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Original Article

MEK1 expression and its relationship with clinical pathological features in hepatocellular carcinoma

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Abstract: Background: MEK1 is overexpressed in various human carcinomas, but the role of MEK1 is not well unknown in hepatocellular carcinoma (HCC). In the present study, we aimed to explore MEK1 expression of in HCC tissues, and to evaluate its relationship with clinical pathological features. Methods: Expressions of MEK1 were detected by western blot assay, real-time quantitative PCR and immunohistochemical (IHC) staining in 30 HCC tissues and their adjacent normal tissues. Pearson Chi-square test was used to analyze the relationship between MEK1 expression and clinical pathological features. The survival curve was drawn by Kaplan-Meier method, and survival was analyzed by Lon-rank test. Results: The expression of MEK1 mRNA in HCC tissues was significantly higher than that in adjacent normal tissues and so was the expression of MEK1 protein. In the 30 specimens, 70% was with Tumor/Normal ratio > 2, 10% with Tumor/Normal ratio < 1 and 20% with 1 < Tumor/Normal ratio < 2. The mean survival time in high MEK1 expression group was significantly lower than that in low MEK1 expression group (Log-rank value = 11.946, P < 0.01). Conclusion: MEK1 expressions in HCC tissues were significantly higher than that in adjacent normal tissues, which indicated that MEK1 was involved in the genesis and development of HCC. Moreover, it was closely related to the postoperative survival time of patients with HCC.

Keywords: HCC, MEK1, mRNA, survival analysis

Introduction

Annually, about 700,000 people are diagnosed as hepatocellular carcinoma (HCC), which becomes the third leading cause of cancer death in the world [1, 2]. The major cause of HCC is hepatitis B virus (HBV), and hepatitis C virus (HCV)-associated cases is also increasing [3-6]. HBV- or HCV-related liver fibrillation and even cirrhosis are the risks of HCC [4, 7, 8]. For patients are in late stage, especially when metastasis happens, chemotherapy and radiotherapy are commonly adopted and play a prior role in promoting survival rate [8-10]. However, these options are limited, and the results are disappointing since elapse and progress to the advanced stages with metastases often occurs. Normal cells can also be affected at the same time, which brings many adverse effects such as vomiting, nausea, and alopecia [11]. Therefore, the diagnosis of HCC in early stage is the key in increasing survival rate and improving prognosis. The tumor associated molecular markers is a specific thus become the effective markers in diagnosis of liver cancer in early phase.

MEK/ERK and MPAK14/P38 are both the proteins in mitogenic signaling cascade. MEK1/2 can lead to the phosphorylation and activation of ERK1/2 through MEK kinases (MEKK) [12]. It has been demonstrated that MEK/ERK signaling cascade played a key regulatory role in regulating cell cycle, and activating of the pathway was sufficient to promote malignant transformation [13]. Many studies also indicated that MEK1 was abnormally expressed in many tumors [9, 14-17]. In the present study, we investigated the expression of MEK1 in HCC tissues and cells.

The results demonstrated significantly enhanced expression of MEK1 mRNA and protein in cancer tissues and cells, which were closely related with size, differentiation degree, AJCC stage and metastasis of the tumor. Survival analysis showed that patients with high expression of MEK1 had a relatively poor prognosis.

Materials and methods

Patients and specimens

Thirty patients (22 males and 8 females) arranged from 45 to 66 years were enrolled in the study. Thirty tumor tissues and adjacent normal tissues were obtained July, 2008 to July, 2013. All enrolled patients did not undergo chemotherapy or radiotherapy before operation, but they were diagnosed as HCC by pathological examination. A thorough communication was conducted between with patients before operation and all patients voluntarily joined this study with informed consents. In this study, the gender, age of 30 patients along with the differentiation degree, diameter, AJCC stage, grading and metastasis of tumor tissues were collected.

Chemical reagents

Mouse anti-MEK1 monoclonal antibody was purchased from Cell Signaling Technology Company in America (No. 2352). Working concentration of Western blot was 1:1000 and working concentration of IHC was 1:250. 50 X Citrate Antigen Retrieval Solution and explore developer were purchased from Beyotime Biotechnology Company in Shanghai. Immunohistochemical (IHC) staining kit and concentrated DAB reagent were purchased from Vector Laboratories Company in America. Reverse transcription PCR kit and Fluorescence quantitative PCR kit were purchased from TAKARA Company.

