Plasma exosomal miR-122 regulates the efficacy of metformin via AMPK in type 2 diabetes and hepatocellular carcinoma

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ABSTRACT

Metformin is a drug that has been applied in clinical use for many years for the treatment of type 2 diabetes mellitus (T2DM). It achieves its function through multiple targets and modulation of multiple signaling pathways. To date, the mechanism of the action of metformin is still not fully understood. Along with glycemic control, metformin has shown good inhibitory effects on the development of many tumors. Here, we elucidated that plasma exosomal microRNA-122-5p (miR-122) is closely related to the mechanism of metformin. MiR-122 regulates glycogen-glucose metabolism in hepatocytes or hepatocellular carcinoma cells (HCC) by inhibiting the phosphorylation of AMPK. Since miR-122 and metformin regulate glucose metabolism homeostasis through similar mechanisms, miR-122 can antagonize the effects of metformin. MiR-122 expression increases the sensitivity of hepatocytes or HCC to metformin. Conversely, decreased expression of miR-122 results in hepatocyte insensitivity to metformin. Therefore, significantly elevated levels of miR-122 in plasma exosomes of hepatocellular carcinoma patients could enhance their sensitivity to metformin. The results of the present study revealed a key regulatory role of plasma exosomal miR-122 on the molecular mechanism of metformin. The regulation of key molecules of related signaling pathways by miR-122 may lead to similar glycemic lowering and tumor suppression therapeutic effects as metformin. This provides new ideas for the development of new therapeutic strategies for hepatocellular carcinoma based on the mechanism of miR-122 and metformin.

1. Introduction

Metformin is a biguanide originally derived from the plant Galega officinalis and is widely used to treat hyperglycemia in type 2 diabetes mellitus (T2DM) [1, 2, 3, 4, 5]. It exerts its hypoglycemic effect mainly by reducing hepatic glucose production (HGP) [5, 6, 7]. Although metformin has been applied in clinical use for decades, its precise regulatory mechanism remains relatively unknown. Moreover, recent studies have found that metformin has significant therapeutic effects on a variety of other diseases, especially tumors [8, 9, 10, 11, 12, 13]. This has led to more interest in the mechanism of the action of metformin. Hepatocellular carcinoma (HCC) is a serious health-threatening malignancy and is the fourth most deadly disease in the world. There is no effective treatment available at present. Although T2DM has been documented as one of the risk factors for human HCC [14], the mechanisms associated with the development of T2DM and HCC are also not fully understood. Moreover, patients with HCC are difficult to treat with metformin due to the impairment of liver function [15]. Therefore, determining the molecular mechanisms underlying the effects of metformin on T2DM and HCC will help to develop appropriate treatment strategies.

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In clinical practice, metformin has a limited therapeutic effect in a subset of patients with T2DM. This resistance or insensitivity of metformin is tightly related to its regulatory mechanisms. Consequently, the identification of key molecules of metformin resistance are valuable to identify the precise underlying regulatory mechanisms of metformin. What is known is that metformin regains control of hepatic glucose output mainly by inhibiting gluconeogenesis [5, 6, 7]. At the cellular level, metformin is thought to counteract the insulin signaling pathway by reducing cyclic adenosine monophosphate (cAMP) production or activating AMP-activated protein kinase (AMPK), thereby disrupting the hepatic glucose metabolic process [7, 16, 17, 18, 19, 20]. Therefore, it can be expected that metformin resistance should be tightly related to the metabolic balance of hepatic glycogen and the corresponding signaling pathways [20, 21, 22].

MicroRNAs (miRNAs) are small non-coding RNA molecules containing about 22–25 nucleotides. They are widely present in plants, animals, and viruses and function through post-transcriptional regulation of RNA silencing and gene expression [23, 24, 25, 26, 27]. MiRNAs are functionally complex and their dysfunction is often closely associated with changes in cellular behavior, especially in diseases [23, 24, 25]. Changes in circulating miRNAs in plasma are potential biomarkers for a variety of diseases, such as tumors, and suggest potential therapeutic targets for diseases [23, 28, 29, 30, 31, 32]. In recent years, several studies showed significant changes in some circulating miRNAs in diabetic patients. For example, one of the first studies found changes in plasma miR-126-5p levels in both type 2 diabetic patients and mouse models [33, 34]. Other studies also revealed that plasma miR-30d-5p, miR-122-5p, miR-146a-5p, miR-192-5p and miR-375-5p are associated with diabetes [31, 32, 33, 34, 35, 36, 37, 38].

Circulating miRNAs in plasma are usually transported via extracellular vesicles of nanometers to micrometers in diameter, which are key carriers for intercellular communication [39, 40, 41, 42]. A large number of studies in recent years have shown that miRNAs on extracellular vesicles are mediators of intercellular signaling and important biomarkers for many diseases, drug resistance, etc [41, 42, 43, 44]. In this study, we first compared and analyzed miRNA expression profiles in plasma exosomes of type 2 diabetic patients and hepatocellular carcinoma patients by deep sequencing. MiR-122 was found to be closely associated with sensitivity to metformin treatment in type 2 diabetic patients by these analyses and subsequent testing of clinical samples. Subsequent mechanistic studies confirmed that miR-122 regulates glyco-regulation metabolism in hepatocytes by inhibiting AMPK phosphorylation in primary hepatocytes, hepatocyte lines 68 or multiple hepatoma cell lines. Since miR-122 and metformin regulate glucose metabolism homeostasis through similar mechanisms, miR-122 can antagonize the effects of metformin. MiR-122 expression increases the sensitivity of hepatocytes or HCC to metformin. Knockdown of miR-122 results in hepatocyte insensitivity to metformin. Therefore, significantly elevated levels of miR-122 in plasma exosomes of hepatocellular carcinoma patients could enhance their sensitivity to metformin. Recent studies have demonstrated that metformin shows good therapeutic effects in various malignancies [5, 9, 10, 11, 12, 13]. However, in the treatment of hepatocellular carcinoma, direct application of metformin in patients with hepatocellular carcinoma is not practical due to the impairment of liver function. The results of this study reveal a key regulatory role of plasma exosome miR-122 on the molecular mechanism of metformin and provides a new idea to develop new treatment strategies for hepatocellular carcinoma based on the mechanism of action of metformin.

