Utility of miR-133a-3p as a diagnostic indicator for hepatocellular carcinoma: An investigation combined with GEO, TCGA, meta-analysis and bioinformatics

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Abstract. Increasing evidence has demonstrated that microRNA (miR)-133a-3p is an important regulator of hepatocellular carcinoma (HCC). In the present study, the diagnostic role of miR-133a-3p in HCC, and the potential functional pathways, were both explored based on publicly available data. Eligible microarray datasets were collected from NCBI Gene Expression Omnibus (GEO) database and ArrayExpress database. The data related to HCC and matched adjacent normal tissues were also downloaded from The Cancer Genome Atlas (TCGA). Published studies reporting the association between miR-133a-3p expression and HCC were reviewed from multiple databases. By combining the data derived from three sources (GEO, TCGA and published studies), the authors analyzed the comprehensive relationship between miR-133a-3p expression and clinicopathological features of HCC. Eventually, putative targets of miR-133a-3p in HCC were selected for further bioinformatics prediction. A total of eight published microarray datasets were gathered, and the pooled results demonstrated that the expression of miR-133a-3p in the tumor group was lower than that in normal groups [standardized mean difference (SMD)=-0.54; 95% confidence interval (CI), -0.74 to -0.35; P<0.001]. Consistently, the level of miR-133a-1 in HCC was reduced markedly compared to normal tissues (P<0.001) based on TCGA data, and the AUC value of low miR-133a-1 expression for HCC diagnosis was 0.670 (P<0.001). Furthermore, the combined SMD of all datasets (GEO, TCGA and literature) suggested that significant difference was observed between the HCC group and the normal control group, and lower miR-133a-3p expression in HCC group was noted (SMD=-0.69; 95% CI, -1.10 to -0.29; P=0.001). In addition, the authors discovered five key genes of the calcium signaling pathway (NOS1, ADRA1A, ADRA1B, ADRA1D and TBX2A2R) that may probably be targeted by miR-133a-3p in HCC. The study reveals that miR-133a-3p may function as a tumor suppressor in HCC. The prospective novel pathways and key genes of miR-133a-3p could offer potential biomarkers for HCC; however, the predictions require further confirmation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies in the world with extremely poor prognosis. HCC is also the second top cause of cancer-related death and its incidence ranked fifth in diagnosed cancers globally (1). Moreover, HCC is highly prevalent in many Asian countries owing to its high endemicity of hepatitis B virus (HBV) infection, especially in China (2–4). Multiple treatment possibilities are available for HCC, such as curative resection, liver transplantation, radiofrequency ablation, systemic targeted agents like sorafenib, whereas the management of advanced HCC still presents as a therapeutic challenge over the years (5–9). Despite these developments in surgery and other strategies, the prognosis of HCC patients is still unsatisfactory due to the non-curable stages at the time of diagnosis (10–14). Therefore, early diagnosis of HCC is expected and the molecular pathogenesis of HCC needs to be better elucidated.

MicroRNAs (miRNAs) are a class of highly conserved, endogenous, small non-coding RNA molecules of 18-23 nucleotides in length, which act as post-transcriptional regulators by binding to 3'-untranslated regions (3'-UTR) of mRNA for target genes (15–19). In recent years, researchers have focused on the importance of miRNAs in carcinogenesis (20–22). A

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The data were sequenced based on Illumina Genome Analyzer II (preservation) and 1.12.0 (data version) on October 16, 2016. TCGA data portal (https://tcga-data.nci.nih.gov) was utilized to acquire the potential target miRNAs of miR-133a-3p.

Materials and methods

Dataset sources

**GEO and ArrayExpress database searching.** Firstly, the microarrays related to HCC were obtained from GEO database (www.ncbi.nlm.gov/geo) and ArrayExpress (www.ebi.ac.uk/arrayexpress). The search terms included ‘malignant’ or ‘cancer’ or ‘tumor’ or ‘neoplasm’ or ‘carcinoma’) and (hepatocellular or liver or hepatic or HCC) and (miR-133a or miRNA-133a or microRNA-133a or miR133a or mir133a or miRNA133a or miRNA133a-3p or 'miR 133a' or 'miRNA 133a' or 'microRNA 133a' or 'miRNA-133a' or 'miRNA-133a-3p' or 'miRNA133a-3p' or 'microRNA133a-3p' or 'miRNA133a-3p' or 'microRNA133a-3p').

**Study identification.** Studies including microarrays or publications were considered eligible if they met following criteria: i) Study on human HCC tissues; ii) miR-133a-3p expression in tissues was measured and analyzed; iii) literatures were published in Chinese or English; iv) when data of the same patients were published in more than one article, only the latest publication was included; and v) as to microarrays, both HCC patients and normal samples were included in each dataset, and each group contained more than two samples.

**Data extraction.** All the eligible studies were carefully reviewed and then the information was extracted from the inclusive studies independently by two investigators (Hai-Wei Liang and Xia Yang). Disagreements were negotiated by a third investigator (Gang Chen) and agreements were reached by discussion. With the included criteria, the following characteristics were obtained from eligible articles: First author's name or series (prefix, GSE) accession number, year of publication, country, number of patients and means and standard deviations of miR-133a-3p expression level.

**Prediction of targeted genes of miR-133a-3p.** An online prediction software miRWalk, which included 12 silico databases [such as TargetScan (www.targetscan.org), miRanda (www.mirnada.org) and PicTar (pictar.mdc-berlin.de)] was utilized to acquire the potential target miRNAs of miR-133a-3p.
A total of 3,995 target genes were predicted initially, and only those genes appearing in more than five prediction software programs were essential for the present study.

