Association between microRNA-527 and glypican-3 in hepatocellular carcinoma

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Abstract. The present study aimed to identify the specific microRNAs (miRNAs/miRs) and their corresponding target genes involved in hepatocellular carcinomas (HCCs). Microarray analysis was performed to examine the miRNA expression profiles of four paired HCC and corresponding non-cancerous (N) liver tissues using 985 miRNA probes. The Human miRNA Target database was used to identify the target genes of differentially expressed miRNAs between the HCC and N tissues. The protein expression levels of target genes in the HCC tissues and cell lines were evaluated using western blotting. miRNA-mediated suppression of target gene expression was evaluated by transiently transfecting the miRNA into the HCC cell lines. Of the 985 miRNAs evaluated, four miRNAs were differentially expressed (three upregulated and one downregulated miRNAs). Of these four miRNAs, miRNA-527 was highly downregulated in the HCC tissues. Glypican-3 (GPC-3) was predicted as a target gene of miRNA-527. Western blotting revealed that GPC-3 protein is highly expressed in the HCC tissues and HCC cell lines compared with N and normal cell lines. Transfection with miR-527 resulted in suppression of GPC-3 protein expression in the Cos7 cells. Furthermore, transfection with miR-527 also inhibited the intrinsic expression of GPC-3 in the Huh-7 cell line. This indicated that miR-527 in the HCC tissues may be an important novel miRNA that targets the GPC-3 gene expression. GPC-3, whose expression is regulated by miR-527, may be involved in the development and progression of HCC.

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Introduction

Hepatocellular carcinomas (HCCs) are one of the fourth most common cause of cancer-associated death worldwide (1,2). Globally, there is an increased incidence of HCC (1,2). The risk factors for HCCs are well understood, such as chronic hepatitis or cirrhosis due to persistent infection with hepatitis B and C virus, fatty liver disease, diabetes and excessive alcohol intake (3); however, the molecular mechanisms in HCC have not been elucidated.

microRNAs (miRNAs/miRs) are small, endogenous non-coding RNAs (4,5) that are reported to be involved in gene regulatory mechanisms. miRNAs are reported to be widely involved in regulatory networks, such as proliferation, differentiation and inflammation (5-8). One miRNA is estimated to regulate >200 target genes (5-8). In animals, single-stranded miRNA binds to the 3' untranslated region (3'-UTR) of the target mRNAs through a homologous sequence, which inhibits translation or degrades the mRNAs (5-8).

The importance of these regulators has been recognized; however, the precise function of these regulatory non-coding RNAs is not completely understood. Various human malignancies (9-13), including HCC, are associated with aberrant expression of miRNAs. This indicates that the miRNAs function as oncogenes or tumor suppressor genes (13). miRNAs have an important role in carcinogenesis as half of the known aberrantly expressed miRNA genes are located in the cancer-associated genomic regions or fragile sites (14,15). Changes in miRNA expression have been reported in various

malignancies, such as Burkitt lymphoma (16), colorectal cancer (17,18) and lung cancer (19).

Several studies have reported that the HCC cells or malignant liver tissues exhibit aberrant expression of specific miRNAs, such as miR-26a, let-7a, let-7b, let-7c, let-7d, let- f-1 and let-g, when compared with non-malignant hepatocyte tissues in the previous reports (20-27), which indicates an association between miRNA and HCC. Previous studies have demonstrated that the modulation of non-coding RNA expression, especially miRNAs, may contribute to HCC formation (20-27). However, there are no studies that have reported the correlation between miRNA-527 and HCC, to the best of our knowledge. Additionally, the target genes of the aberrantly expressed miRNAs in HCC have not been fully elucidated. The present study aimed to examine these aberrantly expressed miRNAs, which may contribute to HCC pathogenesis by modulating the expression of gene products.

