Diagnostic and Prognostic Values of MANF Expression in Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, and its prognosis is still poor. Mesencephalic astrocyte-derived neurotrophic factor (MANF) plays a key role in endoplasmic reticulum stress. ER stress plays a key role in HCC carcinogenesis. To confirm the clinical and prognostic value of MANF in HCC, we investigated the expression level of MANF in HCC as recorded in databases, and the results were verified by experiment. Survival analysis was probed by the Kaplan–Meier method. Cox regression models were used to ascertain the prognostic value of MANF in HCC tissue microarray. The diagnostic value of MANF in HCC was evaluated by receiver operating characteristic curve analysis. Potential correlation between MANF and selected genes was also analyzed. Results showed that MANF was overexpressed in HCC. Patients with high MANF expression levels had a worse prognosis and higher risk of tumor recurrence. Furthermore, the expression level of MANF had good diagnostic power. Correlation analysis revealed potential regulatory networks of MANF in HCC, laying a foundation for further study of the role of MANF in tumorigenesis. In conclusion, MANF was overexpressed in HCC and related to the occurrence and development of HCC. It is a potential diagnostic and prognostic indicator of HCC.

1. Introduction

Liver cancer is one of the most common human malignant gastrointestinal tumors and the fourth leading cause of cancer-related deaths worldwide [1, 2]. Hepatocellular carcinoma (HCC) characterized by its asymptomatic nature, high malignancy, early metastasis, and poor curative efficacy is responsible for >90% of primary liver cancers [3–5]. Despite recent therapeutic approaches such as surgical resection, radiofrequency ablation, and orthotopic liver transplantation, the prognosis of HCC remains poor. The metastasis and recurrence of HCC significantly reduce the survival rate and quality of life of HCC patients [5–8]. Therefore, novel biomarkers will be substantially beneficial for HCC diagnosis and treatment, and outcomes of HCC patients urgently need to be improved.

Mesencephalic astrocyte-derived neurotrophic factor (MANF), also named arginine-rich mutated in early tumors (ARMET), was first discovered as a new dopaminergic neurotrophic factor in astrocyte-conditioned medium by Petrova et al. in 2003 [9]. Apart from being secreted into the extracellular space, MANF has been found to remain inside the cells and localize in the endoplasmic reticulum (ER) lumen [10, 11]. Induction of ER stress in vitro causes upregulation of endogenous MANF expression [12, 13]. Hakonen et al. have shown that the protective effect of MANF is associated with inhibition of the nuclear factor-κB signaling pathway and alleviation of ER stress. MANF also enhances human beta cell proliferation when transforming growth factor-β signaling is inhibited [14]. In recent studies, ER stress has been shown to mediate HCC promoted by nonalcoholic fatty liver disease, and the NF-κB pathway is...
closely associated with initiation of cancer [15, 16]. So the diagnostic value and clinical significance of MANF in HCC remain to be elucidated.

In this study, we investigated MANF expression in HCC cell lines, HCC tissues, and nontumor tissues by analyzing the data from bioinformation databases and confirmed our findings by Western blotting, polymerase chain reaction (PCR), and immunohistochemical staining. We examined the clinical and prognostic value of MANF in HCC patients.

2. Material and Methods

2.1. Ethics Statement. This study was approved by the Academic Committee of Shandong Provincial Hospital Affiliated to Shandong University and conducted according to the principles expressed in the Declaration of Helsinki. All the datasets were retrieved from the publishing literature, and all written informed consent was obtained. This article does not contain any studies with animals performed by any of the authors.

2.2. Patients and Specimens. A total of 311 patients undergoing hepatectomy between January 2011 and December 2014 were included in the study. HCC samples and paratumor tissues including 45 freshly frozen HCC samples, and 266 tissue microarrays (TMAs) were collected. We summarized their characteristics and study cohort diagram in Table S2.