Functional analyses. To further explore the biological processes and signal pathways in which miR-133a-3p could be involved and also to provide a theoretical foundation for future studies, functional analyses were performed subsequently. Gene Ontology (GO) analysis was conducted, since it demonstrates the predominant functions of the target genes from three aspects: Biological process, molecular function, as well as cellular component. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (www.genome.jp/kegg) was used to excavate remarkable pathways associated with target genes. All GO, KEGG pathway and panther analyses were performed to gain a further insight into the function of these potential target genes of miR-133a-3p in HCC by database for Annotation, Visualization and Integrated Discovery (DAVID). P<0.01 for GO terms and P<0.05 for pathways were considered statistically significant.

Protein-protein interactions (PPIs) network construction. The potential target genes in the top terms of KEGG analysis were employed to construct PPIs network by STRING 10.0 (http://string-db.org/), a Search Tool for the Retrieval of Interacting Genes/Proteins, with the minimum required interaction score 0.4 (44). The related data were downloaded from Tables/Exports module and the complete PPI network was drawn, then hub genes were identified. The proteins interactions information in STRING was derived from four sources, i.e., i) literature-reported protein interactions; ii) high-throughput experiments; iii) genome analysis and prediction; and iv) co-expression studies. The protein product of a gene serves as a node in the PPI network, and the connectivity degree denotes the interplayed protein numbers of the specific protein.

Statistical analysis. As for data obtained from the TCGA database, the miR-133a expression values were presented in a way as means ± standard deviation. Receiver operating characteristic (ROC) curves were performed to judge the diagnostic value of miR-133a in HCC. The area under the curve (AUC) and P-value were calculated. Student's t-test was applied to verify the difference between two corresponding groups of various clinical features. Box-plots were drawn when significant difference existed in two compared ones. All above analyses were carried out via SPSS software (version 22.0; IBM, SPSS, Armonk, NY, USA). With respect to meta-analysis, standardized mean difference (SMD) and 95% confidence interval (CI) were counted for pooled values. Sensitivity analysis was conducted to assess the stability of included studies. Cochran's Q and I² test were used to evaluate heterogeneity, and P<0.1 indicated significant heterogeneity. Publication bias was estimated by the Egger's and Begg's test. Meta-analysis was conducted using STATA 12.0 (StataCorp LLC, College Station, TX, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Signatures of GEO datasets and meta-analysis. The authors first searched GEO datasets to assess the expression of miR-133a-3p between HCC and non-cancer tissues. miR-133a-3p levels in eight datasets GSE57555 (Japan), GSE69580 (Taiwan), GSE54751 (USA), GSE41874 (Japan), GSE40744 (USA), GSE21362 (Japan), GSE22058 (USA) and GSE12717 (USA) which included 217 HCC and 219 normal tissues are presented in Fig. 3. One dataset about recurrence (GSE64989, Germany) and two for invasiveness (GSE67138; GSE67139, USA) were also presented. Then meta-analysis containing eight microarrays was conducted to evaluate the diagnostic value of miR-133a-3p in HCC. SMD with 95% CIs from involved datasets were pooled, and default fixed-effects model was first used. The pooled results revealed that noticeable difference was observed between tumor and normal groups (SMD=-0.54; 95% CI, -0.74 to -0.35; P<0.001; Fig. 4). No significant heterogeneity was found (I²=36.5%; P=0.138).

Clinical role of miR-133a in HCC analyzed with TCGA data. In order to gain a larger number samples and strengthen the reliability of our results, the authors subsequently searched and downloaded TCGA data for further analysis. Before adding to meta-analysis, they calculated the clinical significance
of miR-133a-1 in HCC with SPSS 20.0, since no data of the mature miRNA, e.g., miR-133a-3p-1 were available for HCC in TCGA. The results demonstrated that level of miR-133a-1 in HCC was 3.48±1.93, which was reduced markedly when compared to that in non-cancerous liver tissues (4.44±1.12, P<0.001; Fig. 5A). The AUC value of low expression miR-133a-1 for HCC diagnosis was 0.670 (P<0.001; Fig. 5B).

Regarding the difference of miR-133a-1 expression between two corresponding groups of various clinical features, the authors revealed that miR-133a-1 expression in vascular invasion group (3.32±1.82) demonstrated a significant reduced pattern compared to non-invasiveness group (3.77±1.75, P=0.04; Fig. 5C). The relationship between miR-133a-1 and vascular invasion was confirmed by Spearman correlation test (r=-0.127; P=0.029). The data of miR-133a-2 was not sufficient for analysis in TCGA database (data not shown).
data derived from TCGA, PubMed, literature (38) and the PCR data in house based on 95 HCC and matched adjacent normal tissue (data on file) were finally merged in the present meta-analysis. In total, 719 HCC tissues and 404 normal tissues were involved. The number of HCC patients ranged from 5 to 367 per dataset. The involved datasets signatures were showed in Table I. The result generated by fixed-effects model indicated that significant heterogeneity existed among individual datasets \(I^2=83.9\%, P<0.001; \text{Fig. 6}\). Thus, a random-effects model was selected to evaluate the pooled SMD with 95% CI. The combined SMD suggested that remarkable difference was noted between HCC group and normal control group \(\text{SMD}=-0.69; 95\% \text{ CI}, -1.10 \text{ to } -0.29; P=0.001; \text{Fig. 7}\). In other words, miR-133a-3p expression in HCC patients was remarkably lower than that in non-cancerous controls.

