) The mRNA level of dynamin 1, 2, and 3, were detected by qRT-PCR in 14 paired tissue samples of HCC and normal adjacent liver tissue.

had significantly lower OS rates compared with female patients (P=0.069).

Independent prognostic factors were determined by multivariate analysis

The verified prognostic variables in the log-rank test were selected and analyzed by the Cox-regression model to identify the independent prognostic factors (Table 4). The prognostic factors with a P-value <0.5 underwent multivariate analysis and included the tumor size, vascular invasion, T stage, and dynamin 1 expression. TNM stage was excluded from the model because the above factors subsequently identified it. Dynamin 1 was verified as an independent prognostic biomarker of HCC (P=0.001; HR=2.06; 95% CI, 1.36–3.12). Also, T stage was identified as an independent prognostic factor (P<0.001; HR=2.9; 95% CI, 1.80–4.69). Tumor size was not confirmed to be an independent prognostic factor because it was one of the determinants of the T stage. Vascular invasion was an independent factor, but with low significance (P=0.060).

Discussion

The aims of this study were to investigate the expression of dynamin 1, 2, and 3 in tissue sections of human hepatocellular carcinoma (HCC) using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry, and to

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Table 2. Correlation between Dynamin isoforms and the other characters.

Characters	Dynamin 1		P*	Dynamin 2		P*	Dynamin 3		P*
	Low	High		Low	High		Low	High	
Sex									
Female	14	5	0.221	13	6	0.798	17	2	0.662
Male	99	74		122	51		159	14	
Age									
<60	85	63	0.491	105	43	0.711	137	11	0.371
≥60	28	16		30	14		39	5	
Tumor size (cm)									
≤5	62	36	0.241	69	29	0.976	91	7	0.608
>5	51	43		66	28		85	9	
Tumor number									
Single	101	70	1.000	120	51	1.000	160	11	0.019
Multiple	12	9		15	6		16	5	
Histopathological grade									
I+II	73	50	0.876	87	36	0.871	111	12	0.423
III	40	29		48	21		65	4	
Vascular invasion									
Negative	81	46	0.053	89	38	1.000	115	12	0.584
Positive	32	33		46	19		61	4	
HBsAg									
Negative	27	23	0.504	36	14	0.858	43	7	0.133
Positive	86	56		99	43		133	9	
HCV									
Negative	108	71	0.149	127	52	0.473	164	15	1.000#
Positive	5	8		8	5		12	1	
AFP									
<500 ng/ml	75	34	0.001	77	32	0.909	100	9	0.965
≥500 ng/ml	38	45		58	25		76	7	
T stage									
I+II	91	48	0.003	99	40	0.724	128	11	0.733
III+IV	22	31		36	17		48	5	
Lymph invasion									
Negative	113	77	0.168#	135	55	0.087#	174	16	0.554#
Positive	0	2		0	2		2	0	
TNM stage									
I+II	92	48	0.002	100	40	0.579	129	11	0.695
III+IV	21	31		35	17		47	5	

* Analyzed with Chi-square test, # analyzed with the Fisher test. HBsAg – hepatitis B surface antigen; HCV – hepatitis C virus; AFP – alpha fetoprotein.

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Table 3. Univariate analysis was performed to screen prognostic characters.

Characters	5-year OS(%)	P*
Sex		
Female	73.7	0.069
Male	45.6	
Age		
<60	45.8	0.215
≥60	56.3	
Tumor size (cm)		
≤5	57.6	<0.001
>5	39.2	
Tumor number		
Single	48.6	0.438
Multiple	42.9	
Histopathological grade		
I+II	50.7	0.249
III	46.1	
Vascular invasion		
Negative	55.8	0.001
Positive	33.4	
HBsAg		
Negative	50.6	0.949
Positive	48.4	
HCV		
Negative	48.5	0.521
Positive	46.2	
AFP		
<500 ng/ml	48.9	0.097
≥500 ng/ml	49.6	
T stage		
I+II	59.7	<0.001
III+IV	18.7	
Lymph invasion		
Negative	48.9	0.108
Positive	0.0	
TNM stage		
I+II	59.2	<0.001
III+IV	19.0	
Dynamin 1		
Low	58.4	<0.001
High	34.0	
Dynamin 2		
Low	46.5	0.585
High	52.6	
Dynamin 3		
Low	48.5	0.594
High	43.8	