2. Results

2.1. The demographics and clinical characteristics of patients enrolled in the study

A total of 102 individuals (54–68 years old) diagnosed without (n = 30, NOR, control group), or with T2DM (n = 40, T2DM group), or with HCC (n = 32, HCC group) were recruited from Wuhan Third Hospital (Wuhan, China) from 2018 to 2019. All T2DM patients were treated with glucose-lowering drugs such as metformin, insulin agonists, glycosidase inhibitors, Dipeptidyl peptidase IV inhibitors (DPP4-i), thiazolidinedione (TZD), and sodium-glucose cotransporter-2 inhibitors (SGLT2-i) according to the 2020 Chinese CDS guidelines for T2DM prevention and treatment. All these groups were comparable in terms of mean age, gender distribution, BMI, post-control glucose, blood pressure, etc. Individual clinical characteristics such as fasting blood glucose, glycosylated hemoglobin, total cholesterol, triglycerides, low-density lipoprotein cholesterol, alanine aminotransferase, aspartate aminotransferase, and total bilirubin are shown in Table 1 (Figure 1).

2.2. Isolate and characterize human plasma exosomes from T2DM patients

Plasma samples were collected from these individuals. Then, plasma exosomes were isolated and characterized by the revised procedure following extracellular vesicles study guideline (MISEV2018) [40, 45, 46]. Exosomes are membrane enclosed nano-sized (30–150 nm in diameter) vesicles with the characteristic cup-shaped morphology that are released from essentially all cell types [23, 24, 25, 26, 27]. Isolated plasma exosomes were visualized by transmission electron microscopy (TEM) and found exosomes of the typical size and morphology (Figure 2A). The size distribution of plasma exosomes was analyzed by dynamic light scattering (DLS) which confirmed that they were 60–200 nm with the peak at 100 nm in diameter (Figure 2B). Isolated plasma exosomes were further verified by exosome-specific biomarker CD63 and Alix (Figure 2D) and quantified by nanoparticle Tracking Analyzer (NTA) (Figure 2C). As shown in Figure 2C, the concentration of plasma exosomes are around 1.1 × 10^8 exosomes per mL. These results indicate our capability of isolating and characterizing human plasma exosomes.

2.3. MiR-122 is essential for metformin resistance in T2DM patients

MicroRNA and non-coding RNA are main component in plasma exosomes. We exploited the miRNA expression profiles of isolated plasma exosomes to determine their potential involvement in T2DM and HCC. Deep sequencing identified 768 miRNAs, including 262 novel ones (Figure 3A and B), in the HCC group. Among them, 79 annotated miRNAs were altered with log2-fold change (FC) < 1 or > 1 and P < 0.05. This left 15 up-regulated miRNAs and 5 down-regulated miRNAs, including miR-

| Table 1. The demographics and clinical characteristics of patients. |
|-------------|----------------|----------------|
| NOR (n = 30) | T2DM (n = 40) | HCC (n = 32) |
| Female/Male  | 16/14          | 22/18         |
| Mean age     | 64.2 ± 4.1     | 64.8 ± 3.6    |
| BMI          | 22.7 ± 1.8     | 23.2 ± 2.1    |
| DBP/SBP, mmHg| 72.2 ± 6.5/129.3| 76.4 ± 7.2/133.3|
| clinical characteristics | mean ± SD | 7.4 ± 6.8 | 75.3 ± 7.4/131.3 ± 6.2 |
| FBG, mmol/L  | 4.89 ± 0.34    | 8.57 ± 0.73   |
| HbA1c, %     | 5.21 ± 0.30    | 8.16 ± 0.69   |
| TC, mmol/L   | 4.22 ± 0.28    | 5.96 ± 0.41   |
| TG, mmol/L   | 1.42 ± 0.10    | 4.15 ± 0.28   |
| LDL-c, mmol/L| 2.67 ± 0.19    | 3.39 ± 0.21   |
| ALT, U/L     | 35.8 ± 2.1     | 32.6 ± 2.3    |
| AST, U/L     | 28.6 ± 1.5     | 26.8 ± 1.7    |
| ALB, g/L     | 35.8 ± 2.1     | 32.6 ± 2.3    |
| TTR, g/L     | 62.1 ± 0.83    | 10.2 ± 0.68   |

NOR, Normal group; T2DM, Type 2 diabetes mellitus; HCC, Hepatocellular carcinoma; BMI, Body Mass Index; DBP/SBP, Diastolic blood pressure/Systolic blood pressure; FBG, Fasting blood glucose; HbA1c, Hemoglobin A1c, glycosylated hemoglobin; TC, Total cholesterol; TG, Triglycerides; LDL-c, Low-Density Lipoprotein Cholesterol; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; TTR, Total bilirubin; SD, standard deviation.
122-5p, miR-451a, miR-126-3p, and miR-486-5p etc (Figure 3C).

Because of the large variation between individual samples within the T2DM group, deep sequencing of this group did not give reliable dataset with significantly altered miRNAs. Therefore, we chose to use RT-PCR to examine the expression level of these altered miRNAs identified from HCC group. As shown in Figure 3D, 20 plasma exosome samples from T2DM patients were examined and, indeed, average expression levels of these miRNAs are close to control (NOR) group. However, further analysis of the data suggested that certain annotated miRNAs, such as miR-122, were significantly under-expressed in plasma exosome samples from a subgroup of T2DM patients (n = 8) who were not sensitive to metformin (Figure 3E, column set 1). This finding was of great interest to us because miR-122 plays a key role in the development of metabolic diseases of the liver, especially tumors [31, 32, 33, 34, 35, 36, 37, 38].