Patients grouping

Tumor tissues and adjacent normal tissues were divided in 30 groups, and the expression of MEK1 and mRNA were examined. In addition, further validation confirmations were taken in three HCC cell lines (Hun7, HepG3B and 7402). Clinical pathological features of 30 patients and their relationship with MEK1 mRNA were also explored. The prognosis of patients with high expression of MEK1 was also analyzed.

IHC procedures

The expressions and intracellular localization of MAPK/ERK in HCC and adjacent normal tissues were tested immunohistochemically. HCC tissues and adjacent normal tissues were stained in SABC method, embedded in paraffin and then underwent successive section in 5 μm. After deparaffinization in xylene and rehydration in ascending grades of alcohol, antigen retrieval was conducted in boiled water for 2 h. Nonspecific sites were blocked and the tissues were incubated with primary antibody at 4°C overnight. Tissues were washed with PBS and then incubated with biotinylated secondary antibody at room temperature. After incubated with SABC, which was prepared within 30 min before, a DAB Chromagen Substrate Kit was carried out. Tissues were washed and staining with hematoxylin. After dehydrated in ascending series of ethanol, cleared in xylene and sealed with resinene tablets, the tissue sections were put under microscopic examination. All tissue sections were stained under similar conditions to ensure equal staining intensity. PBS served as negative control and the company supplied a positive control.

Western blot analysis

Whole-cell extracts were prepared from HCC tissues and liver carcinoma cell lines. After incubation, cells were harvested and re-suspended in lysis buffer, washed with ice-cold PBS and lysed in extraction buffer supplemented with protease inhibitor. The concentration of total protein was tested with BCA method. The protein (50 μg) was separated on 5 X SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (TBS) at room temperature for 1 hour, and then incubated with primary antibody (1:1,000) or anti-β-actin antibody (1:500) in TBS containing 5% non-fat milk for 12 h at 37°C. Tissues were washed with PBS and incubated with secondary antibody in TBS containing 5% non-fat milk for 1 h at room temperature. Antigen-antibody complexes were detected using chemiluminescence kit.

Real time-quantitative PCR (RT-qPCR)

Total RNA were extracted and reverse transcribed to cDNA. Duplicate samples were subjected to RT-qPCR and mRNA was quantified. ABL mRNA from each sample was quantified as an endogenous control of internal RNA. Relative mRNA expression was calculated and untreated cells were used as calibrators.