2.3. Real-Time PCR. Total RNA from liver tissues was isolated by the TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA), and 1 μg mRNA was reverse transcribed to cDNA using the PrimeScript RT Reagent Kit Perfect Real Time (Takara Bio, Japan) according to the manufacturer’s instructions. Reverse transcription- (RT-) PCR was conducted using the LightCycler 480 II Real-Time PCR System (Roche, Switzerland) with SYBR Green PCR Master Mix (Toyobo, Osaka, Japan). An initial denaturation at 95°C for 10 min was followed with PCR cycling: 94°C (30 s), 60°C (30 s), and 72°C (60 s) for 40 cycles. The primers of MANF were as follows: forward—5′-GTGCACGGGACCGATTT GTAG-3′, reverse—5′-GAAAAGCTCCAGGTTCACA-3′. The primers of β-actin were as follows: forward—5′-GAAGAGCTACGAGCTGCCTGA-3′, reverse—5′-GTGCACGGGACCGATTT GTAG-3′. The primers of β-actin were as follows: forward—5′-GAAGAGCTACGAGCTGCCTGA-3′, reverse—5′-CAGCACGTGTGGCCG-3′. Products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no byproducts were formed. Results were expressed relative to the number of β-actin transscripts used as an internal control.

2.4. Western Blot Analysis. Liquid nitrogen frozen liver tissues were immersed in RIPA-added phenylmethylsulfonyl fluoride (100:1) (Beyotime, China) supplemented with protease and phosphatase inhibitors and sonicated on ice to obtain a homogenate. Specimens were centrifuged at 15,000 × g for 15 min, and the supernatant was used for Western blotting and ELISA. Concentration of the protein was assessed by BCA protein assay kit (Beyotime). Proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. After incubation with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature, signals were detected by chemiluminescent reagents (Millipore, USA) and β-actin served as an internal control. The primary antibodies were as follows: rabbit anti-ARMET (Abcam, Cambridge, MA, USA; diluted 1:1000) and rabbit anti-β-actin (Cell Signaling Technology, Danvers, MA, USA; diluted 1:1000). Immunoreactivity was detected using the FluorChem Chemiluminescent Western Blot Imaging System (Cell Biosciences, Santa Clara, CA, USA).

2.5. Immunohistochemical (IHC) Detection of Tissue Microarray (TMA). Two hundred and sixty-six HCC patients, including 259 who had follow-up information, were analyzed. For immunohistochemistry, 5 μm tissue sections were prepared from each block. Tissue sections were deparaffinized, rehydrated, and rinsed in distilled water. After heating the sections in 10 mmol/L citrate buffer for antigen retrieval, the sections were incubated with primary antibody against ARMET (Abcam; dilution at 1:100) at 4°C, followed by secondary antibody for 1 h at room temperature. An

Table 1: The significant changes of MANF expression in transcription level of HCC vs. normal tissues (ONCOMINE database).

| Tissue types   | Fold change | P value  | t-test   | References                                      | Overexpression gene rank |
|---------------|-------------|----------|----------|------------------------------------------------|--------------------------|
| HCC vs. normal| 1.945       | 1.54E-53**| 1.77E+01 | Roessler Liver 2 Statistics (Roessler et al., Cancer Res 2010/12/15) | 386 of 12,624 measured genes (in top 4%) |
| HCC vs. normal| 1.398       | 0.0000514**| 3.984    | Chen Liver Statistics (Chen et al., Cancer Res 2010/12/15) | 1850 of 10,802 measured genes (in top 18%) |
| HCC vs. normal| 1.435       | 0.011*   | 2.391    | Roessler Liver Statistics (Roessler et al., Cancer Res 2010/12/15) | 3333 of 12603 measured genes (in top 27%) |
| HCC vs. normal| 1.482       | 0.122    | 1.236    | Wurmbach Liver Statistics (Wurmbach et al., Hepatology 2007/04/01) | 7466 of 19,574 measured genes (in top 39%) |
| HCC vs. normal| -2.279      | 1        | -6.273   | Mas Liver Statistics (Mas et al., Mol Med 2008/12/21) | 12189 of 12,603 measured genes (in top 97%) |

Notes: *P < 0.05; **P < 0.01.
| Analysis type by cancer | Cancer vs. normal |
|-------------------------|------------------|
| Bladder cancer          | 3                |
| Brain and CNS cancer    | 1                |
| Breast cancer           | 6                |
| Cervical cancer         | 2                |
| Colorectal cancer       | 3                |
| Esophageal cancer       | 1                |
| Gastric cancer          | 1                |
| Head and Neck cancer    | 5                |
| Kidney cancer           | 2                |
| Leukemia                | 3                |
| Liver cancer            | 2                |
| Lung cancer             | 1                |
| Lymphoma                | 2                |
| Melanoma                | 3                |
| Myeloma                 | 6                |
| Other cancer            | 1                |
| Ovarian cancer          | 1                |
| Pancratic cancer        | 2                |
| Prostate cancer         | 1                |
| Sarcoma cancer          | 1                |

(a) The gene expression profile across all tumor samples and paired normal tissues. (Bar plot)

(b) LIHC
(num(T) = 369; num(N) = 50)

(c) Figure 1: Continued.
characteristics such as cohort ID, RNA-seq platform, samples size (nontumor and tumor samples), publication year, and country in Table S1.