2.4. MiR-122 level correlates with metformin sensitivity in patients with T2DM or HCC

To confirm the correlation between miR-122 expression and metformin insensitivity in T2DM patients, a total of 80 individuals (aged 46–68 years) were recruited from Wuhan Third Hospital (Wuhan, China) between 2018 and 2019. Among them, 30 were diagnosed with HCC (n = 18, HCC group) or no HCC (n = 12, NOR, control group). 50 T2DM patients were diagnosed as metformin-sensitive (n = 18, Met-S T2DM group), metformin-insensitive (n = 20, Met-IS T2DM group), or HCC (n = 12, HCC-T2DM group). The clinical characteristics of the patients are listed in Table 2 (Figure 4A). As shown in Figure 4B, miR-122 levels were indeed down-regulated in the plasma exosomes of these metformin-insensitive patients. MiR-122 levels were more than 5-fold higher in the T2DM group than in the Met-ins T2DM group (Figure 4B, column 2 vs 3). We also noted that miR-122 levels were approximately 50% higher in the HCC group than in the HCC-T2DM group, although their metformin sensitivity was not known (Figure 4B, column 6 vs 7). For both groups, HCC tissue and pathologically confirmed adjacent healthy liver tissue (distance from the tumor margin, ≤3 cm) were collected by performing tumor resection. As shown in Figures 4C and 4D, the expression of miR-122 was significantly lower in liver tissue from HCC-T2DM patients than in samples from HCC patients, both in hepatocellular carcinoma tissue and in paracancerous tissue (Figures 4C and 4D, column 2 vs 3).

2.5. MiR-122 affects glucose metabolism in hepatocyte and HCC

To further explore the correlation between miR-122 levels and metformin treatment sensitivity, we first examined the expression levels of miR-122 in hepatocytes and hepatocellular carcinoma cell lines. The effect of different concentrations of metformin treatment on the glucose metabolism of these cells was also examined. As shown in Figure 5A, miR-122 in the exosomes and cytoplasm of primary hepatocytes, hepatocytes WRL-68 and hepatic stellate cells LX-2 were roughly comparable. In contrast, the miR-122 levels in different hepatocellular carcinoma cell lines were very different. The hepatoma cell lines Hep3B and MHCC97H had lower exosomal miR-122 than primary hepatocytes, WRL-68, and LX-2, whereas the levels of miR-122 in exosomes and cytoplasm of PLC/
PRF/5, Huh7, and HepG2 cells had much higher miR-122 levels in exosomes and cytoplasm than these hepatocytes (Figure 5A). Glucose metabolism was significantly higher in these hepatocellular carcinoma cells than in these hepatocytes (Figure 5B). When glycogen was used to activate glucose metabolism in hepatocytes in vitro in cell culture, 100 μM metformin treatment significantly inhibited gluconeogenesis in hepatocytes within 1 h (Figure 5C). Meanwhile, glucagon (100 nM) did stimulate glucose synthesis in primary hepatocytes, WRL-68, LX-2, and hepatocellular carcinoma cells (Figure 5D). Similarly, as shown in Figure 5D, the same concentration of metformin treatment had less effect on glucose metabolism in Hep3B and MHCC97H cells (column set 4 and 5 from left, Figure 5D) than in primary hepatocytes, WRL-68, LX-2, Huh7, and HepG2 cells (column 1–3, 6, and 7 from left, Figure 5D). That is, Hep3B and MHCC97H cells were insensitive to metformin, which was negatively correlated with their miR-122 levels in exosomes or cytoplasm.

Metformin is known to inhibit the conversion of glycogen to glucose by altering mitochondrial function and activating phosphorylation of AMPK. We first measured the phosphorylation levels of AMPK in these cell lines in the absence of metformin treatment. The phosphorylation level of their AMPK was found to be roughly negatively correlated with the expression of miR-122 in these cell lines (Figure 5E). 100 μM metformin treatment significantly increased AMPK phosphorylation in primary hepatocytes, hepatic stellate cells LX-2, and hepatoma cells Huh7.
Figure 3. Plasma exosomal miR-122 is critical for metformin sensitivity in T2DM patients. (A–C) Differential expression of miRNAs in circulating plasma exosomes of HCC patients. (A) Scatter plot of expression levels of differentially expressed miRNAs in plasma exosomes of HCC patients before and after tumor resection surgery. (B) Heat map of differentially expressed miRNAs in plasma exosomes of HCC patients before and after tumor resection surgery. (C) List of miRNAs with increased and decreased expression in plasma exosomes of HCC patients before and after tumor resection surgery, arranged in descending order of significant changes. (D and E) Expression levels of the significantly differentially expressed miRNAs screened in (A–C) in plasma exosomes of T2DM patients and control group (NOR group) were detected by qRT-PCR. Data were calculated as $2^{-\Delta\Delta C(t)}$ and expressed as the means ± SD, ***$p$ < 0.001. (E) miR-122-5p were significantly under-expressed in plasma exosome samples from a subgroup of T2DM patients who were not sensitive to metformin (column set 1). Data represent mean ± SEM of 8 experiments.
Figure 4. miR-122 level correlates with metformin sensitivity in patients with T2DM or HCC. (A) The demographics and clinical characteristics of individuals diagnosed with HCC (HCC group), no HCC (NOR, control group), T2DM with metformin-sensitive (Met-S group), T2DM with metformin-insensitive (Met-IS group), or T2DM and HCC (HCC-T2DM group). All these groups were comparable in terms of mean age, gender distribution, BMI, post-control glucose. Individual clinical characteristics such as fasting glucose, glycosylated hemoglobin, total cholesterol, triglycerides, and LDL cholesterol are shown in Table 2. (B) Relative miR-122 level in plasma exosomes from control group (NOR group), T2DM with metformin-insensitive (Met-IS group), T2DM with metformin-sensitive (Met-S group), T2DM group, HCC group, or T2DM and HCC (HCC-T2DM group) were determined by RT-PCR respectively. MiR-122 level in plasma exosomes from Met-IS group is lower than in Met-S group (*: P < 0.05). The experiments were repeated 6 times and data represent mean ± SEM. (C) Relative miR-122 level in HCC tissue from control group (NOR group), HCC group, or T2DM and HCC (HCC-T2DM group) were determined by RT-PCR respectively. MiR-122 level in HCC-T2DM group is lower than in HCC group (*: P < 0.05). Data represent mean ± SEM of 6 experiments. D. Relative miR-122 level in paracancerous tissue from control group (NOR group), HCC group, or T2DM-HCC group were determined by RT-PCR. MiR-122 level in HCC-T2DM group is lower than in HCC group (*: P < 0.05). Data represent mean ± SEM of 6 experiments.
Figure 5. MiR-122 affects glucose metabolism in hepatocyte and HCC. (A) Relative miR-122 level in exosomes and cytoplasm of primary hepatocytes, hepatocytes WRL-68, hepatic stellate cell LX-2, hepatocellular carcinoma cell Hep3B, MHCC97H, PLC/PRF/5, Huh-7, and HepG2 were determined by RT-PCR. MiR-122 level in these cells were presented as relative ‘fold’ compared to miR-122 level in primary hepatocytes. Data represent mean ± SEM of 6 experiments. (B) Relative glucose production of various hepatocytes or hepatocellular carcinoma cells in Figure 5A were determined by hepatic glucose production Assay. Glucose production of these cells were presented as relative ‘fold’ compared to glucose production of primary hepatocytes. Data represent mean ± SEM of 4 experiments. (C) Glucose production of primary hepatocytes pre-treated with or without metformin (100 μM) for different time periods were determined by hepatic glucose production assay (✽: P < 0.05). Data represent mean ± SEM of 4 experiments. (D) Glucose production of various hepatocytes or hepatocellular carcinoma cells in Figure 5A pre-treated with or without metformin for 1 h were determined by hepatic glucose production assay (△: P > 0.05; ✽: P < 0.05). Data represent mean ± SEM of 4 experiments. (E) The whole cell lysates of primary hepatocytes, WRL-68, Huh-7, HepG2, Hep3B, and MHCC97H cells (10 μg/samples) were immunoblotted with antibodies against phosphorated AMPK (p-AMPK), AMPK, and GAPDH. Relative protein level of p-AMPK/AMPK were quantified from Western blot images (bottom graph). (F) Primary hepatocytes, WRL-68, Huh7, and MHCC97H cells were treated without or with metformin (100 μM) for 1 h. The whole cell lysates of these cells (10 μg/samples) were immunoblotted with antibodies against phosphorated AMPK (p-AMPK), AMPK, and GAPDH. Relative protein level of p-AMPK/AMPK were quantified from Western blot images (bottom graph).
(lane 1 vs lane 2, lane 3 vs lane 4, lane 5 vs lane 6, Figure 5F). In addition, hepatoma cells with lower miR-122 levels, such as MHCC97H cells, showed limited elevation of AMPK phosphorylation by metformin treatment because their AMPK phosphorylation levels were already high (lane 7 vs lane 8, Figure 5F). This is consistent with the effect of metformin treatment on glucose metabolism in these cell lines (Figure 5D).

