Up-Regulated Maternal Embryonic Leucine Zipper Kinase Predicts Poor Prognosis of Hepatocellular Carcinoma Patients in a Chinese Han Population

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Background: Maternal embryonic leucine zipper kinase (MELK) has been implicated in various types of tumors, but its expression profile and clinicopathologic significance in hepatocellular carcinoma (HCC) in Chinese Han people remains unknown. Therefore, this study attempted to investigate the expression pattern of MELK in HCC tissues obtained from a Chinese Han population.

Material/Methods: The expression of MELK, from RNA to protein levels, in HCC or disease-free human liver tissues was evaluated using quantitative real-time polymerase chain reaction assays and immunohistochemistry staining, and its prognostic significance was determined based on its impact on HCC patients’ survival.

Results: We found that HCC tissues expressed a higher level of MELK RNA than non-tumor tissues in tumor-related public databases (P<0.001). Hence, we assessed MELK mRNA expression within 32 HCC samples and their adjacent non-tumorous liver tissues in our center. Subsequently, MELK protein expression was evaluated within 101 HCC specimens and 40 disease-free liver tissues. Notably, it revealed that high MELK protein expression was significantly related with tumor number, tumor size, higher pathological tumor-nodule-metastasis stage, vascular invasion, and recurrence (P<0.05, all). Furthermore, elevated MELK protein expression was correlated with decreased overall survival and disease-free survival (P=0.004 and P=0.002, respectively). Univariate and multivariate analysis results show that MELK protein may serve as an independent prognostic indicator for determining prognosis of HCC patients.

Conclusions: We found that, in a Chinese Han population, MELK was highly expressed within HCC tissues from RNA to protein levels, and may be a potential independent prognostic biomarker for HCC patients.

MeSH Keywords: Carcinoma, Hepatocellular • Genes, vif • Tumor Markers, Biological

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**Background**

Human cancers are a major public health problem in China and many other parts of the world [1–3]. Especially in China, cancer is the leading cause of death [3]. Among all cancer types, hepatocellular carcinoma (HCC) is the fourth most common; it is a major cause of cancer-related death and has a high rate of tumor recurrence and extremely poor prognosis [3]. Although great advances in diagnosis and treatment have been made, long-term survival of HCC patients remains low [4,5]. It was revealed that aberrant activation and dysfunction of essential genes could cause progression of HCC. Unfortunately, researchers are still unable to fully identify specific cancer-related genes and elucidate the molecular mechanisms serving as clinical or prognostic factors. In this regard, it is crucial to explore novel biomarkers directly associated with HCC for early diagnosis and efficiently predicting the prognosis of HCC patients.

Maternal embryonic leucine zipper kinase (MELK), a new member of the Snf1/AMPK family of kinases, was initially cloned from mice (Mus musculus) [6]. It was first reported to be expressed in a wide range of early embryonic cellular stages, and as a result has been shown to be involved in embryogenesis and cell cycle control [6]. Additionally, it was proved to be correlated with cell proliferation, apoptosis, cell renewal, and oncogenesis [7]. In recent years, although 2 studies reported MELK was not necessary for the proliferation or fitness of basal-like breast cancer cells [8,9], numerous studies indicated MELK plays a crucial role in regulating the genesis and development of several human malignancies, including ovarian [10], breast [11], gastric [12], hepatocellular [13], and colorectal [14] cancers. Moreover, increased MELK expression was closely related to the poor prognosis of patients with these cancers [10,12,13,15]. Notably, China has about 18% of the world’s population. MELK gene expression pattern and its prognostic role in HCC of Chinese patients have not been fully determined. It is well known that many human diseases, including tumors, have racial specificity. In the present study, we mainly explored MELK gene expression profiles and revealed its contributions to prognosis of HCC patients in a Chinese Han population.