2.7. Statistics for Meta-analysis. Stata 12.0 was utilized to analyze the pooled diagnostic value of MANF with the data from the GEO dataset. $I^2$ was used to evaluate the heterogeneity of those studies, which indicated significant heterogeneity at $I^2 > 50\%$. The random effects model was used, and subgroup analysis was performed to explore the source of heterogeneity, while heterogeneity was conspicuous between those studies. Publication bias was determined by Begg’s funnel plot and Egger’s test.

2.8. ONCOMINE Analysis. ONCOMINE (http://www.oncomine.org/), an online cancer microarray database, was used to analyze differential expression classification in different cancers with their respective normal tissues and their clinical and pathological characteristics. MANF expression in HCC samples was compared with that in nontumor samples. The $P$ value was generated utilizing Students’ $t$-test. The cut-off $P$ value and fold change were defined as 0.01 and 2, respectively.
2.9. GEPIA Dataset. The online database Gene Expression Profiling Interactive Analysis (GEPIA), providing customizable functions, is a newly developed interactive web server for analyzing the RNA sequencing expression data and prognostic value. Tumors and nontumor specimens in the GEPIA database were derived from The Genotype-Tissue Expression (GTEx) and The Cancer Genome Atlas (TCGA) projects (http://gepia.cancerpku.cn/index.html) [17]. Tumor/nontumor differential expression analysis, patient survival analysis, and correlation analysis were explored using the GEPIA database. We selected the median as the group cut-off for survival plots.

2.10. CCLE Dataset. Cancer Cell Line Encyclopedia (CCLE) project is a collaboration concentrated on a detailed genetic and pharmacological characterization of a large panel of human cancer cell lines, in order to develop integrated computational analyses that link distinct pharmacological vulnerabilities to genomic patterns and to translate cell line integrative genomics into clinical application. Genomic data, analysis, and visualization providing by CCLE for around 1000 cell lines are available for public access [18]. CCLE gene expression data of MANF were downloaded and collected from https://portals.broadinstitute.org/ccle/data.

2.11. LinkedOmics Dataset. LinkedOmics is a user-friendly bioinformatics web in the software ecosystem for disseminating data from large-scale cancer omics projects. It uses preprocessed and normalized data from the Broad TCGA Firehose and CPTAC data portal to reduce redundant efforts and focuses on exploration and interpretation of attribute associations and thus complements existing cancer data portals [19]. Correlation analysis data were collected and downloaded from http://www.linkedomics.org/admin.php.

2.12. EMBL-EBI Dataset. EMBL-EBI (https://www.ebi.ac.uk) is a user-friendly bioinformatics web and programmatic tool framework providing free and open access to a range of bioinformatics applications for sequence analysis [20]. The expression data of MANF in HCC cell lines was collected from the EMBL-EBI dataset.

2.13. Data Analysis and Statistics. SPSS version 22.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA) were used.
Note: weights are from random effects analysis