2.6. MiR-122 affects metformin sensitivity through AMPK

To verify the role of miR-122 in the activation of AMPK phosphorylation signaling pathway by metformin, we constructed plasmids over-expressing miR-122 or knocking down miR-122 (miR-122 KD), respectively. We found that overexpression of miR-122 slightly reduced

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**Figure 6.** MiR-122 affects metformin sensitivity through AMPK. (A) Primary hepatocytes were transfected with empty vector (control), plasmid expressing miR-122 (miR-122), or plasmid expressing siRNA targeting miR-122 (miR-122 KD). The relative level of miR-122 in these cells were determined by RT-PCR (Left two graphs). These cells were pre-treated without or with metformin (100 μM) for 1 h. The whole cell lysates of these cells (10 μg/samples) were immunoblotted with antibodies against phosphorylated AMPK (p-AMPK), AMPK, and GAPDH (Middle panels). Relative protein level of p-AMPK/AMPK were quantified from Western blot images (Right graph). (B) Primary hepatocytes were transfected with empty vector (control), plasmid expressing miR-122 (miR-122), or plasmid expressing siRNA targeting miR-122 (miR-122 KD). These cells were treated without or with metformin (100 μM) for 1 h. Glucose production of these cells were measured by hepatic glucose production assay (△: p > 0.05; *: p < 0.05). Data represent mean ± SEM of 4 independent experiments. (C) MHCC97H cells were transfected with empty vector (control) or plasmid expressing miR-122 (miR-122). The relative level of miR-122 in these cells were determined by RT-PCR (Left graph). These cells were pre-treated without or with metformin (100 μM) for 1 h. The whole cell lysates of these cells (10 μg/samples) were immunoblotted with antibodies against phosphorylated AMPK (p-AMPK), AMPK, and GAPDH (Middle panels). Relative protein level of p-AMPK/AMPK were quantified from Western blot images (Right graph). (D) Glucose production of those cells in Figure 6C were measured by hepatic glucose production assay (△: p > 0.05; *: p < 0.05). (E) Huh-7 cells were transfected with empty vector (control) or plasmid expressing siRNA targeting miR-122 (miR-122 KD). The relative level of miR-122 in these cells were determined by RT-PCR (Left graph). These cells were pre-treated without or with metformin (100 μM) for 1 h. The whole cell lysates of these cells (10 μg/samples) were immunoblotted with antibodies against phosphorylated AMPK (p-AMPK), AMPK, and GAPDH (Middle panels). Relative protein level of p-AMPK/AMPK were quantified from Western blot images (Right graph). (F) Glucose production of these cells in Figure 6E were measured by hepatic glucose production assay (△: p > 0.05; *: p < 0.05). Data represent mean ± SEM of 4 independent experiments.
AMPK phosphorylation in primary hepatocytes (lane 1 vs 2, Figure 6A), and significantly reduced AMPK phosphorylation in MHCC97H cells (lane 1 vs 2, Figure 6C). Consistent with this, overexpression of miR-122 showed an increased sensitivity of glucose production to metformin in primary hepatocytes (column 1 vs 2, Figure 6B). Similarly, miR-122 overexpression increased the sensitivity of glucose production to metformin from insensitive to sensitive in MHCC97H cells (column 1 vs 2, Figure 6D). Corroborating with this, metformin treatment significantly elevated AMPK phosphorylation (lane 2 vs 3, Figure 6C). This should be due to the relatively low level of endogenous miR-122 in MHCC97H cells with significant AMPK phosphorylation (lane 6, Figure 5E), while overexpression of miR-122 significantly inhibited AMPK phosphorylation. When miR-122 was knocked down in primary hepatocytes or Huh-7 cells, AMPK phosphorylation was significantly increased in primary hepatocytes or Huh-7 cells (lane 1 vs 3, Figure 6A; lane 1 vs 2, Figure 6E). Since AMPK was already significantly phosphorylated, metformin treatment was unable to further elevate AMPK phosphorylation in primary hepatocytes or Huh-7 cells (lane 3 vs 4, Figure 6A; lane 2 vs 3, Figure 5E). Consistent with this, knockdown of miR-122 caused gluconeogenesis in primary hepatocytes and Huh-7 cells to become insensitive from metformin-sensitive (column 3 vs 4, Figure 6B; column 1 vs 2, Figure 6F). The above results suggest that miR-122 regulates metformin sensitivity through the inhibition of AMPK phosphorylation.