* Analyzed with log-rank test. HBsAg – hepatitis B surface antigen; HCV – hepatitis C virus; AFP – alpha fetoprotein.

correlate the tissue expression of dynamin proteins and mRNA with clinicopathological factors and overall survival (OS) rates. Of the three members of the dynamin family studied, upregulation of dynamin 1 expression at the protein and mRNA level was an independent prognostic biomarker of reduced OS in patients with HCC.

Previous studies have shown that dynamin is a key protein in endocytosis, but the studies on carcinogenesis and cancer progression are limited. Recently published studies have shown that dynamins are capable of affecting many processes during tumor progression, including chemo-resistance, cell migration, and metastasis [14,20,21]. There have been some previous studies on the expression of dynamin in hepatocellular carcinoma (HCC). Sun et al. showed that mitochondrial fission promoted cell migration in HCC through the Ca2+/CaMKII/ERK/FAK pathway [22]. However, these authors did not investigate whether dynamin was involved in cancer progression as a key modulator of mitochondrial fission.

Previously reported studies have shown that the methylation of the dynamin 3 promoter was capable of downregulating its expression and was correlated with a worse prognosis in patients with HCC [23]. Also, dynamin 3 has previously been shown to reduce HCC tumor growth by activating P53 [24]. However, the down-regulation of dynamin 2 was previously reported to facilitate the invasion of HCC cells by promoting epidermal growth factor receptor (EGFR) signaling [25].

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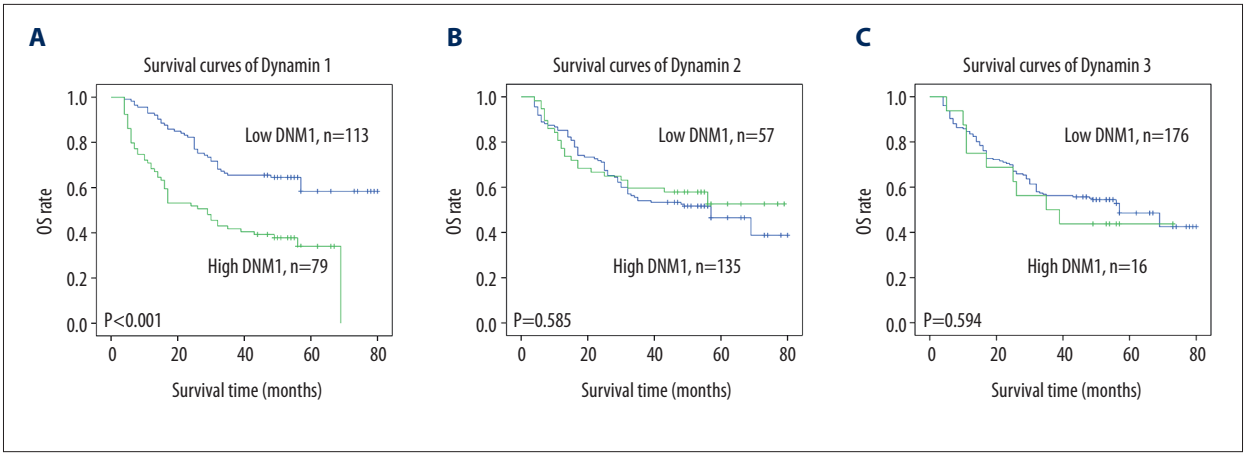

Figure 2. The prognostic significance on overall survival (OS) of dynamin 1, 2, and 3 expression in tissue sections of hepatocellular carcinoma (HCC) and adjacent normal liver tissues. (**A–C**) The correlations between dynamin 1, 2, and 3 expression and the OS curves, evaluated using the Kaplan-Meier method. The prognostic significance was evaluated with the log-rank test.