| Study ID         | OR (95% CI)       | Weight% |
|------------------|-------------------|---------|
| GSE17548 (2013)  | 2.75 (0.72, 10.48)| 2.02    |
| GSE20140 (2011)  | 8.02 (2.74, 23.51)| 3.13    |
| GSE29722 (2011)  | 5.44 (0.80, 36.87)| 0.99    |
| GSE31370 (2012)  | 6.00 (0.53, 67.65)| 0.62    |
| GSE36411 (2015)  | 6.25 (2.42, 16.11)| 4.03    |
| GSE39791 (2012)  | 7.78 (3.71, 16.33)| 6.58    |
| GSE41804 (2013)  | 5.44 (1.41, 21.05)| 1.98    |
| GSE45050 (2017)  | 15.00 (1.03, 218.30)| 0.50    |
| GSE45267 (2014)  | 5.52 (2.20, 13.87)| 4.27    |
| GSE47595 (2014)  | 2.51 (1.21, 5.20)| 6.83    |
| GSE57958 (2014)  | 20.90 (6.57, 66.43)| 2.70    |
| GSE62232 (2014)  | 27.51 (5.57, 485.37)| 0.44    |
| GSE63898 (2015)  | 3.90 (2.55, 5.95)| 20.11   |
| GSE64041 (2014)  | 3.65 (1.74, 7.65)| 6.61    |
| GSE75285 (2016)  | 4.70 (0.49, 45.03)| 0.71    |
| GSE76311 (2017)  | 6.24 (2.82, 13.83)| 5.71    |
| GSE76427 (2017)  | 5.58 (2.64, 11.79)| 6.47    |
| GSE84006 (2017)  | 10.38 (3.61, 29.90)| 3.23    |
| GSE84402 (2017)  | 3.24 (0.69, 15.20)| 1.51    |
| GSE84598 (2017)  | 7.11 (1.89, 26.80)| 2.05    |
| GSE98383 (2018)  | 1.98 (0.53, 7.34)| 2.10    |
| GSE102083 (2018) | 6.93 (3.94, 12.19)| 11.34   |
| GSE112791 (2019) | 7.33 (1.61, 33.41)| 1.57    |
| GSE121248 (2018) | 5.60 (2.29, 13.72)| 4.50    |

Overall (I-squared = 0.0%, P = 0.489) Weight% = 5.28 (4.37, 6.39) 100.00

Note: weights are from random effects analysis

Figure 3: Continued.
for statistical analyses. We select the median expression level for splitting the high-expression and low-expression cohorts. Samples with expression level higher than this threshold are considered the high-expression cohort. Samples with expression level lower than this threshold are considered the low-expression cohort. The \( \chi^2 \) test was used to explore the correlation between MANF expression levels and the clinicopathological parameters. Survival analysis was performed by the Kaplan–Meier method. The relationship between different variables and survival was determined by the multivariate Cox proportional hazards method. The pooled diagnostic value of MANF in HCC was analyzed via receiver-operating characteristic (ROC) curves. The linear association between two variables was evaluated by Pearson’s correlation. All of the data of samples are presented as the mean ± standard deviation (SD). The differences between tumor and nontumor samples were determined with nonparametric tests. In all cases, \( P < 0.05 \) was considered to be statistically significant.

3. Results

3.1. MANF Overexpression in HCC Was Explored by Analyzing Bioinformation Databases. We analyzed MANF mRNA expression in HCC tissues and paired nontumor tissues using the ONCOMINE and GEPIA databases. Compared to nontumor samples, ONCOMINE demonstrated that MANF was significantly upregulated in HCC samples \( (P < 0.01) \), while the other two statistics had no significance in this regard (Table 1). We compared transcriptional levels of MANF in cancer with those in normal tissues using ONCOMINE (Figure 1(a)). GEPIA showed that mRNA of MANF was significantly overexpressed in HCC samples and many other types of cancer (Figures 1(b)–1(d)).

We used the EMBL-EBI bioinformatics website to measure the expression of MANF in HCC cell lines, which indicated that MANF was upregulated in 21 HCC cell lines (Figure 2(a)). The CCLE database showed that MANF was highly expressed in a variety of cell lines originated from different tissue types (Figure 2(b)).

To explore further whether MANF expression was higher in HCC tissues than in nontumor tissues, 24 HCC microarrays from the GEO database were subjected to meta-analysis. Like the forest plot in Figure 3(a), higher MANF expression was found in HCC tissues than in the nontumor tissue \( \chi^2 \) test, Egger’s test, and funnel plots (Figures 3(b)–3(d)). There were no significant publication bias and heterogeneity. As shown in the sensitivity analysis, there were no significant differences between these microarrays (Figure 3(e)). Hence, high expression of MANF in HCC samples was identified by meta-analysis.