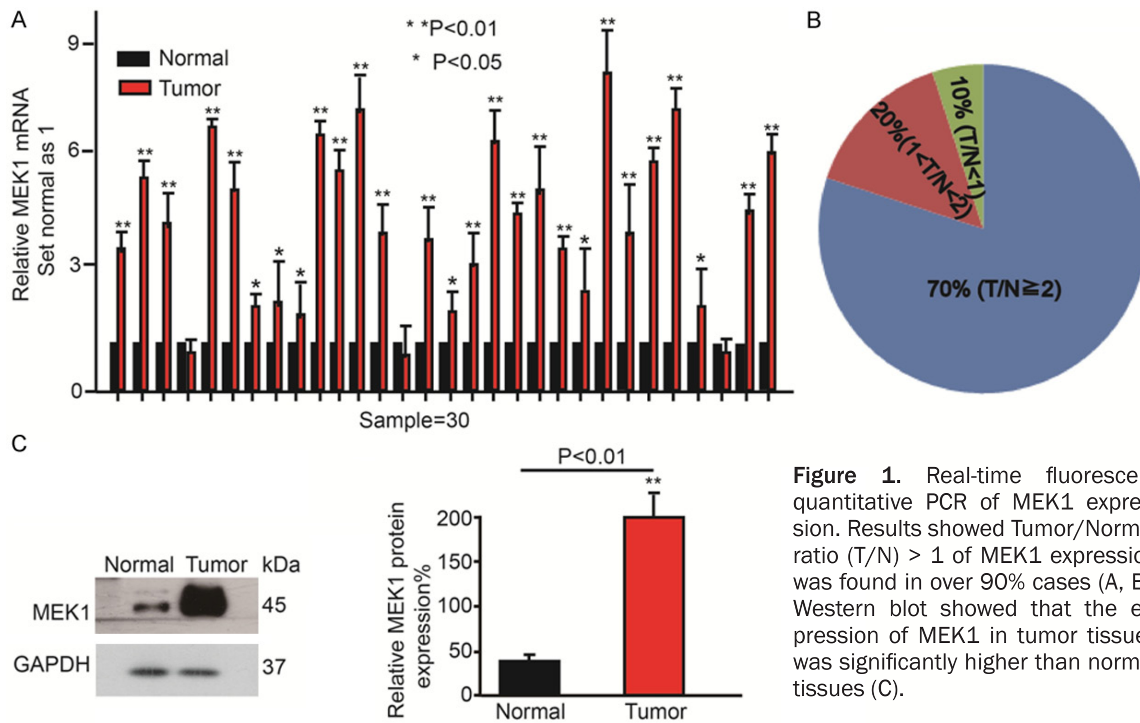

Figure 1. Real-time fluorescent quantitative PCR of MEK1 expression. Results showed Tumor/Normal ratio (T/N) > 1 of MEK1 expression was found in over 90% cases (A, B). Western blot showed that the expression of MEK1 in tumor tissues was significantly higher than normal tissues (C).

Outcome assessment

Two pathologists were in charge of judging the outcome in independent double-blind evaluation. The brown or sepia granules were the mark of the positive outcomes. The scale and power in MEK1 IHC staining were evaluated. Zero indicated negative, while 1-4 scores indicated week positive, 5-8 scores indicated positive and 9-12 scores indicated strong positive. In another way, 0-4 scores indicated low expression and 5-12 scores indicated high expression.

Follow up

All the patients were followed up by telephone or clinic after operations, and the median follow-up period was 13 months. The end of follow-up was July, 2014 except for the one who was lost and the one who died during the follow up before the given time.

Statistical analysis

All statistical analyses were made with SPSS17.0. Pearson Chi-Square test was used in analyzing the relation between the expression of MEK1 and mRNA and pathological characteristics of the patients. T-test was taken to analyze the expression of mRNA in tumor tissues and normal tissues beside them. Survival curve was made by Kaplan-Meier method. Lonrank test was used to analyze survival situation: $P < 0.05$ was considered statistically significant.

Results

MEK1 expression in HCC tissues

Real-time fluorescent quantitative PCR (RT-qPCR) demonstrated that Tumor/Normal ratio (T/N) > 1 of MEK1 expression was over 90% of those tumor tissues (**Figure 1A**, **1B**). Western blot showed that the expression of MEK1 in tumor tissues was of significantly higher than normal tissues ($P < 0.01$) (**Figure 1C**). IHC exhibited that MEK1 mostly expressed in nuclear, and seldom expressed in cytoplasm. In HCC staining tissues, 30% exhibited low expression, 70% demonstrated high expression. Of note, in adjacent normal tissues, IHC staining were negative (**Figure 2C**). The expressions of MEK1 protein and mRNA in three liver cell lines (Hun7, HepG3B and 7402) were significantly higher than 293T ($P < 0.01$, $P < 0.05$) (**Figure 2A**, **2B**).

MEK1 expression and clinical pathological features

Positive MEK1 mRNA expression was detected to be significantly correlated with differentiation ($\chi^2 = 29.731$, $P < 0.01$), tumor size ($\chi^2 =$