Sensitivity analysis. To identify the reason why notable heterogeneity occurred in meta-analysis, the authors conducted a sensitivity analysis (Fig. 6). According to the results, dataset from Zhang et al (38) reported obvious deviation from the estimate. Heterogeneity was found significantly reduced following omission of the dataset of Zhang et al \(I^2=18.5\%; P=0.273\), revealing heterogeneity may be perplexed by the present study. The combined SMD with 95% CI after removal of data from Zhang et al \(\text{SMD}=-0.54, 95\% \text{ CI}, -0.68 \text{ to } -0.39, P<0.001\) is presented in Fig. 8.

Publication bias. The authors performed both Begg’s funnel plots and Egger’s tests for all datasets to evaluate the publication bias. The results generated a Begg’s test score of \(P=0.755>0.05\) (Fig. 9) and an Egger’s test score of \(P=0.827>0.05\), which suggested the absence of publication bias in the present meta-analysis (Fig. 9).

Targets prediction and functional enrichment. Both DEGs from TCGA and target genes of miR-133a-3p predicted from 12 prediction algorithms were obtained. In total, 4,159 DEGs were retrieved from TCGA. In order to increase the reliability, 3,995 potential target genes were identified by at least five prediction algorithms. Then genes from both approaches (DEGs from TCGA and target genes predicted) were
Table I. Characteristics of studies included in the meta-analysis.

| Study     | Year (publication) | Country | Age (years) | Sex | n | Mean ± standard deviation |
|-----------|-------------------|---------|-------------|-----|---|---------------------------|
|           |                   |         | <60 | ≥60 | Male | Female | HCC | Normal control | HCC | Normal |
| GSE57555  | 2015              | Japan   | 6   | 15  | 18   | 3 | 5 | 16 | -0.042±0.006 | -0.053±0.018 |
| GSE69580  | 2015              | Taiwan  | -   | -   | -   | - | 5 | 5 | 1.854±1.106 | 1.697±0.937 |
| GSE54751  | 2014              | USA     | 6   | 14  | 10   | 10 | 13 | 7 | 0.001±0.001 | 0.005±0.009 |
| GSE41874  | 2013              | Japan   | 9   | 0   | 6    | 3 | 6 | 4 | 1.032±0.206 | 1.043±0.346 |
| GSE40744  | 2013              | USA     | 16  | 5   | 15    | 6 | 9 | 12 | 1.741±0.171 | 1.862±0.247 |
| GSE21362  | 2011              | Japan   | -   | -   | -    | - | 73 | 73 | 2.632±1.514 | 3.316±1.571 |
| GSE22058  | 2010              | USA     | -   | -   | -    | - | 96 | 96 | -0.748±0.369 | -0.530±0.216 |
| GSE12717  | 2008              | USA     | 16  | 0   | 4    | 12 | 10 | 6 | 6.475±1.395 | 7.997±0.219 |
| Zhang et al | 2015          | China   | -   | -   | 24   | 16 | 40 | 40 | 0.510±0.110 | 1.050±0.240 |
| PCR       | 2016              | China   | -   | -   | 75   | 20 | 95 | 95 | 3.285±2.379 | 4.497±2.195 |
| TCGA       | -                 | -       | 172 | 204 | 255  | 122 | 367 | 50 | 3.484±1.928 | 4.444±1.116 |

HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.

Figure 6. Sensitivity analysis of the 11 evaluable datasets assessing microRNA-133a-3p expression in hepatocellular carcinoma. Significant heterogeneity existed among individual datasets (I²=83.9%; P<0.001), and the dataset from Zhang et al (38) presented an obvious deviation from the estimate.

Figure 7. Forest plot of the 11 evaluable datasets assessing microRNA-133a-3p expression in HCC. A random-effects model was selected to evaluate the pooled SMD with 95% CI. The combined SMD suggested that remarkable difference was noted between HCC group and normal control group (SMD=-0.69; 95% CI, -1.10 to -0.29; P=0.001). SMD, standardized mean difference; 95% CI, 95% confidence intervals; HCC, hepatocellular carcinoma.
overlapped and 828 putative targets were selected for further functional analyses.

In the three categories of GO pathway analyses, target genes were statistically enriched in biological process (BP), in which 372 terms were identified and FDR were <0.05 in 21 terms. The top enriched BP term was cell-cell signaling (GO: 0007267), which contained 70 genes, and the FDR reached 4.02x10^{-9}. Besides, the top term of cellular components (CC) and molecular functions (MF) were the plasma membrane part (GO: 0044449; FDR=2.42x10^{-10}) and calcium ion binding (GO: 0005509; FDR=0.014), respectively. The top 10 terms of each category are listed in Table II, and the GO pathway map is presented in Fig. 10.

The KEGG pathways were also identified, many of which were related to signaling pathway, such as the calcium signaling pathway (hsa04020; P=2.94x10^{-4}), neuroactive ligand-receptor interaction (hsa04080; P=0.001) and the Hedgehog signaling pathway (hsa04340; P=0.005). The target genes were significantly enriched in PANTHER terms of Synaptic-vesicle-trafficking (P05734; P=4.32x10^{-5}), the

Figure 8. Forest plot of 10 evaluable datasets assessing microRNA-133a-3p expression in hepatocellular carcinoma. The combined SMD with 95% CI following removal of the data from Zhang et al (SMD=-0.54; 95% CI: -0.68 to -0.39; P<0.001; I^2=18.5%; P=0.273) presented a noticeable difference between tumor and normal groups. SMD, standardized mean difference; 95% CI, 95% confidence intervals.