3.2. MANF Upregulated in HCC Was Confirmed by Experiments. To confirm the expression level of MANF, we examined mRNA and protein levels of MANF in HCC and paired nontumor samples, utilizing quantitative RT-PCR and Western blotting. MANF expression in HCC tissues \( (n = 45) \) was higher than that in nontumor tissues \( (n = 45) \) (PCR, \( P < 0.05 \); Western blotting, \( P < 0.01 \) (Figures 4(a)–4(c)). We characterized MANF protein expression in human HCC and nontumor specimens by TMA. We analyzed...
MANF protein expression by immunohistochemical staining of HCC and paired nontumor tissues and found that MANF was significantly upregulated in HCC tissues compared with adjacent nontumor tissues \((n = 266)\) \((P < 0.01)\) (Figures 4(d) and 4(e)).

3.3. Diagnostic Value of MANF. The diagnostic value of MANF in identifying HCC and nontumor samples was evaluating by ROC curve analysis. Areas under the curve (AUCs) from GEO databases were as follows: GSE39791, 0.811 (95% CI: 0.740–0.882, \(P < 0.001\); Figure 5(a)) with cut-off point, and respective specificities and sensitivities were 10.075, 0.722, and 0.806; GSE63898, 0.677 (95% CI: 0.625–0.730, \(P < 0.001\); Figure 5(b)) with cut-off point, and respective specificities and sensitivities were 10.4102, 0.544, and 0.821; GSE64041, 0.710 (95% CI: 0.619–0.801, \(P < 0.001\);
Figure 5: Continued.
Figure 5(c)) with cut-off point, and respective specificities and sensitivities were 9.1892, 0.631, and 0.750; GSE76427, 0.749 (95% CI: 0.668–0.830, \( P < 0.0001 \); Figure 5(d)) with cut-off point, and respective specificities and sensitivities were 3357.82, 0.722, and 0.750; GSE102083, 0.782 (95% CI: 0.727–0.838, \( P < 0.0001 \); Figure 5(e)) with cut-off point, and respective specificities and sensitivities were 9.542, 0.618, and 0.848. AUCs from immunohistochemistry of TMA were 0.570 (95% CI: 0.522–0.619, \( P < 0.01 \); Figure 5(f)) with cut-off point, and respective specificities and sensitivities were 152.7620, 0.643, and 0.5. Results indicate that MANF was a reliable diagnostic marker in HCC.

To analyze expression levels of MANF in dysplastic nodules, the GEO database was searched. GSE98620 was the only database that meets the retrieval requirements. The result showed that higher MANF expression was found in HCC tissues than in dysplastic nodules (\( P < 0.001 \)) (Figure 5(g)), and there was no statistical difference between normal tissues and dysplastic nodules.

Correlation analysis of MANF expression and TNM staging was performed using LinkFinder of LinkedOmics. There was no significant correlation between high MANF expression and TNM pathological stage (\( P > 0.05 \)) (Figures 5(h)–5(k)).
3.4. Prognostic Value of MANF. To investigate further the prognostic role of MANF in HCC patients, GEPIA database and supporting clinical data of TMA were analyzed. We analyzed TCGA prognostic data and MANF transcriptional level of HCC (n = 364) using the GEPIA database. The overall survival rates of HCC patients with high expression of MANF
were significantly lower \( (P < 0.05) \) (Figure 6(a)) than those of patients with low expression of MANF. Disease-free survival did not differ significantly (Figure 6(b)). Beyond that, the TMA analysis of 259 HCC patients showed that patients with high MANF expression had shorter disease-free survival \( (P < 0.05) \) (Figure 6(d)) compared with patients with low expression of MANF. No significant difference was found in overall survival (Figure 6(c)). Therefore, high expression of MANF is a prognostic factor for HCC.

Patients with high MANF expression levels had a significantly higher risk of tumor recurrence \( (P < 0.05) \) (Table 2). There was no correlation of MANF expression with age, sex, \( \alpha \)-fetoprotein (AFP) levels, hepatitis B virus infection, cirrhosis, tumor size, tumor number, TNM stage, differentiation grade, and venous invasion. Univariate Cox regression analysis showed that tumor number \( (P < 0.01) \), AFP level \( (P < 0.05) \), TNM stage \( (P < 0.05) \), and venous invasion \( (P < 0.001) \) were independent prognostic factors for HCC patients. Multivariate Cox regression analysis showed that only venous invasion \( (P < 0.001) \) was an independent prognostic factor for HCC (Table 3).