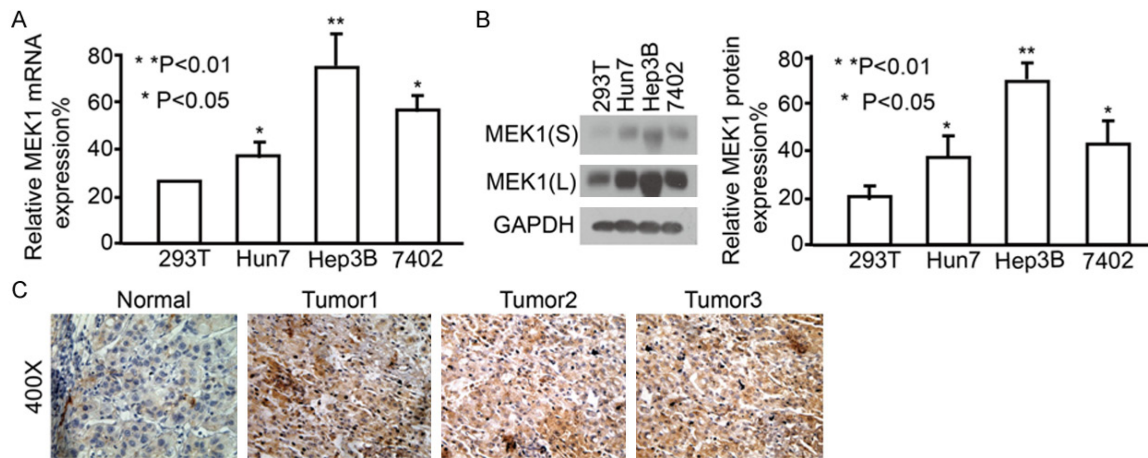

Figure 2. PCR detection and immunohistochemical staining for MEK1 expression in HCC cell lines. The expression of MEK1 mRNA (A) and protein (B, C) in three liver cell lines (Hun7, HepG3B and 7402) were significantly higher than controls (***P* < 0.01, **P* < 0.05).

Table 1. The relationship between expression of MEK1 and clinical pathological features

Clinical pathological indicators	Patients (N = 30)	MEK1 (n, %)		χ^2	P
		High expression	Low expression		
Gender					
Male	22	10 (45.5)	12 (54.5)	0.613	1.235
Female	8	4 (50)	4 (50)		
Age					
≤ 60	18	10 (55.6)	8 (44.4)	0.023	2.461
> 60	12	5 (41.7)	7 (58.3)		
Differentiation					
High	5	1 (20)	4 (80)	29.731	< 0.01
Moderate	15	11 (73.3)	6 (26.7)		
Low	10	8 (80)	2 (20)		
Tumor size					
≤ 5 cm	17	4 (23.5)	13 (76.5)	20.142	< 0.01
> 5 cm	13	10 (76.9)	3 (23.1)		
AJCC stage					
I-II phase	10	3 (30)	7 (70)	45.671	< 0.01
III-IV phase	20	15 (75)	5 (25)		
Tumor grading					
1	3	1 (33.3)	2 (66.7)	0.248	0.545
2	9	4 (44.4)	5(55.6)		
3	18	10 (55.6)	8 (44.4)		
Metastasis					
Yes	14	10 (71.4)	4 (28.6)	10.547	< 0.05
No	16	6 (37.5)	10 (62.5)		

AJCC: American Joint Committee on Cancer.

20.142, P < 0.01), AJCC stage (χ^2 = 45.671, P < 0.01), and tumor metastasis (χ^2 = 10.541, P < 0.05). However, negative relations were detected between positive expression with gender (χ^2 = 0.613, P = 1.235), age (χ^2 = 0.023, P = 2.461) and tumor stage (χ^2 = 0.023, P = 2.461) (**Table 1**).

Survival analysis

Kaplan-Meier was used to analyze the survival of enrolled patients. It is demonstrated that patients with high expression of MEK1 had average survival time (AST) of 7 months and median survival time (MST) of 7 months. However, patients with low expression of MEK1 exhibited AST of 27 months, and MST of 26 months. There was significant difference (Log-rank = 20.635, P < 0.01) in AST between patients with high and low expression of MEK1 (**Figure 3**).

Discussion

The present study revealed a significant increase of MEK1 expression in tumor tissues at both mRNA and protein level (**Figures 1**, **2**). Survival analysis showed a poor prognosis in patients with high MEK1 expression (**Figure 3**). Chi-square test also demonstrated positive correlation between MEK1 high expression and tumor size, differentiation degree, AJCC stage and metastasis (**Table 1**). In the present study,