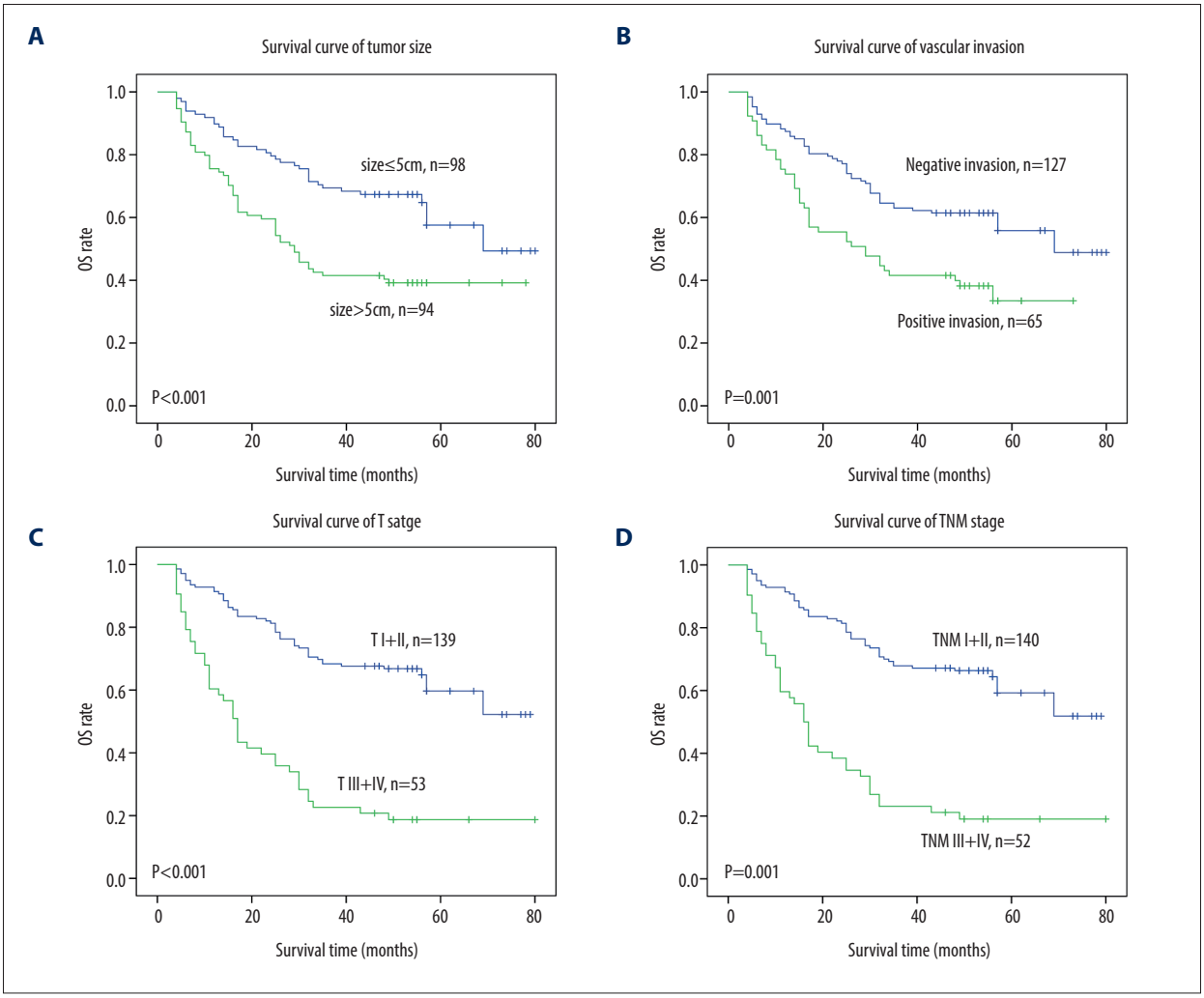

Figure 3. The prognostic significance of tumor size, vascular invasion, T stage, and TNM stage. The correlations between the tumor size (**A**), vascular invasion (**B**), T stage (**C**), TNM stage (**D**), and the overall survival (OS) curves were evaluated using the Kaplan-Meier method. The differences between the subgroups were evaluated with the log-rank test.