**Material and Methods**

Liver tissue samples, patient characteristics, and ethics

We obtained 101 HCC samples from patients (ages 35–71 years, median age 48 years) who were histologically and clinically diagnosed with HCC. Forty normal (disease-free) liver tissues were collected from Chinese donors (all males, age 41.5±8.5 years). All patients were of Chinese Han ethnicity. We also collected their clinicopathologic characteristics. Surgeries were performed at the Department of General Surgery, the Second Affiliated Hospital of Jiaxing College (Jiaxing, Zhejiang, China) between June 2005 and December 2011. All patients were approached for participation in the project following written informed consent. This study was conducted according to the Declaration of Helsinki and its amendments, and was approved by the Ethics Committee of the Second Affiliated Hospital of Jiaxing College (Ethics Committee number CZ 2016-236; Chairperson professor Liqin Jiang) on 17 November 2016. None had received radiotherapy, chemotherapy, or other related anti-tumor treatments before surgery. All liver tissue samples were immediately cryopreserved in liquid nitrogen, and then kept in a freezer at −80°C until needed for further study. A portion of each specimen was fixed with 10% paraformaldehyde and embedded in paraffin blocks.

RNA isolation and qRT-PCR

Total RNA of 32 fresh HCC tissues and the adjacent non-tumor liver tissues, collected during the period of 2016, were extracted using TRIzol (Carlsbad, USA) according to the manufacturer’s recommendations. First, single-stranded cDNAs were synthesized with a SuperScript VILO cDNA Synthesis Kit (Fermentas, USA) following the manufacturer’s instructions. Subsequently, quantitative real-time polymerase chain reaction (qRT-PCR) assays were performed using 4 ml of cDNA (1:10 dilution) and 2×SYBR Green qPCR mix (TaKaRa, Japan) in a total volume of 20 ml, using the ABI 7900HT Real-time PCR System (ABI, USA). The fold change of mRNA copies was expressed as the relative quantification, and was calculated by the 2^-ΔΔCt method. The data were normalized to β-non-actin (β-actin, a housekeeping gene) mRNA. Primers sequences of β-actin gene were as following: forward 5'-AAGGTGACAGCAGTCGGTT-3' and reverse 5'-TGTTTCCCGGTCTCGCTAT-3'. Total RNA isolation and qRT-PCR

Tissue microarray and IHC staining and evaluation of staining and scoring

For immunohistochemistry (IHC) analysis, the tissue samples were fixed with 10% paraformaldehyde, embedded in paraffin blocks, and sectioned consecutively at 4-mm thickness, as previously described [16]. Tissue microarray (TMA) was constructed from HCC tissues (n=101) in collaboration with Shanghai Biochip (Shanghai, China).

IHC assay was performed using anti-MELK primary antibody (1: 200 dilution, Cat. NO. 11403-1-AP, Proteintech, Illinois, USA), and incubated overnight at 4°C. The secondary antibody was goat anti-rabbit immunoglobulin G (Golden Bridge Biotechnology Co., Beijing, China). As the negative controls for
all the experiments, the primary antibody was omitted. IHC scoring was performed independently in a blinded manner by 2 pathologists. MELK protein staining was scored in terms of the staining intensity and percentage of cell staining, as previously described [16].

Statistical analysis

All data were analyzed using the SPSS 19.0 statistical software package (SPSS Inc., Chicago, USA) and GraphPad Prism 6.02 (GraphPad Software, San Diego, CA). MELK mRNA expression in fresh HCC tissues and paired non-tumor tissues were performed using the t test (2-sided). The chi-square (χ²) test or Fisher’s exact test was employed to evaluate the associations between MELK expression and clinicopathological parameters of HCC patients. Cumulative overall survival (OS) and disease-free survival (DFS) were plotted by Kaplan-Meier analysis and the log-rank test. Furthermore, univariate and multivariate analysis were performed using the Cox proportional hazard regressions models. Significance was established at the conventional P<0.05 level.
Elevated expression of MELK in HCC tissues

We first analyzed the expression of MELK RNA in independent public datasets using the Oncomine analysis system (https://www.oncomine.org/resource/main.html). It was shown that MELK expression in HCC tissues was higher than normal liver tissues [17,18] (Figure 1A, 1B). In addition, we analyzed “The Cancer Genome Atlas (TCGA)” database [19], and verified that MELK RNA was expressed much higher in liver cancer tissues than in non-tumor liver tissues and the difference was significant (Figure 1C). Moreover, over-expressed MELK in 8 types of human tumors predicted poor prognosis (Figure 1C). The expression of MELK RNA in HCC tissues was markedly higher than in normal liver tissues (P<0.001, Figure 1D). Interestingly, high expression of MELK was associated with low overall survival time (P=0.001, Figure 1E). Notably, the above observations were not obtained from Chinese populations.