2.7. MiR-122 affects metformin resistance by regulating glucose synthesis via AMPK

As previously mentioned, a retrospective clinical study showed that levels of plasma exosomal miR-122 were significantly lower in T2DM patients who were not sensitive to metformin. We therefore subsequently investigated whether exosomal miR-122 modulates glucose synthesis in cells receiving exosomes via AMPK, thereby altering the sensitivity of these cells to metformin. Because of the high miR-122 content in exosomes released from Huh-7 cells, we selected it as the donor cells for exosomal miR-122 (column 2, Figure 7A). The siRNA against miR-122 (miR-122 KD) was also used to knock down miR-122 in Huh-7 cells, resulting in miR-122 knockdowned exosomes (miR-122 KD Eso) (column 3, Figure 7A). Exosomes released from Huh-7 cells were isolated, purified, and quantified. Subsequently, 10 μg per ml of exosomes were incubated with primary hepatocytes or MHCC97H cells. Exosomes derived from Huh-7 cells significantly elevated the miR-122 levels within both primary hepatocyte and MHCC97H cells. In contrast, the miR-122 knockdowned exosomes, as a control, had no such effect (column 4 and 5, Figure 7A). This result indicates that the elevated intracellular miR-122 levels do originate from the delivery of exosomes. We then examined the effect of treating primary hepatocytes or MHCC97H cells with these exosomes from Huh-7 cells on AMPK phosphorylation. As shown in Figure 7B, exosomal miR-122 significantly inhibited AMPK phosphorylation in primary hepatocytes or MHCC97H cells (lane 1 and 2 vs 3, lane 4 and 5 vs 6, Figure 7B). Consistent with above results, exosomal miR-122 did enhance the sensitivity of glucose production to metformin in primary hepatocytes or MHCC97H cells. These results suggest that small extracellular vesicles (exosomes) can regulate glucose homeostasis in cells receiving exosomes, altering the sensitivity of these cells to metformin and thus possibly affecting the therapeutic effect of metformin (Figures 7C and D). These effects are accomplished by exosomal miR-122 regulating the phosphorylation status of AMPK in cells receiving exosomes. Since exosomal miR-122 is significantly elevated in the plasma of patients with liver disease, especially hepatocellular carcinoma. Therefore, the results of this study suggest that exosomal miR-122 can regulate glucose homeostasis in normal or hepatocellular carcinoma cells in the tumor microenvironment of hepatocellular carcinoma and improve the sensitivity to metformin treatment. Figure 7E depicts the effect of exosomal miR-122 on the efficacy of metformin through inhibition of AMPK phosphorylation. MiR-122 expression is elevated in HCC tumor cells, which results in significantly higher levels of miR-122 in plasma exosome from HCC patients. Exosomal miR-122 is transported into hepatocytes and lead to altered glycogen-glucose metabolic homeostasis in hepatocytes or hepatocellular carcinoma cells (HCC) by inhibiting AMPK phosphorylation. This effect is antagonistic to the activation of AMPK phosphorylation by metformin. This antagonistic effect is manifested by plasma exosomes leading to a significant increase in miR-122 levels in hepatocytes, which eventually enhances hepatocyte sensitivity to metformin. Whether this mechanism contributes to the therapeutic effect of metformin on tumors, in addition to lowering blood glucose, needs to be further investigated.

3. Discussion

It is well known that T2DM and HCC are metabolic diseases that seriously threaten health and affect quality of life. The determinants of T2DM consist of a matrix of genetic, epigenetic and lifestyle factors which interact with one another [1]. HCC is mainly caused by a variety of incentives, such as viruses, alcohol, drugs, and genes and epigenetics [47]. The liver maintains a relatively stable blood glucose concentration mainly through the synthesis and decomposition of hepatic glycogen and gluconeogenesis in glucose metabolism. Therefore, the relationship between T2DM and HCC is extremely close and is mediated by a variety of factors. In general, the association between type 2 diabetes and HCC is mediated primarily by chronic inflammatory states. HCV clearance by DAA treatment reduces T2DM incidence more likely by restoring the HCV-activated alteration of glucose homeostasis mechanisms [48, 49, 50]. We acknowledge that chronic inflammation plays an important role in the relationship, but it wasn’t addressed in this study. Instead, we focused on the association between the therapeutic mechanism of metformin, which is commonly used clinically to treat diabetes, and the delivery of miR-122 on extracellular vesicles. A retrospective analysis of clinical data found that about 18% of T2DM patients showed to be nonsensitive to metformin, while this proportion should be significantly lower in HCC patients. Since metformin is not used in cancer patients with poor liver function in clinical treatment, we cannot clearly count and explain the proportion of HCC patients who are not sensitive to metformin. However, in our study, it has been verified that the sensitivity to metformin is significantly increased in liver cancer cell lines from the perspective of cell biology.

As we know, metformin is a drug that has been in clinical use for many years to treat type 2 diabetes. It is a natural compound originally derived from a plant rather than a targeted drug with a single target of action. It achieves its function through multiple targets and modulation of multiple signaling pathways. The corresponding molecular mechanisms are also complicated. To date, the mechanism of action of metformin is still not fully understood. In recent years, researchers have found that metformin not only controls blood glucose, but also has a good inhibitory effect on the progression of many tumors [51, 52]. Therefore, the molecular mechanism of its action has attracted great interest. Since metformin cannot be applied to patients with hepatic impairment. It cannot be directly used in attempts to treat patients with hepatocellular carcinoma. In the present study, we further revealed a key regulatory role of plasma exosomal miR-122 on the molecular mechanism of metformin. This work not only helps us to gain insight into the relationship between type 2 diabetes and hepatocellular carcinoma progression, but also provides new ideas and treatment strategies for the treatment of diabetes as well as hepatocellular carcinoma.