Figure 9. Funnel blot and Egger’s plot for the publication bias test of 11 studies. SMD, standardized mean difference.

Figure 10. Gene Ontology pathway interaction maps were drawn using the Cytoscape v3.4.0 BiNGO plug-in. Color and size intensity denotes the significance of functional pathway and the enrichment of gene, dark color represents the most significant pathway terms, and the larger size represents more enrichment of genes.
### Table II. GO functional annotation for most significantly related targets of miR133a-3p.

| GO ID      | GO term                  | Count (%) | P-value         | Benjamini FDR | Genes                                                                 |
|------------|--------------------------|-----------|-----------------|----------------|------------------------------------------------------------------------|
| Biological process |                        |           |                 |                |                                                                        |
| GO:0007267 | Cell-cell signaling      | 70 (0.9)  | 2.24x10^{-12}   | 6.42E-09       | SYT1, PDGFA, SLC6A2, SLC6A4, EFNA3, LPAR3, ILDR2, GABBR2, ENPep, GJA5 |
| GO:0007268 | Synaptic transmission    | 40 (0.5)  | 4.78x10^{-9}    | 8.56E-06       | PPFIA3, SYT1, RAB3A, SLC6A2, OPRK1, SLC6A4, LPAR3, OXTR, GABBR2, DFNb31 |
| GO:0019226 | Transmission of nerve    | 43 (0.6)  | 1.63x10^{-8}    | 1.55E-05       | PPFIA3, SYT1, RAB3A, SLC6A2, OPRK1, SLC6A4, LPAR3, OXTR, GABBR2, DFNb31 |
| GO:0048666 | Neuron development       | 41 (0.6)  | 5.71x10^{-8}    | 4.08E-05       | RAB3A, CDK5R1, PAX2, CXCL12, EPHB1, EPHB2, TGFB2, DFNb31, LINGO1, BDNF |
| GO:0030182 | Neuron differentiation   | 47 (0.6)  | 1.99x10^{-7}    | 1.14E-04       | RAB3A, CDK5R1, NNAT, BRSK1, PAX2, CXCL12, EPHB1, TGFB2, EPHB2, DFNb31 |
| GO:0009002 | Cell morphogenesis       | 41 (0.6)  | 2.17x10^{-7}    | 1.04E-04       | RAB3A, CDK5R1, TFCP2L1, BRSK1, SOX6, SOX9, PAX2, CXCL12, EPHB1, EPHB2 |
| GO:0031175 | Neuron projection        | 33 (0.4)  | 3.37x10^{-7}    | 1.38E-04       | RAB3A, CDK5R1, PAX2, CXCL12, EPHB1, EPHB2, LINGO1, BDNF, PVRL1, ANK3  |
| GO:0009004 | Cell morphogenesis       | 32 (0.4)  | 3.62x10^{-7}    | 1.29E-04       | RAB3A, CDK5R1, SOX9, PAX2, CXCL12, EPHB1, EPHB2, TGFB2, BDNF, PVRL1  |
| GO:003030  | Cell projection          | 41 (0.6)  | 5.20x10^{-7}    | 1.65E-04       | RAB3A, CDK5R1, CROCC, PDGFA, LPAR3, PAX2, CXCL12, EPHB1, EPHB2, DFNb31 |
| GO:0048667 | Cell morphogenesis       | 28 (0.4)  | 1.48x10^{-6}    | 4.23E-04       | RAB3A, CDK5R1, PAX2, CXCL12, EPHB1, EPHB2, BDNF, PVRL1, ANK3, BAI1    |
|           | involved                 |           |                 |                |                                                                        |
|           | Cellular component       |           |                 |                |                                                                        |
| GO:0044459 | Plasma membrane part    | 176 (2.4) | 1.74x10^{-13}   | 6.66E-11       | SYT1, ENAH, ADORA3, SGMS2, SLC6A20, SLC6A2, SYT2, EFNA3, SYT3, SLC6A4 |
| GO:0005887 | Integral to plasma      | 108 (1.5) | 2.56x10^{-11}   | 4.88E-09       | ADORA3, SGMS2, SLC6A20, SLC6A2, SLC6A4, EFNA3, SLC7A8, CSPG4, LPAR3, ENPep |
| GO:0031226 | Intrinsic to plasma     | 109 (1.5) | 4.46x10^{-11}   | 5.68E-09       | ADORA3, SGMS2, SLC6A20, SLC6A2, SLC6A4, EFNA3, SLC7A8, CSPG4, LPAR3, ENPep |
| GO:0005886 | Plasma membrane         | 239 (3.2) | 7.37x10^{-8}    | 7.04E-06       | SYT1, ADORA3, GLDN, SGMS2, SYT2, EFNA3, SYT3, LPAR3, SYT9, SYP         |
| GO:0045202 | Synapse                 | 40 (0.5)  | 7.75x10^{-7}    | 5.92E-05       | RAB3A, SYT1, CDK5R1, ENAH, RAB3B, LZTS1, RAB3C, SYT2, SYT3, SYT9       |
| GO:0016021 | Integral to membrane    | 299 (4.0) | 3.61x10^{-5}    | 0.002295       | CYP3A4, SYT1, ADORA3, SGMS2, GLDN, SYT2, SLC9A2, EFNA3, SYT3, SYT9    |
Table II. Continued.