Subsequently, the expression level of MELK mRNA was tested in 32 randomly selected and paired HCC samples using qRT-PCR method. HCC tissues expressed higher levels of MELK than did matched non-tumor tissues (P<0.001, Figure 2A) in Chinese Han people, consistent with the aforementioned results derived from public datasets.

Associations between IHC staining of MELK and patient clinicopathological parameters

The expression of MELK protein in a set of 32 paraffin-embedded HCC tissues and 10 disease-free liver tissues were

Figure 2. Elevated expression of MELK mRNA and protein in liver tissues. (A) MELK mRNA expression in 32 HCC tissues and adjacent liver tissues using qRT-PCR (P<0.001). (B) The strong immunostaining profiles of MELK protein represented high MELK expression in HCC tissues. (C) The weak staining one represented low MELK expression in HCC tissues. (D) The weak staining one indicated low MELK expression in normal liver tissues. MELK – maternal embryonic leucine zipper kinase; HCC – hepatocellular carcinoma; qRT-PCR – quantitative real-time polymerase chain reaction.
evaluated using IHC staining. We found that 56.25% (18/32) of 32 HCC specimens showed higher MELK expression according to median HCC tissues samples, and 80.0% (8/10) of normal liver tissues showed weak MELK expression. The panels in Figure 2B and 2C represented the strong and weak immunostaining profiles of MELK protein in HCC tissues. Figure 2D indicates weak immunostaining profiles of MELK expression in normal liver tissues. Additionally, it showed that MELK protein is primarily localized in the cytoplasm of hepatocytes.

Encouraged by the above observations, 101 HCC tissues and 40 disease-free liver tissues were collected to further assess the expression patterns of MELK protein. Consequently, 50.5% (51/101) of HCC tissues showed high MELK expression (Figure 3A1, 3A2), 49.5% showed low expression (Figure 3B1, 3B2), and 72.5% (29/40) of normal liver tissues showed weak MELK expression (Figure 3C1, 3C2). Subsequently, we evaluated the associations between the clinicopathological characteristics and MELK expression levels (Table 1). HCC patients were divided into 2 groups (low MELK and high MELK) in terms of median of MELK expression (staining score=3). Interestingly, increased MELK expression was strongly related to tumor number, vascular invasion, higher tumor-node-metastasis (TNM) stage, and tumor recurrence. However, no correlations were found between MELK expression and sex, age, Child-Pugh grade, preoperative alpha-fetoprotein (AFP) level, tumor distribution, or tumor differentiation (Table 1). These findings indicate that MELK overexpression is related with the clinical progression in Chinese Han people.

**Survival analysis and prognostic significance of MELK expression in HCC patients after curative surgery**

Next, we analyzed the influence of MELK protein level on HCC patient survival time in a Chinese Han population. Among 101 HCC patients, up-regulated MELK protein had significantly shorter OS and DFS than low MELK protein ($P=0.004$ and $P=0.002$, Figure 4A, 4B), suggesting that high MELK expression is correlated with tumorigenesis of HCC at the protein level. In addition, univariate and multivariate analysis showed that tumor TNM stage, vascular invasion, and MELK expression were each recognized as independent prognostic factors in HCC patients ($P<0.05$, Tables 2, 3), suggesting that MELK has potential clinical value as a predictive biomarker for disease outcome in HCC.