MicroRNAs (miRNAs) are small non-coding RNA molecules containing approximately 22–25 nucleotides. Through post-transcriptional regulation of RNA silencing and gene expression, they play key regulatory roles in various cellular behaviors, functions, and diseases, including diabetes [23, 24, 25, 26, 27]. In this study, we first analyzed miRNA expression profiles on plasma exosomes from patients with type 2 diabetes or hepatocellular carcinoma by RNA sequencing and identified miRNAs with significantly altered expression on plasma exosomes from patients with hepatocellular carcinoma. While difficulties arose in analyzing samples from patients
Figure 7. MiR-122 affects metformin resistance by regulating glucose synthesis via AMPK. (A) Huh-7 cells were transfected with empty vector (control) or plasmid expressing siRNA targeting miR-122 (miR-122 KD). Exosomes with high level of miR-122 (Exosome) or exosomes with miR-122 knock-downed (miR-122 KD Exo) were isolated from these cells and quantified. The relative level of miR-122 in these exosomes were determined by RT-PCR (Left graphs). Primary hepatocytes or MHCC97H cells were incubated with these exosomes (10 μg exosome protein/ml) for 1 h. The relative level of miR-122 in receipt cells were determined by RT-PCR (Right graphs). Data represent mean ± SEM of 6 experiments. (B) The whole cell lysates of primary hepatocytes (Left panels) or MHCC97H cells (Right panels) incubated with Exosome or miR-122 KD Exo immunoblotted with antibodies against phosphorated AMPK (p-AMPK), AMPK, and GAPDH. Relative protein level of p-AMPK/AMPK were quantified from Western blot images (Bottom graph). (C) Primary hepatocytes incubated with Exosome or miR-122 KD Exo were pre-treated without or with metformin (100 μM) for 1 h. Glucose production of these cells were measured by hepatic glucose production assay (△: p > 0.05; ✤: p < 0.05). Data represent mean ± SEM of 4 experiments. (D) MHCC97H cells incubated with Exosome or miR-122 KD Exo were pre-treated without or with metformin (100 μM) for 1 h. Glucose production of these cells were measured by hepatic glucose production assay (△: p > 0.05; ✤: p < 0.05). Data represent mean ± SEM of 4 experiments. (E) Diagram depicts that exosomal miR-122 affects the efficacy of metformin via AMPK. MiR-122 expression is elevated in HCC tumor cells, which results in significantly higher levels of miR-122 in plasma exosomes (a). Exosomal miR-122 is transported to hepatocytes and inhibits the phosphorylation of AMPK (b). Exosomal miR-122 eventually led to altered glycogen-glucose metabolic homeostasis in hepatocytes (c), which in turn enhances hepatocyte sensitivity to metformin.
with type 2 diabetes, mainly because the differences between individual patients were too large to obtain statistically significant differences results. Therefore, we changed our approach and examined the differential miRNAs screened in samples from patients with hepatocellular carcinoma directly by RT-PCR. We found that miRNA-122, which is transported on plasma exosomes, is very lowly expressed on plasma exosomes of metformin-insensitive type 2 diabetic patients, suggesting that miRNA-122 can serve as a biomarker of metformin insensitivity and is closely related to the mechanism of the action of metformin. Since the activation of AMPK by metformin is its main molecular mechanism for lowering blood glucose, we investigated the regulation of AMPK by miRNA-122 and found that miRNA-122 could indeed negatively regulate AMPK and in this way antagonize the effect of metformin on glucose synthesis in hepatocytes. When miRNA-122 is lowly expressed, AMPK is activated and the presence of metformin cannot further activate the phosphorylation of AMPK. Therefore, the inhibitory effect of metformin on the conversion of glycogen to glucose in hepatocytes is blocked by miRNA-122, which should account for the insensitivity of these diabetic patients to metformin. However, it is still unclear whether miRNA-122 acts directly on AMPK. By analyzing the target molecules of miRNA-122, we tend to think that miRNA-122 is indirectly inhibiting AMPK. It is also important to note that in HCC patients, activation of AMPK is no longer sufficient to control blood glucose within reasonable limits by inhibiting the conversion of glycogen to glucose. For this group of patients, their blood glucose was not completely out of control, so how did they achieve control of their blood glucose? It is possible that this is more dependent on AMPK-independent signaling pathways.

In a retrospective study of nearly 1,000 patients with diabetes, we found that approximately 18% of patients were insensitive to metformin. Since type 2 diabetes is a chronic disease, when patients are found to be insensitive to metformin, better outcomes can be achieved by changing medications or adding other medications. Therefore, early determination of sensitivity to metformin is an urgent need. Nevertheless, the results of this study suggest that miRNA-122 is intimately involved in glucose regulation in hepatocytes. MiRNA-122 itself is one of the more intensively studied miRNAs, and recent studies have found that it is expressed at high level in the liver [53]. In addition, it has a very significant elevated expression in the blood and liver tissues of patients with hepatocellular carcinoma. It is an important regulatory molecule in the hepatocellular carcinoma process, affecting many aspects of hepatocellular carcinoma, such as proliferation metastasis apoptosis. Thus, our findings link the balance of glucose metabolism in hepatocytes, especially the conversion of glycogen to glucose, to the progression of hepatocellular carcinoma. Key molecules in the mechanism of action of miRNA-122 are also potential drug targets in the treatment of hepatocellular carcinoma. Of course, further studies are needed to elucidate the detailed mechanisms and upstream and downstream relationships between them.

Since miRNA-122 is highly expressed in hepatocellular carcinoma, we can expect AMPK phosphorylation to be inhibited in hepatocellular carcinoma, and by testing different hepatocellular carcinoma cell lines, we found that this is indeed the case in a certain percentage of hepatocellular carcinomas. Thus, would such hepatocellular carcinoma cells be more sensitive to metformin? Is metformin the preferred hypoglycemic agent for patients with hepatocellular carcinoma with abnormal blood glucose? In experiments using cell lines, we have found that this does seem to be the case. Certainly, in clinical applications, metformin would not be used in cancer patients with poor liver function. Then, whether miRNA-122 or the corresponding regulatory pathway can be targeted for inhibition, or whether a cancer treatment regimen is undertaken, should also take into account the concomitant effects on patient glycemins. Therefore, the results of this study are of value for all these clinical applications.