| GO ID       | GO term                     | Count (%) | P-value       | Benjamini | FDR          | Genes¹         |
|-------------|-----------------------------|-----------|---------------|-----------|--------------|----------------|
| GO:0034702  | Ion channel complex         | 25 (0.3)  | 3.75x10⁻⁵     | 0.002043  | 0.051895     | KCNA3, CACNB3, KCNJ14, KCNJ13, TTYH3, KCNQ4, BEST1, TTYH2, KCNE1, CHRFAM7A |
| GO:0031224  | Intrinsic to membrane       | 307 (4.1) | 5.18x10⁻⁵     | 0.002469  | 0.071712     | CYP3A4, SYT1, ADORA3, SGMS2, GLDN, SYT2, SLC9A2, SYT3, EFNA3, SYT9 |
| GO:0030054  | Cell junction               | 46 (0.6)  | 5.59x10⁻⁵     | 0.002369  | 0.077386     | SYT1, ENAH, LZZT1, SYT2, SYT3, FERT1, SYT9, OXTR, BRSK1, GABBR2 |
| GO:0043005  | Neuron projection           | 34 (0.5)  | 7.87x10⁻⁵     | 0.003003  | 0.109018     | SYT1, CDK5R1, LZZT1, TACR1, GABBR2, KLC2, TGFB2, DFN1B1, PCSK1, KCNQ4 |
|             | Molecular function          |           |               |           |              | KCNA3, CACNB3, KCNJ14, KCNJ13, TTYH3, KCNQ4, BEST1, TTYH2, KCNE1, CHRFAM7A |
| GO:0005509  | Calcium ion binding         | 73 (1.0)  | 9.04x10⁻⁶     | 0.007564  | 0.013963     | SYT1, LDLR, MASP1, SYT2, MMP9, SYT3, SYT9, ENPEP, PCDHGA4, MMP3 |
| GO:0004935  | Adrenoceptor activity       | 6 (0.1)   | 4.55x10⁻⁵     | 0.018938  | 0.070301     | ADRB1, HTR4, ADRA1B, ADRA1A, ADRA2B, ADRA1D |
| GO:0005003  | Ephrin receptor activity    | 7 (0.1)   | 8.10x10⁻⁵     | 0.022426  | 0.125062     | EPHA7, EFN3B, EFN3A, EPHA10, EFNA4, EPHB1, EPHB2 |
| GO:0022832  | Voltage-gated channel activity | 23 (0.3) | 1.16x10⁻⁴     | 0.024163  | 0.179776     | HCN2, KCND3, CACNA1I, CACNG4, KCNA3, KCNJ10, CACNB3, KCNK13, KCNJ14, KCD7 |
| GO:0005244  | Voltage-gated ion channel activity | 23 (0.3) | 1.16x10⁻⁴     | 0.024163  | 0.179776     | HCN2, KCND3, CACNA1I, CACNG4, KCNA3, KCNJ10, CACNB3, KCNK13, KCNJ14, KCD7 |
| GO:0022843  | Voltage-gated cation channel activity | 19 (0.3) | 1.71x10⁻⁴     | 0.02837  | 0.264299     | HCN2, KCND3, CACNA1I, CACNG4, KCNA3, KCNJ10, CACNB3, KCNK14, KCD7, KCNJ13 |
| GO:0005261  | Cation channel activity     | 28 (0.4)  | 2.23x10⁻⁴     | 0.030778  | 0.344365     | KCNA3, KCNJ10, CACNB3, KCNK13, KCNJ14, KCNK10, KCNJ13, KCNQ4, GP4M6A, KCNE |
| GO:0015267  | Channel activity            | 37 (0.5)  | 2.31x10⁻⁴     | 0.027299  | 0.355692     | KCNA3, KCNJ10, CACNB3, KCNK13, KCNJ14, KCNK10, KCNJ13, MIP, TTYH3 |
| GO:0022803  | Passive transmembrane transporter activity | 37 (0.5) | 2.41x10⁻⁴     | 0.024983  | 0.371552     | KCNA3, KCNJ10, CACNB3, KCNK13, GI5, KCNJ14, KCNK10, KCNJ13, MIP, TTYH3 |
| GO:0022838  | Substrate specific channel activity | 36 (0.5) | 2.48x10⁻⁴     | 0.022846  | 0.3818       | KCNA3, KCNJ10, CACNB3, KCNK13, KCNJ14, KCNK10, KCNJ13, MIP, TTYH3, KCNQ4 |

KEGG, Kyoto Encyclopedia of Genes and Genomes; "gene, the top 10 genes of the pathway are listed, Benjamini, a corrected P-value which is corrected for multiple hypothesis testing using the Benjamini-Hochberg method."
| KEGG ID  | KEGG term                           | Count (%) | P-value       | Benjamini FDR | Genes                                                                 |
|---------|-------------------------------------|-----------|---------------|---------------|----------------------------------------------------------------------|
| hsa04200 | Calcium signaling pathway           | 21 (0.3)  | 2.94E-04      | 0.049092      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04340 | Neuroactive ligand-receptor interaction | 25 (0.3)  | 0.001306      | 0.096932      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04360 | MAPK signaling pathway              | 23 (0.3)  | 0.009768      | 0.001202      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04370 | Axon guidance                       | 13 (0.2)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04371 | Pathways in cancer                  | 25 (0.3)  | 0.027097      | 0.051044      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Fatty acid metabolism               | 6 (0.1)   | 0.042643      | 0.049092      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Basal cell carcinoma                | 7 (0.1)   | 0.048923      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Hedgehog signaling pathway          | 9 (0.1)   | 0.052995      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Wnt signaling pathway               | 13 (0.2)  | 0.060234      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Wnt signaling pathway               | 13 (0.2)  | 0.060234      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Wnt signaling pathway               | 13 (0.2)  | 0.060234      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
| hsa04377 | Whole signaling pathway             | 23 (0.3)  | 0.022042      | 0.051123      | PTGER3, NOS3, TACR1, CACNA1A, HTR4, OXR, AVPR2, ADORA3, TACR1, OPRK1, PTHR1, PTH1R,  |
Alzheimer disease-presenilin pathway (P00004, P=0.002), the Alzheimer disease-amyloid secretase pathway (P00003; P=0.016). All KEGG and PANTHER pathway terms are listed in Table III and Fig. 11.