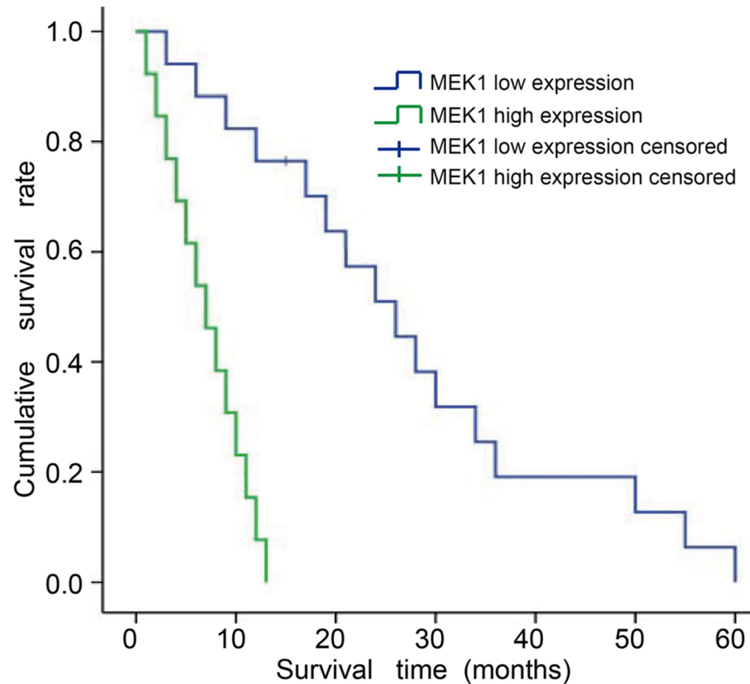

Figure 3. Kaplan-Meier was used to analyze the survival of enrolled patients. It is demonstrated that patients with high expression of MEK1 longer average survival time than patients with low expression of MEK1.

we found that MEK1 played a crucial role in the genesis and development of live tumor.

MEK1 is on the 15th chromosome of human and the DNA it encodes is 1182 bp in length. The protein it encodes belongs to serine/threonine family and is of MAPK activity [18]. MAPK signal pathway is crucial in controlling the apoptosis, proliferate and differentiation of cells. MEK1 and MEK2 are both ERKs, but only MEK1 can induce the phosphorylation of ERK1/2. MEK2 just decrease the negative feedback from ERK1/2 thus decrease the inhibition to MEK1 [19]. MAPK signaling cascade is important in mediating the effect of growth factor and cytokine, most importantly, it plays a significant role in malignant transformation of cells [13]. MEK1 is a kinase in the upstream of MAPK cascade, it can accelerate cell proliferation through EGF-EGFR-Ras-MEK-MAPK cascade [20].

Up to now, studies indicated that MEK1 was highly expressed in many kinds of tumor cells, and it could promote malignant transformation of mammal cells. Gilbert [21] revealed that MEK1/2 accelerated the proliferation and prohibited the apoptosis of melanoma, and the inhibition of MEK1/2 could slow the process of melanoma. Stinchcombe [15] also found an increased MEK1 expression in small cell lung cancer and that brought an increase in cell proliferation. It also noted that MEK1 inhibitors combined with chemotherapy significantly enhanced tumor cells sensitivity to chemotherapy. Liang [22] found that baicalein could retard the proliferation and induce apoptosis of liver cells by inhibiting MEK-EKR signal pathway. It is also exhibited that MEK1 inhibitors could effectively inhibit the proliferation of tumor cells and accelerate apoptosis. This injuring effect to tumor could be enhanced when combined with chemotherapy drugs.

In our study, MEK1 mRNA of tumor tissues and adjacent normal tissues were tested and 90% tumor tissues showed a significant increase in the expression. Western blot test also exhibited a stronger expression of MEK1 in tumor tissues, and it was of significant difference. The increased expression of MEK1 mRNA and protein were also detected in three liver tumor cell lines (Hun7, Hep3B and 7402) when compared to normal 293T cell line. IHC also indicated that MEK1 showed stronger expression in HCC. These evidences above indicated that MEK1 may play important roles in crucial progress of tumor cells including growth, differentiation and metastasis. Survival analysis also demonstrated that survival time significantly decreased in patients with high expression of MEK1. The research on MEK1 expression in liver tumor really helps in figuring out the molecular mechanism of HCC, thus provide a target for new effective drugs and provide a new guidance in clinical medication and prognosis evaluation.

Disclosure of conflict of interest

None.

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