**Discussion**

Human HCC is a malignant carcinoma that is prevalent worldwide and causes a huge economic burden for society [2,3,20].
Table 1. Association between maternal embryonic leucine zipper kinase expression and clinicopathological characteristics in hepatocellular carcinoma patients (n=101).

| Variables                        | Total No. of patients | High MELK expression (n) | Low MELK expression (n) | \( \chi^2 \) value | \( P \) values |
|----------------------------------|-----------------------|--------------------------|-------------------------|---------------------|----------------|
| **Sex**                          |                       |                          |                         |                     |                |
| Female                           | 88                    | 44                       | 44                      | 0.067               | 0.796          |
| Male                             | 13                    | 7                        | 6                       |                     |                |
| **Age (year)**                   |                       |                          |                         |                     |                |
| <50                              | 48                    | 29                       | 19                      |                     |                |
| ≥50                              | 53                    | 32                       | 31                      |                     |                |
| **Child-Pugh grade**             |                       |                          |                         |                     |                |
| A–B                              | 85                    | 43                       | 42                      |                     |                |
| C                                | 16                    | 8                        | 8                       |                     |                |
| **Preoperative AFP level (ng/ml)**|                       |                          |                         |                     |                |
| ≤20                              | 46                    | 20                       | 26                      |                     |                |
| >20                              | 55                    | 31                       | 24                      |                     |                |
| **Tumor number**                 |                       |                          |                         |                     |                |
| ≤3                               | 84                    | 38                       | 46                      |                     |                |
| >3                               | 17                    | 13                       | 4                       |                     |                |
| **Tumor distribution**           |                       |                          |                         |                     |                |
| Single                           | 77                    | 35                       | 42                      |                     |                |
| Multiple                         | 24                    | 16                       | 8                       |                     |                |
| **Tumor size (cm)**              |                       |                          |                         |                     |                |
| ≤5                               | 50                    | 18                       | 32                      |                     |                |
| >5                               | 51                    | 33                       | 18                      |                     |                |
| **Tumor TNM stage**              |                       |                          |                         |                     |                |
| I–II                             | 54                    | 20                       | 34                      |                     |                |
| III–IV                           | 47                    | 31                       | 16                      |                     |                |
| **Tumor differentiation**        |                       |                          |                         |                     |                |
| I–II                             | 71                    | 32                       | 39                      |                     |                |
| III–IV                           | 30                    | 19                       | 11                      |                     |                |
| **Vascular invasion**            |                       |                          |                         |                     |                |
| No                               | 46                    | 15                       | 31                      |                     |                |
| Yes                              | 55                    | 36                       | 19                      |                     |                |
| **Recurrence**                   |                       |                          |                         |                     |                |
| No                               | 59                    | 21                       | 38                      |                     |                |
| Yes                              | 42                    | 30                       | 12                      |                     |                |

MELK – maternal embryonic leucine zipper kinase; HCC – hepatocellular carcinoma; AFP – preoperative alpha-fetoprotein; TNM – tumor-nodule-metastasis.
Increasing evidence proves that various genes up-regulated or down-regulated in hepatocellular liver tissues can be considered as special prognostic markers and therapeutic targets [21]. Recent studies reported that MELK expression is increased in many digestive malignant tumors, such as gastric [12], hepatocellular [13], and colorectal [14] tumors. Additionally, high MELK expression indicates poor clinical outcome [12,13]. Notably, a study recently revealed that MELK mRNA in HCC tissues, collected from a Japanese population, exhibited significantly high expression levels compared with paired normal liver tissues. Moreover, low expression of MELK mRNA predicted longer OS and DFS in HCC patients [13]. Similarly,

Figure 4. The impact of MELK expression at the protein level on HCC patients’ OS and DFS. (A) Significant difference between aberrant expression of MELK protein and OS was observed in HCC patients (P=0.004). (B) High expression of MELK protein was connected with low DFS (P=0.002). MELK – maternal embryonic leucine zipper kinase; HCC – hepatocellular carcinoma; OS – overall survival; DFS – disease-free survival.