Glypican (GPC)-3 is an oncofetal glycoprotein attached to the cell membrane by a glycophosphatidylinositol anchor (28). GPC-3 is overexpressed in some tumors, especially HCC (29). GPC-3 may be involved in the regulation of Wnt, hedgehog, bone morphogenic protein, and fibroblast growth factor (FGF) signaling pathways (30). Therefore, GPC-3 contributes to cell proliferation and apoptosis in certain cell types, including malignant cells (29). Moreover, overexpression of GPC-3 is reported to be associated with poor prognosis in patients with HCC (31,32). The present study aimed to investigate GPC-3 as a target gene of miR-527.

Materials and methods

Chemicals. All chemicals were obtained from Sigma-Aldrich; Merch KGaA.

Cell lines and culture. All HCC cell lines (Huh7, HLE, HLF, Alexander (code no. IFO50069), Li-7 and Hep3B (code no. 86062703) and Cos7 cells, one of the most commonly used cell system for mammalian transient expression, were obtained from the Japanese Cancer Research Resources Bank and KAC Co., Ltd. Cos7 cells do not express GPC-3. The normal human hepatocytes were obtained from DS Pharma Biomedical (Gibco; Thermo Fisher Scientific, Inc.). The HCC cell lines were cultured in RPMI-640 medium (Gibco; Thermo Fisher Scientific, Inc.), while the normal hepatocytes were cultured using the CS-c COMPLETE Medium kit R (Sumitomo Dainippon Pharma Co., Ltd.). Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc.). All media were supplemented with 10% fetal bovine serum (cat. no. 533-69545; FUJIFILM Wako Pure Chemical Corporation) and penicillin-streptomycin (100 mg/l; Invitrogen; Thermo Fisher Scientific, Inc.). All the cells were cultured in a humidified atmosphere at 5% CO_2 and 37°C.

Human tissues. HCC tissues were limited to patients who had not received any treatment, including any chemotherapy, prior to partial hepatectomy. Partial hepatectomy surgery was performed from May 2015 to September 2015. Four tissue samples were collected during partial hepatectomy of patients with HCC in Kagawa University Hospital (Kita-gun, Japan).

Histology of tissues were pathologically analyzed using a light microscope. These tissues were analyzed retrospectively. The HCC tissue samples were frozen on dry ice within 30 min of collection. The samples were preserved in the freezer at -70°C.

The histological analysis of the surrounding non-cancerous tissues (N) revealed that the tissues were cirrhotic. Of the four patients, three were male and one was female (mean age 60.5±6.98 years; range, 51-69). All patients tested positive for hepatitis C virus and negative for hepatitis B surface antigen. The HCC tissues from all patients were moderately differentiated. The study was approved by The Human Subjects Committee of Kagawa University (approval no. 22-163). Additionally, informed written or verbal consent was provided by all patients.

Tissue and cell line lysate. The samples were homogenized in TNE buffer (10 mmol/l Tris-HCl [pH 7.4], 10 mmol/l Na_3VO_4, 50 mmol/l, Na_2MoO_4, 1% Nonidet P-40 and 100 U/ml aprotinin). The tissue lysate was centrifuged at 2,900 x g and 4°C for 60 min. The protein concentration in the supernatant was measured by bicinchoninic acid protein assay.

Analysis of miRNA microarrays. miRNAs expression in the cancerous and surrounding N tissues was evaluated using microarray analysis. Total RNA was extracted from the liver tissues using the miRNeasy Mini kit (Qiagen AB) as described previously (33,34). The OD_{260} nm/OD_{280} nm ratio of the total RNA samples used in this study ranged from 1.8 to 2.0. The samples were labeled using the miRCURY Hy3 Power Labeling kit (Exiqon; Qiagen AB). miRNA expression was measured by hybridizing the RNA sample on a human miRNA Oligo chip, version 14.0 (Toray Industries, Inc.) as described previously (33,34). The chip was scanned using a 3D-Gene Scanner 3000 (Toray Industries, Inc.). The raw intensity of the image was measured using the 3D-Gene extraction software (version 1.2; Toray Industries, Inc.). The raw data were analyzed using GeneSpringGX (version 10.0; Agilent Technologies, Inc.) and subjected to quantile normalization (35). The fold changes in the miRNA expression levels were evaluated between the cancerous and surrounding N tissues. Hierarchical clustering was performed using the furthest neighbor method and Pearson's product-moment correlation coefficient as a metric, with P<0.01 and false discovery rate (FDR) <0.05.