PPIs network of calcium signaling pathway. Proteins rarely act alone as their functions tend to be regulated. PPIs refer to lasting or ephemeral physical contacts of high specificity which was established between two or more protein molecules as a result of biochemical events steered by electrostatic forces including the hydrophobic effect. Commonly they refer to physical contacts with molecular associations between chains that occur in a cell or in a living organism in a specific biomolecular context. Thus, the authors decided to further analyze the most significant pathway of KEGG by constructing PPIs network. So ‘Calcium signaling pathway’ with 21 genes was included for the PPI analysis. As a result, a total of five key genes with the number of more than eight edges indicating higher connectivity degree were identified, including NOS1, ADRA1A, ADRA1B, ADRA1D and TBXA2R. PPIs network were presented in Fig. 12.

Validation of some potential target gene expression based on TCGA dataset. Among all the significant pathways in the present study, the Calcium signaling pathway (KEGG ID: hsa04020) was selected for validation. There were 21 genes involved in the pathway. The authors then analyzed the relative expression (on a log2 scale) of these 21 predicted genes of miR-133a-3p by using TCGA HCC dataset. Eight genes (NOS1, PRKCB, ADRA1A, ADRB1, TBXA2R, CAMK4, ADRA1B, CAMK2B) were downregulated and they could not be real targets of miR-133a-3p due to same trend of the expression in HCC (Table IV). It was interesting that 11 among the rest 13 genes presenting extremely significant overexpressed pattern in liver cancers as compared to that in the adjacent liver tissues (P<0.01; Table IV; Fig. 13A-K), such as CACNA1E, HTR4, CACNAIC, CHRM3, OXTR, CACNA1I, CACNA1A, CHRFAM7A, PTGER3, TACR1, CAMK2A.

Discussion

As the investigation surrounding miRNA function is currently very significant in research, more and more miRNAs have been reported to exert functions in HCC and development (45-54). Downregulated miR‑133a‑3p has been identified in several types of cancers, such as bladder (55), colorectal (15), osteosarcoma (56-58), non-small-cell lung (36,59) and esophageal cancers (60). However, the expression of miR-133a-3p in HCC tissues remains controversial. Most pilot studies proposed that miR-133a-3p may play as a tumor suppressor in HCC, while several studies present inconsistency (GSE57555, GSE69580). How miR-133a-3p exerts its function is still unclear. Comparing the data collected from GEO datasets, TCGA and literature, the authors found that the uneven sample size from different studies may contribute to the contradictory results. In this meta-analysis, miR-133a-3p expression was pooled from eight microarray datasets from GEO and the other three studies from literature, TCGA and the authors' own PCR data. The result generated by fixed-effects model indicated that significant heterogeneity was present among individual datasets (I²=83.9%; P<0.001; Fig. 6). The significant heterogeneity may result from the following reasons: Firstly, different
From the First Hospital of Jilin University (Changchun, China). The tissue samples of the study of Zhang et al. (38) were collected. Their result indicated that miR-133a-3p could function as a tumor suppressor in HCC, which was consistent with our own PCR data used different means to detect the expression of miR-133a-3p. The studies from the literature and our own data used different means to detect the expression of miR-133a-3p. A total of 40 pairs of HCC tissue samples of the study of Zhang et al. (38) were collected from the First Hospital of Jilin University (Changchun, China). Compared to other case-control studies, the authors hypothesize that the limited sample sizes could be the main reason for the current heterogeneity. Wang et al. (61) investigated 10 primary HCC samples as well as their matched normal adjacent hepatic tissues, and demonstrated that miR-133a-3p level was reduced in HCC. However, this study was not included into this meta-analysis, for the means and standard deviation or original data could not be collected. Furthermore, Wang et al. (61) also stated that overexpression of miR-133a-3p suppressed cell growth, migration and invasion, whereas the overexpression of miR-133a-3p enhanced apoptosis in HCC. Though, some evidence reported that miR-133a-3p suppressed cell growth, migration and invasion, whereas the overexpression of miR-133a-3p enhanced apoptosis in HCC cells. In their study, the authors identified a miRNA classifier (CMI) containing miR-133a, miR-143, miR-145, miR-192, and miR-505) was notably expressed in the serum of HCC. In their study, the authors identified a miRNA classifier (CMI) containing miR-133a, miR-143, miR-145, miR-192, and miR-505) was notably expressed in the serum of HCC. In their study, the authors identified a miRNA classifier (CMI) containing miR-133a, miR-143, miR-145, miR-192, and miR-505) was notably expressed in the serum of HCC. In their study, the authors identified a miRNA classifier (CMI) containing miR-133a, miR-143, miR-145, miR-192, and miR-505) was notably expressed in the serum of HCC. In their study, the authors identified a miRNA classifier (CMI) containing miR-133a, miR-143, miR-145, miR-192, and miR-505) was notably expressed in the serum of HCC.