### Table 2: The relationship between MANF status and clinicopathological features of HCC (tissue microarray).

| Clinicopathological features | Number of cases (n) | MANF expression, n (%) | \( P \) value |
|-----------------------------|---------------------|------------------------|--------------|
|                             | High | Low  |  |
| **Age**                     |      |      |  |
| \( \geq \text{Median} \)     | 133  | 64 (48.1) | 69 (51.9) | 0.54  |
| \(< \text{Median} \)        | 133  | 69 (51.9) | 64 (48.1) |      |
| **Gender**                  |      |      |  |
| Male                        | 241  | 123 (51)  | 118 (49)  | 0.293 |
| Female                      | 25   | 10 (40)   | 15 (60)   |      |
| **HBV**                     |      |      |  |
| Positive                    | 245  | 124 (50.6) | 121 (49.4) | 0.495 |
| Negative                    | 21   | 9 (42.9)  | 12 (57.1) |      |
| **Cirrhosis**               |      |      |  |
| Positive                    | 220  | 108 (49.1) | 112 (50.9) | 0.517 |
| Negative                    | 46   | 25 (54.3) | 21 (45.7) |      |
| **Tumor size**              |      |      |  |
| \( \geq 5 \)                | 221  | 106 (48)  | 115 (52)  | 0.141 |
| \(< 5 \)                    | 45   | 27 (60)   | 18 (40)   |      |
| **Tumor number**            |      |      |  |
| Single                      | 153  | 73 (47.7) | 80 (52.3) | 0.385 |
| Multiple                    | 113  | 60 (53.1) | 53 (46.9) |      |
| **AFP**                     |      |      |  |
| \( \geq 20 \)               | 201  | 95 (47.3) | 106 (52.7) | 0.117 |
| \(< 20 \)                   | 65   | 38 (58.5) | 27 (41.5) |      |
| **TNM stage**               |      |      |  |
| Stage I-II                  | 124  | 68 (54.8) | 56 (45.2) | 0.104 |
| Stage III-IV                | 142  | 65 (45.8) | 77 (54.2) |      |
| **Differentiation grade**   |      |      |  |
| Grade 1-2                   | 182  | 94 (51.6) | 88 (48.4) | 0.429 |
| Grade 3-4                   | 84   | 39 (46.4) | 45 (53.6) |      |
| **Vasoinvasion**            |      |      |  |
| Yes                         | 53   | 28 (52.8) | 25 (47.2) | 0.645 |
| No                          | 213  | 105 (49.3) | 108 (50.7) |      |
| **Tumor recurrence**        |      |      |  |
| Yes                         | 110  | 64 (58.2) | 46 (41.8) | 0.025 * |
| No                          | 156  | 69 (44.2) | 87 (55.8) |      |

Notes: *\( P < 0.05 \); **\( P < 0.01 \).

### 3.5. Coexpression Genes Correlated with MANF in HCC.

MANF association results were confirmed using LinkFinder of LinkedOmics to analyze mRNA sequencing data from 367 HCC patients in the TCGA via Pearson’s correlation test. The volcano plot (Figure 7(a)) shows that there were 3773 genes positively correlated with MANF (marked by red dots).
and 4404 genes negatively correlated (marked by green dots) ($P < 0.01$, FDR < 0.01). The top 50 significant gene sets positively and negatively correlated with MANF are shown in the heat map (Figures 7(b) and 7(c)). As it turns out, MANF has extensive influence on the transcriptome.

We examined the correlations between MANF and the top 10 genes with the highest expression multiples in HCC.

MANF was significantly correlated with $UBD$, $MDK$, and $AKRIB10$ and had some degree of correlation with other genes (Figures 8(a)–8(c)).

To confirm the role of MANF expression in the development of cancer, we used LinkFinder or LinkedOmics to analyze the relationship with common oncogenes and tumor suppressor genes. There were negative correlations between