4. Materials and methods

4.1. Patient recruitment and clinical investigation

Clinical observations were initiated from April 2015 to November 2019 in the Department of Endocrinology, Wuhan Third Hospital, Wuhan, China. All subjects submitted their informed consent for participation before the study initiated. The present study The clinical characteristics of the patients are presented in Table 1 and Table 2. Most of the patients were newly diagnosed with T2DM or HCC. Patients who suffered from other complications, such as diarrhea, were excluded. The blood samples of patients were collected and blood profiles were determined, including Hba1c, blood glucose, insulin, glucagon, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride, and free fatty acid. HCC tissues and adjacent healthy hepatic tissues (distance from tumor margin, <3 cm) confirmed by pathology were collected by performing a tumorectomy. Once the tissues were collected, the patients received first-line chemotherapy. All protocols of clinical study were approved by the Ethics Committee of Wuhan Third Hospital, Wuhan, China (approval no. QX-2019-020).

4.2. Reagents

All plasmids were provided by HanBio Bio-technology Co., Ltd (Shanghai, China). Phospho-AMPK (p-AMPK) (50081s) and AMPK (5831s) antibodies were obtained from Cell Signaling (Danvers, MA). The Annexin (PA5-27315), Alix (10628D), and CD-63 (MA1-83977) antibodies were from Invitrogen (Waltham, MA). The GADPH (60004-1) and β-actin (66009-1) antibodies were from Proteintech Group (Rosemont, IL, USA). The Sterilflip PVDF filters (0.1 or 0.22 μm pore size) and centrifugal filters, were from Millipore. Various primers for RT-PCR were purchased from RiboBio (Guangzhou, China). MiR-122 mimics and miR-

| Table 2. The demographics and clinical characteristics of patients. |
|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
|                        | NOR (n = 12)            | HCC (n = 18)            | T2DM Met-S (n = 18)      | T2DM Met-IS (n = 20)    | T2DM + HCC (n = 12)     |
| Female/Male            | 6/6                     | 9/9                     | 10/8                    | 11/9                    | 6/6                     |
| Mean age               | 64.0 ± 3.8              | 63.0 ± 4.0              | 64.6 ± 3.6              | 65.2 ± 3.8              | 63.3 ± 4.0              |
| BMI (kg/m²)            | 22.5 ± 2.3              | 22.1 ± 2.1              | 23.2 ± 2.2              | 23.1 ± 2.0              | 22.9 ± 2.4              |
| clinical characteristics mean ± SD |
| FBG, mmol/L            | 4.76 ± 0.42             | 5.76 ± 0.42             | 8.76 ± 0.73             | 8.58 ± 0.64             | 8.45 ± 0.74             |
| Hba1c, %               | 5.24 ± 0.40             | 5.46 ± 0.41             | 8.22 ± 0.58             | 8.17 ± 0.64             | 8.20 ± 0.59             |
| TC, mmol/L             | 4.28 ± 0.33             | 6.65 ± 0.47             | 5.92 ± 0.38             | 5.96 ± 0.40             | 5.94 ± 0.41             |
| TG, mmol/L             | 1.46 ± 0.13             | 2.08 ± 0.22             | 4.12 ± 0.32             | 4.18 ± 0.30             | 4.10 ± 0.30             |
| LDL-c, mmol/L          | 2.75 ± 0.23             | 3.01 ± 0.26             | 3.34 ± 0.29             | 3.37 ± 0.27             | 3.40 ± 0.27             |

NOR, Normal group; T2DM, Type 2 diabetes mellitus; HCC, Hepatocellular carcinoma; T2DM Met-S, T2DM with metformin sensitive; T2DM Met-IS, T2DM with metformin insensitive; BMI, Body Mass Index; FBG, Fasting blood glucose; Hba1c, Hemoglobin A1c, glycosylated hemoglobin; TC, Total cholesterol; TG, Triglycerides; LDL-c, Low-Density Lipoprotein Cholesterol; SD, standard deviation.
122 RNAi constructs (plasmid serial number for target vector: pc034-pCDNA3.1-CMV-MCS-EF1-ZsGreen-T2A-puro) were ordered from Hanbio (Shanghai, China).

4.3. Cell culture and transfections

The primary hepatocytes, hepatocytes WRL-68 and hepatic stellate cells LX-2 were free from mycoplasma contamination, and the cells were grown in DMEM medium (C11965118BT, Gibco, USA) containing 10% FBS (Gibco, Australia), in an incubator of 5% CO₂ at 37 °C. The medium was replaced every 3 days. The cells were passaged by trypsin treatment when they reached ~80% confluency. Cells were seeded in individual plates. According to the manufacturer’s instructions, expression constructs were transfected into cells with Lipofectamine 2000 (Invitrogen). 1× PBS wash cells, replace the medium with complete medium 4 h after transfection.

4.4. Isolation of exosomes

Plasma samples were collected and partially clarified by 3 consecutive centrifugations at 3000×g for 15 min at 4 °C. The supernatant was immediately diluted with PBS at a ratio of 1:20 and then the isolated exosomes were lysed or resuspended in PBS.

4.4.1. Light scattering

The size and purity of exosomes isolated from plasma were measured using the Zetapizer µV (Malvern) at the Core Facility Center for life Sciences, University of Science and Technology of China.

4.5. Transmission electron microscope (TEM)

To observe the morphology of exosomes, 20- to 40-µl suspension of exosomes was placed onto carbon-coated formvar grids for 10 min and stained with phosphotungstic acid (pH 6.8) for 5 min. The sample was then observed and photographed under transmission electron microscope (TEM, FEI, Tecnai G² 120KV).

4.6. Nanoparticle tracking analysis (NTA)

The quantification and purity of plasma exosome samples were measured using the NanoSight NS300 Instrument ZetaView® BASIC NTA (Particle Metrix) at Cancer Research Center, University of Science and Technology of China.