### Table IV. Expressions of 21 genes based on The Cancer Genome Atlas dataset (374 HCC tissues and 50 normal liver tissues).

| Gene name     | HCC tissue (Mean ± SD) | Normal liver tissue (Mean ± SD) | t     | P-value   | FC   |
|---------------|------------------------|-------------------------------|-------|-----------|------|
| CACNA1E       | 3.86±2.16              | 1.81±1.33                     | -5.97 | 3.64x10^-6a | 2.05 |
| HTR4          | 3.52±1.89              | 2.13±1.14                     | -5.82 | 5.36x10^-7a | 1.4  |
| CACNA1C       | 6.5±1.65               | 5.38±1.03                     | -6.62 | 2.81x10^-6a | 1.12 |
| CHRM3         | 6.3±2.12               | 5.23±1.4                      | -4.7  | 1.02x10^-5a | 1.07 |
| OXTR          | 4.91±1.79              | 3.86±1.66                     | -3.83 | 1.47x10^-4a | 1.04 |
| CACNA11       | 3.51±2.18              | 2.51±1.24                     | -4.03 | 1.60x10^-4a | 1.00 |
| CACNA1A       | 3.73±1.3               | 2.85±0.82                     | -6.44 | 7.71x10^-9a | 0.88 |
| CHRFAM7A      | 2.25±1.35              | 1.38±0.48                     | -4.22 | 7.54x10^-4a | 0.86 |
| PTGER3        | 4.88±1.73              | 4.03±1.42                     | -3.23 | 1.35x10^-3a | 0.84 |
| TACR1         | 3.21±1.7               | 2.38±0.91                     | -4.65 | 1.46x10^-5a | 0.84 |
| CAMK2A        | 3.6±2.19               | 2.82±1.03                     | -3.94 | 1.34x10^-4a | 0.78 |
| ADRA1D        | 3.61±2.26              | 3.27±1.74                     | -0.72 | 4.69x10^-1  | 0.34 |
| PTGFR         | 5.92±3.04              | 5.6±1.21                      | -1.37 | 1.74x10^-1  | 0.32 |
| NOS1          | 2.64±1.84              | 2.97±0.98                     | 1.66  | 9.98x10^-2  | -0.33|
| PRKCB         | 6.67±1.65              | 7.78±1.43                     | 4.56  | 6.62x10^-6a | -1.12|
| ADRA1A        | 5.78±2.62              | 6.95±4.59                     | 1.76  | 8.35x10^-2  | -1.17|
| ADRB1         | 3.71±1.76              | 5.03±1.49                     | 5.71  | 2.37x10^-6a | -1.33|
| TBX2A2R       | 6.87±1.09              | 8.32±1.17                     | 8.28  | 1.46x10^-11a| -1.45|
| CAMK4         | 5.25±1.44              | 7±1.09                        | 10.17 | 1.09x10^-15a| -1.74|
| ADRA1B        | 6.55±1.86              | 8.47±0.74                     | 13.47 | 8.19x10^-28a| -1.92|
| CAMK2B        | 5.32±2.63              | 8.55±0.89                     | 17.13 | 3.33x10^-41a| -3.23|

FC, fold change; SD, standard deviation; HCC, hepatocellular carcinoma. *P<0.001 vs. normal tissue.
The different expression trend of miR-133a-3p between tissue and serum might because of the different patient samples and detection methods. For example, the matched controls of this study included 51 healthy controls, 51 patients with chronic hepatitis B, and 47 with HBV-induced liver cirrhosis; miRNAs level were detected by reverse transcription-quantitative PCR. Thus, more explorations are required to fig. out the miR-133a-3p level in the serum of HCC patients.

Even though eight microarray gene sets did not show noticeable heterogeneity in the meta-analysis, two of them (GSE57555 and GSE69580) reported that miR-133a-3p expression was higher in HCC than in normal tissues. One (GSE41874) demonstrated no significant difference between HCC and normal tissues. Having analyzed the three micro-arrays (GSE57555, GSE69580 and GSE41874), the authors noticed that the number of normal samples (n=16) was obviously larger than that of HCC tissues (n=5) in GSE57555, while both tumor and normal samples were small in GSE69580 (n=5 for HCC, n=5 for control) and GSE41874 (n=6 for HCC, n=4 for control). It should be noted that, CIs intervals showed the same trend, which provides more reliable evidence that miR-133a-3p serves a suppressive role in HCC. Furthermore, miR-133a-3p could also be a diagnostic marker for HCC.

In the present study, the authors observed that miR-133a-1 expression in vascular invasion group (3.32±1.82) revealed a significantly reduced pattern compared to the non-invasive group (3.77±1.75; P=0.04; Fig. 5) from the TCGA data. Similarly, in one GEO dataset (GSE67138), the expression of miR-133a-3p in the vascular invasion group also showed significant reduced pattern compared to without vascular invasion group (P=0.0072; Fig. 3J). It was indicated that decreased expression of miR-133a-3p was closely correlated with vascular invasion. Meanwhile, the present functional analyses indicated that several target genes of miR-133a-3p were both involved in the calcium signaling pathway and the mitogen-associated protein kinase (MAPK) signaling pathway; from which the calcium signaling pathway may involve in the vascular invasion of HCC (63), and the MAPK signaling pathway also has been reported to promote cell growth and invasion in HCC (64). Thus, miR-133a-3p may exert its effect of vascular invasion by targeting CACNA1A, CACNA1C, CACNA1E, CACNA1I and PRKCB in the calcium signaling pathway and the MAPK signaling pathway.