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Table 4. Multivariate analysis was performed to confirm the independent prognostic factors.

Characters	HR	95% CI	P*
Tumor size (cm)			
≤5	1		
>5	1.21	0.74–1.98	0.446
Vascular invasion			
Negative	1		
Positive	1.51	0.98–2.31	0.06
T stage			
I+II	1		
III+IV	2.90	1.80–4.69	<0.001
Dynamin 1			
Low	1		
High	2.06	1.36–3.12	0.001

* Analyzed with the Cox-regression model. HBsAg – hepatitis B surface antigen; HCV – hepatitis C virus; AFP – alpha fetoprotein; HR – harzrd ratio; CI – confidential incidence.

The three isoforms of dynamin have similar functions, but their expression varies in different tissues [26]. The findings from the present study showed that in HCC tissues, the expression of dynamin was dynamin 1 > dynamin 2 > dynamin 3. This study was the first to report the expression of all the dynamin isoforms with comparison in the same tumor cohort. Gong et al. showed that dynamin 2 was associated with poor prognosis in patients with HCC [25]. This previous finding differed with the findings from the present study, which found that dynamin 2 was not significantly associated with patient prognosis in HCC. This finding may be explained by the different cut-ff values used in the immunohistochemistry scoring systems. However, in the present study, a large study sample size was used, and dynamin 1 expression was confirmed in the cell membrane by immunohistochemistry, which was consistent with its function as a regulator of membrane fission and endocytosis.

Endocytosis is a fundamental process in all cells, including tumor cells. One important feature of tumor cells is the activated secretion of exosomes, which is regulated by endocytosis and endosomal sorting complexes required for transport (ESCRT) [27]. Dynamins have also been reported to have other functions in human cancers. For example, dynamin 2 was shown to stabilize filopodia in non-small cell lung carcinoma (NSCLC) cells and to facilitate cell migration [12]. Also, dynamin 2 was reported to interact with podocalyxin and regulated cytoskeletal dynamics to promote migration in pancreatic cancer cells [21]. These mechanisms of dynamin-mediated tumor progression are independent of endocytosis.

The detailed molecular mechanisms for the roles of dynamins remain poorly understood, and their role in regulating membrane fission and endocytosis remains controversial [28]. In the present study, dynamin 1 expression was significantly associated with vascular invasion, tumor T stage, and alpha fetoprotein (AFP) levels, which indicated that dynamin 1 might be involved in cell proliferation, cell invasion, and AFP secretion by HCC cells. However, the molecular mechanisms of how dynamin 1 results in the poor patient prognosis in HCC were not identified in this study. However, these molecular mechanisms may rely on cell endocytosis regulation, as liver cells have a strong secretory function.

Although breakthroughs in molecular diagnostics such as next-generation sequencing (NGS) have identified some of the molecular characteristics of HCC, there have been no targeted drugs developed to the most common mutations, and only 25% of tumors possess potentially targetable drivers [29]. Until recently, targeted therapies approved by the US Food and Drug Administration (FDA) have been limited compared to other common cancers, such as lung cancer. More efforts should be made to promote the process of more targeted drugs, which rely on the verification of new biomarkers. The findings from the present study showed that dynamin 1, rather than dynamin 2 or 3, was an independent prognostic factor in patients with HCC. Following the findings from this study, further studies on the function of dynamin 1 in cancer progression may confirm whether dynamin 1 should be included in prognostic immunohistochemistry panels for HCC and other types of cancer.

Conclusions

This study aimed to investigate the expression of dynamin 1, 2, and 3 in tissue sections of human hepatocellular carcinoma (HCC) using quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry, and to correlate the tissue expression of dynamin proteins and mRNA with clinicopathological factors and overall survival (OS) rates. The findings showed that the upregulation of dynamin 1 at the protein and mRNA level was an independent prognostic biomarker of reduced OS in patients with HCC.

Conflict of interest

None.

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