| Clinicopathological features | Univariate analysis | Multivariate analysis |
|-----------------------------|---------------------|----------------------|
| | HR 95% (CI) | $P$ value | HR 95% (CI) | $P$ value |
| **MANF expression (T)** | | | | |
| Low | 1.000 | | 1.000 | |
| High | 1.070 | 0.829-1.380 | 0.605 | |
| **MANF expression (NT)** | | | | |
| Low | 1.000 | | 1.000 | |
| High | 0.896 | 0.694-1.158 | 0.401 | |
| **Age** | | | | |
| $<\text{Median}$ | 1.000 | | 1.000 | |
| $\geq\text{Median}$ | 1.014 | 0.786-1.307 | 0.917 | |
| **Gender** | | | | |
| Male | 1.000 | | 1.000 | |
| Female | 0.751 | 0.470-1.202 | 0.233 | |
| **HBV** | | | | |
| Negative | 1.000 | | 1.000 | |
| Positive | 1.169 | 0.731-1.870 | 0.514 | |
| **Cirrhosis** | | | | |
| Negative | 1.000 | | 1.000 | |
| Positive | 0.931 | 0.664-1.304 | 0.677 | |
| **Tumor size** | | | | |
| $<5$ | 1.000 | | 1.000 | |
| $\geq5$ | 1.218 | 0.869-1.705 | 0.252 | |
| **Tumor number** | | | | |
| Single | 1.000 | | 1.000 | |
| Multiple | 1.442 | 1.114-1.867 | 0.005** | 1.278 | 0.955-1.710 | 0.099 |
| **AFP** | | | | |
| $<20$ | 1.000 | | 1.000 | |
| $\geq20$ | 1.360 | 1.013-1.827 | 0.041* | 1.142 | 0.838-1.555 | 0.401 |
| **TNM stage** | | | | |
| Stage I-II | 1.000 | | 1.000 | |
| Stage III-IV | 1.404 | 1.086-1.814 | 0.010* | 0.991 | 0.726-1.353 | 0.957 |
| **Differentiation grade** | | | | |
| Grade 1-2 | 1.000 | | 1.000 | |
| Grade 3-4 | 0.955 | 0.725-1.260 | 0.746 | |
| **Vasoinvasion** | | | | |
| No | 1.000 | | 1.000 | |
| Yes | 2.757 | 1.993-3.813 | $<0.001^{**}$ | 2.521 | 1.769-3.591 | $<0.001^{**}$ |
| **Tumor recurrence** | | | | |
| No | 1.000 | | 1.000 | |
| Yes | 0.815 | 0.630-1.054 | 0.119 | |

Notes: *$P < 0.05$; **$P < 0.01$. T: tumor tissue; NT: nontumor tissue.
MANF expression and *RB1* (Pearson’s correlation = -0.3048, \( P < 0.01 \)) and *BRCA2* (Pearson’s correlation = -0.3493, \( P < 0.01 \)) (Figures 8(d) and 8(e)).

### 4. Discussion

HCC accounts for >90% of the histological types of primary malignant liver tumors, which are highly malignant and have a high recurrence rate and poor prognosis [3, 4]. Therefore, elucidating the molecular mechanisms underlying the progression and initiation of HCC is important for treatment selection.

ER stress can be induced by oncogene activation, such as *B-Raf* proto-oncogene mutations, *H-Ras* proto-oncogene mutations, and *c-Myc* amplification, as well as chemotherapeutic drugs [21]. When the ER functions, only correctly folded proteins can reach their cell compartment and unfolded or misfolded proteins accumulate within the ER.
lumen. Overwhelming cellular demand and shortage of cellular energy availability lead to the accumulation of wrongly folded proteins [22]. Unfolded protein response (UPR) helps cells to reestablish homeostasis by decreasing protein synthesis and increasing the folding and clearance capacity of the ER [23]. Under sustained ER stress conditions, ER homeostasis mediated by UPR cannot be restored and leads to initiation of apoptosis [24]. However, cancer cells have evolved UPR to alleviate ER stress conditions as a survival mechanism for progression [25, 26]. MANF

**Figure 8:** Genes correlated with MANF in HCC. (a–c) Correlations between MANF and the top 10 genes including UBD, MDK, and AKR1B10, which have the highest expression multiples in HCC (\( P < 0.01 \)). (d, e) Correlations between MANF and tumor suppressor genes RB1 and BRCA2 (\( P < 0.01 \)).
protects SH-SY5Y cells against 6-OHDA-induced toxicity by activating the PI3K/Akt/mTOR pathway and alleviating ER stress [27]. ER stress regulated by UPR also plays an important role in mechanisms of chemotherapy or radiation resistance in cancer [28]. MANF is a neurotrophic factor secreted from cells [29]. Kim et al. have indicated that MANF can serve as a urinary biomarker for detecting ER stress in podocytes or renal tubular cells [30]. Expression of MANF has been confirmed to be closely related to ER stress, which is a mediator in the initiation of HCC [16].