The mature miRNA microRNA-133a-3p (miR-133a-3p, previously named miR-133a) can be generated from miR-133a-1 and miR-133a-2 (the precursor-miRNA, pre-miRNA). miR-133a-1 and miR-133a-2 are located on different chromosomes and have different sequences, with diverse functions in the process of transcription (65). Theoretically, miRNAs are transcribed as long primary-miRNA (pri-miRNA) transcripts that are cleaved in the nucleus by the DROSHA (Drosha) enzyme to liberate the precursor-miRNA (pre-miRNA) hairpin. The pre-miRNA is subsequently exported from the nucleus and further processed by the enzyme Dicer1 (Dicer) in the cytoplasm to produce mature miRNAs. miR-133a is encoded by two separate genes in humans (miR-133a-1 and miR-133a-2). miR-133a-1 is located on chromosome 18 (MI0000450), and miR-133a-2 is located on chromosome 20 (MI0000451). The processing of the precursor transcripts by Dicer generates mature miRNAs: miR-133a-3p.

Figure 13. Expressions of the 11 genes with significant fold changes from the calcium signaling pathway in HCC based on TCGA dataset. The TCGA RNAseq profiles of HCC were extracted to explore the relative expressions of 11 selected genes from the calcium signaling pathway in HCC tissue and normal liver tissue (on a log2 scale), **P<0.01, ***P<0.001 as indicated. CACNA1A; HTR4; CACNA1C; CHRM3; OXTR; CACNA1I; CACNA1A; CHRFAM7A; PTGER3; TACR1; CAMK2A. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas.
miR-133a-1, miR-133a-2 and miR-133a-3p have different sequences and different targets bind to their promoter region in the transcription or translation process. Based on the understanding of their diverse functions, the authors intend to understand disease progression more precisely, and this may provide new insights for disease treatment.

The molecular mechanism of miR-133a-3p in HCC is of great importance to understand the carcinogenesis of HCC. Previous studies have reported that miR-133a-3p may target fascin actin-bundling protein 1 (FSCN1), insulin-like growth factor 1 receptor (IGF-1R), epidermal growth factor receptor, transgelin 2, matrix metallopeptidase-9 (MMP-9), ATPase copper transporting β, death associated protein kinase 2, SRY-box 4 and serum response factor, to exert its function in different kinds of tumors. Furthermore, FSCN1, IGF-1R and MMP-9 have been described as targets of miR-133a-3p and influenced cell proliferation, colony formation, migration and invasion in HCC cells. Thus, the authors deduce that miR-133a-3p may target multiple mRNAs in different signaling pathways of HCC. To get the whole picture of miR-133a-3p related potential target networks, they conducted targets prediction and functional pathway analyses. A total of 12 target prediction tools were used for collection of the potential targets genes. In order to minimize false positives, the potential target genes need to be predicted in at least five in silico tools out of 12. Subsequently, predicted targets were overlapped with DEGs from TCGA, which are representative of key genes in the tumorigenesis of HCC, to eventually obtain final target genes of miR-133a-3p in HCC. Then functional analysis was conducted to investigate potential molecular pathway of miR-133a-3p, which will contribute to the understanding of molecular mechanisms of the tumorigenesis and progression of HCC. The possible multiple pathways suggested that miR-133a-3p can potentially regulate many of the required steps in HCC development, from variations in cell morphology to channel activity, as well as various signaling pathways. The molecular functions of these pathways may initiate a new trial on HCC pathogenesis. These pathways could be a prospective source of novel treatment targets and markers. Five key genes NOS1, ADRA1A, ADRA1B, ADRA1D and TBX2AR were obtained from PPIs network of the calcium signaling pathway, which was the most significantly enriched pathway achieved from KEGG, are potentially targeted by miR-133a-3p in HCC. However, these five hub genes have not yet been researched in HCC; thus further experimental validation is needed.

Some limitations should be noted in the present study. Firstly, significant heterogeneity was observed when all data were pooled together in the meta-analysis. Subgroup analyses have not been designed due to the small number of included studies. Other confounding introduced by RNA extraction or individual background may also disturb the result of the meta-analysis. Secondly, the small sample size limited the robustness of the meta-analysis conclusion. Further studies entailing large sample size should be designed to confirm miR-133a-3p expression levels in HCC. Thirdly, the potential target genes and pathways were only predicted by bioinformatics without experimental verification. The authors' group plans to perform in vitro and in vivo experiments to validate these in silico findings in the future.

To better explore the possibility of the target candidates from simple prediction, the authors further inspected the gene expression data from TCGA dataset and confirmed the overexpression of 13 of 21 genes described in the calcium signaling pathway (KEGG ID: hsa04020), which was the most significant KEGG term. Especially, six genes were dramatically upregulated in liver tumor tissues, giving an FC of >1. These genes may have more possibility to be the real targets of miR-133a-3p in HCC due to their noteworthy overexpression in cancer tissues. Of course, the results need to be further confirmed with other experiments.

The present study mined the publicly available data, including GEO, TCGA, published study and the authors' own PCR data together to study the possible clinical role of miR-133a-3p. The results suggested that miR-133a-3p may play as a tumor suppressor in HCC. Furthermore, the prospective novel pathways of miR-133a-3p could offer potential biomarkers for HCC. To the best of the authors' knowledge, this is the first study to combine multiple resources to validate miR-133a-3p suppressive role in HCC and to explore its molecular mechanism by functional pathway analysis. However, the findings need further experimental confirmation.

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