4.7. High-throughput RNA-seq analysis

High throughput RNA-seq analysis of isolated exosome was performed at Ribobio Co. Ltd. Total RNA was isolated by using Trizol (Invitrogen,USA). The quantity and integrity of RNA yield was assessed by using the Qubit®2.0 (Life Technologies, USA) and Agilent 2200 TapeStation (Agilent Technologies, USA) separately. 1µg total RNA of each samples were used to prepare small RNA libraries by NEBNext® Multiplex Small RNA Library Prep Set for Illumina (NEB, USA) according to manufacturer's instructions. The libraries were sequenced by HiSeq 2500 (Illumina, USA) with single-end 50bp at Ribobio Co. Ltd (Ribobio, China). The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

4.8. Western blot

Exosomal protein was lysed with RIPA lysis buffer (Beyotime Biotechnology, China) according to the manufacturer’s protocol. Protein concentrations were determined using the BCA protein assay kit (Beyotime Biotechnology, China). Proteins (10 µg) were separated via 10% SDS-PAGE, and subsequently transferred onto NC membranes (EMD Millipore). After transferred to a NC membrane, following blocking with 5% bovine plasma albumin, the membranes were washed three times with PBS. Subsequently, the membranes were incubated with primary antibodies at 4 °C overnight. After washing with PBS, the membranes were incubated with H&L secondary antibody for 1 h at 25 °C. Protein bands were visualized using the ECL Substrate kit (Thermofisher). Protein expression levels were semi-quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.)

4.9. Real-time quantitative PCR

To verify the accuracy of the miRNA-seq data from exosomes, qRT-PCR was performed. Briefly, according to the manufacturer’s protocol, total RNA was extracted from plasma exosomes using RNAiso reagents (Invitrogen Bio, Inc.), total RNA was isolated from tissues or cells using a TRIzol reagent (15596-018, Invitrogen). Nanodrop (Thermo, USA) was used to quantify the concentration of RNA. cDNA was produced from 1 µg of total RNA using PrimeScript RT reagent KIT with gDNA Eraser (Perfect Real Time) (RR047A; Takara). qRT-PCR was performed in triplicate with the HiScript® II Q RT SuperMix for qPCR (+g DNA wiper) (R223, Vazyme Biotech, Nanjing, China) and the Roche LightCycler 96 System (Roche, Basel, Switzerland). The sequences were obtained from the GenBank nucleic acid sequence database (National Center for Biotechnology Information). Relative expression was calculated according to the 2^(-ΔΔCq) method. GAPDH was used as the internal reference genes.

The designed primer sequences for RT-qPCR were as follows:

| Gene       | Sequence                        |
|------------|---------------------------------|
| mir-122-3p | Forward 5'-TATTGCACCTGGATACGACAAAAAC-3'  |
|           | Reverse 5'-GGCCGCTGATGTCACATGTC3'  |
| mir-451a  | Forward 5'-CACTTCACTGCTGGAAACCGTATTACATC-3'  |
|           | Reverse 5'-CTGTGCTGACGACTGCGCAA-3'  |
| mir-126-3p | Forward 5'-CTTGCTGACGACCGGAAGAG-3'  |
|           | Reverse 5'-GGATATATATCTACGGAAAGG-3'  |
| mir-148a-3p| Forward 5'-AGCAACACCTAAGCAAGGACCAA-3'  |
|           | Reverse 5'-GCACTGCGGATTCTGATC-3'  |
| mir-108-5p | Forward 5'-GGATATATCTAAGACGGAC-3'  |
|           | Reverse 5'-GACATGCTTGTCACTAATC-3'  |
| mir-423-5p | Forward 5'-CTGGTGATGGTTCTGAC-3'  |
|           | Reverse 5'-GTGGCTGGCGGAGTGTG-3'  |
| mir-196a-5p| Forward 5'-CCAGGATGTAGTGTGATGTG-3'  |
|           | Reverse 5'-GGCATGGCTGCGGAGG-3'  |
| mir-486-5p | Forward 5'-CCCGAGGCTGTGAC-3'  |
|           | Reverse 5'-GATCCAGTGGTGGTGCG-3'  |
| GAPDH     | Forward 5'-CGTGCTCTACTGGAGGCGTGC-3'  |
|           | Reverse 5'-GCAAGGTAAGAGGAG-3'  |
| U6        | Forward 5'-GGTTGGGCAGACATACATAAT-3'  |
|           | Reverse 5'-GCCCAAGAATTTGGGTCAT-3'  |

4.10. Hepatic glucose production assay

Primary hepatocytes were seeded to the collagen-coated 6-well plate with 300,000 cells per well. Primary hepatocytes were incubated in low glucose DMEM (2% FBS), 37 °C for 3 h for cell attachment. After
attachment, the medium was replaced by HGP buffer (120 mM NaCl, 5 mM KCl, 2.5 mM KH2PO4, 2.5 mM MgSO4, 2 mM CaCl2, 25 mM NaHCO3, 10 mM HEPES, 0.5%BSA, 10 mM Lactate, 5 mM Pyruvate). Metformin were pretreated in corresponding groups for different time period, followed by 100 nM glucagon treatment for 2 h. Glucose content in medium was determined by AmplexTM Red Glucose/Glucose Oxidase Assay Kit (Thermo Fisher, Waltham, MA, USA) according to manufactory protocol and normalized by protein.

4.11. Data analyses
All results are presented as mean ± SE. P values were calculated using Student’s t-test for the comparison of differences between two groups. Significance among multiple groups was tested using one-way or two-way ANOVA. P < 0.05 was considered statistically significant.

For exosome quantification, and NTA: The data shown represents the mean ± SD from at least three independent experiments.

Declarations
Author contribution statement
Qingsheng Yu, Yilin Hou: Conceived and designed the experiments. Hui Peng, Mei Hou, Jing Wang, and Xiangjin Zhuang: Performed the experiments. Man Zhou, Qianqian Tao, and Jiayu Xing: Analyzed and interpreted the data. Long Huang, Fuhai Zhou, and Shengming Zhang: Contributed reagents, materials, analysis tools or data.
Qiuyi Feng: Conceived and designed the experiments; Wrote the paper.

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Data availability statement
Data will be made available on request.

Declaration of interest’s statement
The authors declare no conflict of interest.

Additional information
No additional information is available for this paper.

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