The liver is an important organ for the synthesis of proteins and lipids, so hepatocyte ER has appropriate adaptive capacity [31]. When the liver is in a state of inflammation for a long time, ER stress is maintained at a high level, which leads to hepatic dysfunction and progression of liver diseases, even HCC [32].

Our study is believed to be the first to explore mRNA expression and prognostic value of MANF in HCC. We analyzed MANF expression in HCC samples using gene expression and clinical prognostic data in the TCGA, CCLE, EMBL-EBI, GEPIA, LinkedOmics, and ONCOMINE databases, clinical specimens from our hospital, and HCC TMA s. We found that MANF was always highly expressed in HCC and many other cancers, indicating the significance of MANF in tumorigenesis.

Although previous studies have shown that MANF is highly expressed in HCC, there is a lack of reliable means to prove the diagnostic value of MANF in HCC. Therefore, we conducted a meta-analysis of MANF expression in previous studies retrieved from the GEO HCC dataset. ROC curves from GEO datasets were used to confirm the satisfactory diagnostic performance of MANF. However, diagnostic performance of MANF in TMA analysis was not entirely satisfactory, which may be caused by the subjectivity of immunohistochemical staining analysis. Overall, MANF was shown to be a potential diagnostic marker for distinguishing between HCC and nontumor tissues.

As shown in the analysis of the GEPIA database and TMA supporting clinical data, MANF is a novel potential prognostic marker for HCC patients. Consistent with these findings, patients with high MANF expression levels had a higher risk of tumor recurrence. Dysfunction of ER stress and UPR signal underlines the resistance of cancer cells to chemotherapy, and ER stress response was inhibited in chemoradiotherapy-resistant cells compared with that in sensitive cells [33]. MANF could alleviate ER stress and reduce ER stress-induced cell death, and ER stress activation could cause upregulation of MANF in vivo and in vitro [13, 34]. The higher recurrence rate and worse prognosis might be due to MANF-ER stress-mediated chemotherapy or targeted drug resistance; it needs further validation.

Our study proved that MANF was upregulated in HCC tissues more than in nontumor tissues. High expression of MANF was also involved in the development and progression of HCC and a potential indicator in the diagnosis, treatment, and prognosis of HCC. In addition, the molecular mechanism involved in MANF expression and occurrence of HCC remains unknown. In order to study further the important role of MANF in occurrence and development of HCC, more in vitro and in vivo experiments should be conducted.

5. Conclusion

MANF was overexpressed in HCC and related to poor prognosis and progression of HCC. Our results showed that MANF is a potential diagnostic and prognostic indicator of HCC.

**Abbreviations**

HCC: Hepatocellular carcinoma

MANF: Mesencephalic astrocyte-derived neurotrophic factor

(ARMET): Arginine-rich mutated in early tumor

GEPIA: Gene Expression Profiling Interactive Analysis

NAFLD: Nonalcoholic fatty liver disease

TCGA: The Cancer Genome Atlas

EMBL-EBI: The European Bioinformatics Institute

CCLE: Cancer Cell Line Encyclopedia

GEO: Gene Expression Omnibus

SMD: Standard mean difference

TMA: Tissue microarray

ROC: Receiver-operating characteristic

**Data Availability**

The PCR, WB, immunohistochemical staining and their supporting clinical data used to support the findings of this study are available from the corresponding author upon request because the data also forms part of an ongoing study. The Microarray Data supporting this META-ANALYSIS are from previously reported studies and datasets, which have been cited. The processed data are available at Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The bioinformation databases data supporting this study are from previously reported studies and datasets, which have been cited. The processed data are available at ONCOMINE (http://www.oncom/ http://ine.org/), Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepi.a.cancer-pku.cn/), Cancer Cell Line Encyclopedia (CCLE) (https://portals.bro http://adinstitute.org/ccle/data), LinkedOmics (http://www.linkedomics.org/admin.php), EMBL-EBI (https://www.ebi.ac.uk).

**Conflicts of Interest**

The authors report no conflicts of interest in this work.

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Supplementary Materials

Supplementary material is the basic characteristics of 24 HCC cohort from GEO supporting meta-analysis in this study. (Supplementary Materials)

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