Table 2. Univariate analyses of factors associated with overall survival and time to recurrence.

| Variables                          | Overall survival | Time to recurrence |
|------------------------------------|------------------|--------------------|
|                                    | Hazard ratio     | P values           |
|                                    | (95% CI)         |                    |
| Sex (Female vs. Male)              | 0.614 (0.245–1.540) | 0.298 | 0.591 (0.236–1.484) | 0.263 |
| Age (Year, <50 vs. ≥50)            | 1.005 (0.591–1.709) | 0.987 | 1.036 (0.609–1.762) | 0.896 |
| Child-Pugh grade (A–B vs. C)       | 1.025 (0.051–2.096) | 0.946 | 1.012 (0.495–2.069) | 0.975 |
| Preoperative AFP level (ng/ml, ≤20 vs. >20) | 1.763 (0.960–2.844) | 0.060 | 1.600 (0.929–2.756) | 0.090 |
| Tumor number (≤3 vs. >3)           | 1.809 (1.105–2.800) | 0.048 | 1.719 (0.714–2.819) | 0.019 |
| Tumor distribution (Single vs. Multiple) | 0.931 (0.491–1.767) | 0.828 | 0.870 (0.502–1.574) | 0.660 |
| Tumor size (≤5 vs. >5)             | 1.903 (1.291–2.724) | 0.027 | 1.924 (1.377–2.257) | 0.030 |
| Tumor TNM stage (I–II vs. III–IV)  | 2.490 (1.449–4.280) | 0.001 | 2.232 (1.470–3.436) | 0.001 |
| Tumor differentiation (I–II vs. III–IV) | 1.799 (1.030–3.141) | 0.039 | 1.738 (1.126–3.044) | 0.041 |
| Vascular invasion (No vs. Yes)     | 3.016 (1.682–5.408) | <0.001 | 3.084 (1.717–5.539) | <0.001 |
| MELK expression (High vs. Low)     | 0.458 (0.266–0.788) | 0.005 | 0.430 (0.249–0.743) | 0.003 |

MELK – maternal embryonic leucine zipper kinase; HCC – hepatocellular carcinoma; AFP – preoperative alpha-fetoprotein; TNM – tumor-nodule-metastasis; 95% CI – 95% confidence interval.
Table 3. Multivariate analyses of factors associated with overall survival and time to recurrence.

| Variables                            | Overall survival | Time to recurrence |
|--------------------------------------|------------------|--------------------|
|                                      | Hazard ratio     | P values           | Hazard ratio     | P values           |
|                                      | (95% CI)         |                    | (95% CI)         |                    |
| Tumor number (≤3 vs. >3)              | 1.942 (0.899–3.198) | 0.091              | 1.598 (0.779–3.277) | 0.102 |
| Tumor size (≤5 vs. >5)                | 1.623 (0.901–2.724) | 0.338              | 1.574 (0.786–2.457) | 0.395 |
| Tumor TNM stage (I–II vs. III–IV)     | 2.092 (1.125–3.891) | **0.020**          | 3.398 (1.300–4.420) | **0.005**          |
| Tumor differentiation (I–II vs. III–IV)| 1.143 (0.588–2.223) | 0.394              | 1.869 (0.982–3.557) | 0.057 |
| Vascular invasion (No vs. Yes)        | 2.534 (1.340–4.793) | **0.007**          | 3.031 (1.617–5.082) | **0.001**          |
| MELK expression (High vs. Low)        | 0.536 (0.304–0.946) | **0.031**          | 0.507 (0.279–0.923) | **0.026**          |

MELK – maternal embryonic leucine zipper kinase; HCC – hepatocellular carcinoma; TNM – tumor-node-metastasis; 95% CI – 95% confidence interval.

A Singapore study demonstrated that MELK was an oncogenic kinase and overexpression of its RNA was strongly correlated with early recurrence and poor patient survival [22]. Unfortunately, they did not fully investigate the expression profiles of MELK protein and the correlation between the expression levels of MELK protein and OS or DFS in HCC patients with a larger sample. Additionally, these results all were obtained from non-Chinese patients.

Therefore, first, we analyzed Oncomine and TCGA public databases and found that the expression of MELK was much higher in HCC tissues than that in normal liver tissues (Figure 1A–1D). Additionally, high MELK expression at the RNA level was associated with low OS and the difference was significant (Figure 1C, 1D). These results are consistent with previous reports [13,22]. Second, we further validated them by qRT-PCR analysis in an independent and random cohort of HCC samples consisting of 32 hepatocellular liver tissues and adjacent liver tissues (Figure 2A). Third, we preliminarily explored the expression features of MELK protein in a group of 32 paraffin-embedded HCC tissues and 10 disease-free liver tissues by IHC staining, showing that the percentage of HCC specimens with high MELK expression was much higher than that of normal liver tissues (56.25% vs. 20.0%, P<0.001). Moreover, it showed that MELK protein was primarily localized in the cytoplasm of liver cells. Encouraged by these results, subsequently, we obtained 101 HCC tissues and 40 normal liver tissues to further determine the expression patterns of MELK protein: 50.5% of HCC tissues showed high MELK expression (Figure 3A1, 3A2) and 72.5% of normal liver tissues showed weak MELK expression (Figure 3C1, 3C2). These results revealed the expression trend of the MELK gene at the protein level was in accordance with that at the RNA level. Interestingly, when investigating the association between the clinicopathological factors and MELK protein expression, we found that increased MELK expression was strongly related to tumor number, vascular invasion, higher TNM stage, and tumor recurrence. However, Hongping Xia et al. [22] reported that the expression of MELK at the RNA level was related to preoperative AFP level. In our opinion, this inconsistency can be easily explained. It is well known that the RNA expression of certain genes is not completely consistent with their protein levels. Moreover, RNA easily degrades in a short period of time, but protein is relatively stable and is easily preserved. Our observations in this study are not based on a relatively large clinical sample set, but to a certain degree, these findings indicate that overexpression of MELK protein or RNA is correlated with recurrence and poor prognosis in HCC.

There is wide evidence that MELK is expressed in several human cancers and stem cell types. Unique spatial and temporal patterns expression within them lead to MELK playing a prominent role in G2/M arrest, cell proliferation, apoptosis, tumorigenesis, invasion, cancer treatment resistance, and recurrence [10,12]. MELK is closely involved in cell migration and epithelial-mesenchymal transition (EMT) [12]. Additionally, since the discovery of MELK, numerous signaling proteins and pathways regulating the action of MELK were revealed. Tumor-specific activation of the JNKs/MELK pathway regulates glioma stem cell growth in a p53-dependent manner [23]. MELK-dependent FOXM1 phosphorylation is important for proliferation of glioma stem and HCC cells [22,24]. It is well known that JNKs, p53, and FOXM1 signaling pathways are all extremely important in a number of human cancers, including HCC. However, Huang et al and Lin et al reported that MELK is not necessary for the proliferation or fitness of breast cancer cells [8,9]. It is worth pointing out that their results were obtained only in breast cancer cells, not HCC cells. Moreover, the occurrence and development of malignant tumors is very complex and related to many genes and signaling pathways, but MELK may still be an important biomarker, even if it is not an oncogene.
Unfortunately, our study has several limitations. First, similar findings have been already reported in HCC [13,22]. However, we additionally explored expression features of MELK at the protein level using liver tissue microarray including HCC and normal liver tissues. Therefore, our results enrich and confirm previous studies. The number of cases examined is not large enough, but we strictly limited the inclusion criteria for HCC patients to avoid bias and assure reliability of our data. Future studies should be conducted in large groups of HCC patients.

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**Conclusions**

In conclusion, based on published reports, we analyzed results obtained from 2 public human tumor-related databases and observations in this study, demonstrating that the MELK gene is highly expressed in HCC tissues from RNA to protein levels. Moreover, in this Chinese Han population, elevated MELK protein was significantly correlated with recurrence and poor prognosis in HCC patients. Therefore, MELK may serve as an independent prognostic indicator in HCC patients and might be a viable target for HCC therapy in the Chinese Han population.