Gel electrophoresis and western blotting. The expression level of GPC-3 was determined in the liver tissue and HCC cell lysates using western blotting. The lysate samples ($10 \mu g$) were subjected to SDS-PAGE following the methods of Laemmli (36). Next, the resolved proteins were subjected to western blotting, following the methods of Towbin *et al* (37). The membrane was incubated with the following primary antibodies: Anti-GPC-3 (1:500) and anti-neomycin phosphotransferase II (1:500) (both Santa Cruz Biotechnology, Inc.). β-actin was used as the loading control in western blot analysis. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Funakoshi Co., Ltd.) on an X-ray film.

Transient transfection. The cDNA of GPC-3 gene was inserted into the pCMV6-XL5 vector (Funakoshi Co., Ltd.), which contains the target DNA sequences, poly A signal and
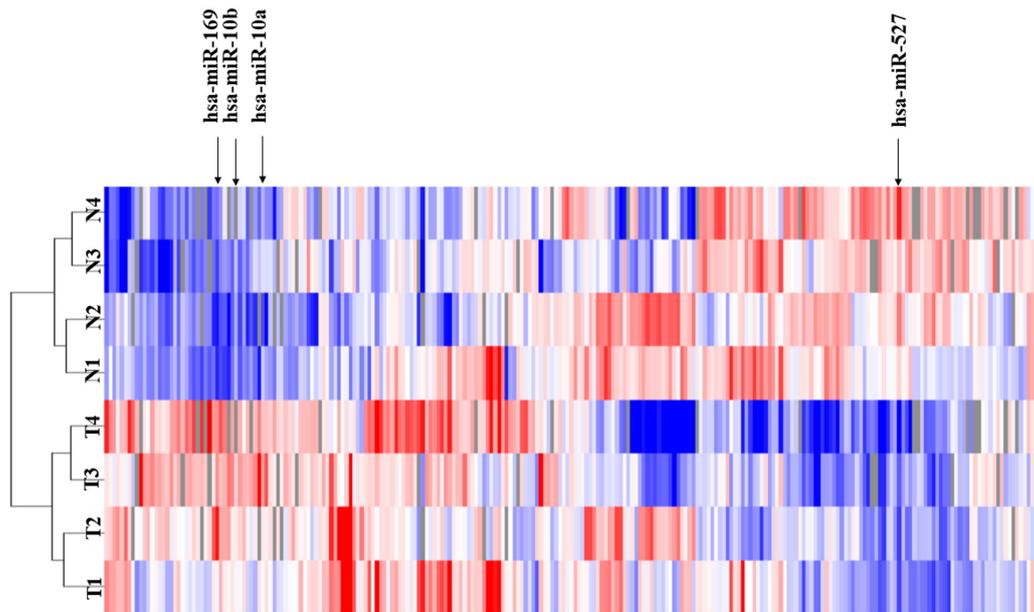
Figure 1. Hierarchical clustering of miRNAs from cancerous (T1-T4) and non-cancerous cirrhotic tissues (N1-N4). Hierarchical clustering was performed for miRNA expression profiles of cancerous (left side) and non-cancerous tissues (right side). Samples are arranged in columns, while miRNAs are arranged in rows. miRNA clustering tree is shown on the left and the sample clustering tree is shown at the top of each heat map. Heat maps show the relative expression intensity for each miRNA in which the base-2 logarithm of the intensity is median-centered for each row. Red indicates high expression and blue indicates low expression level. Overall, four miRNAs (arrows) were differentially expressed in the cancerous and non-cancerous cirrhotic tissues. P<0.01, false discovery rate <0.05. miRNA/miR, microRNA; T, tumor tissues; N, non-cancerous cirrhotic tissues.

CMV promoter. The African green monkey kidney cells, Cos7 cells, were used for transfection as these cells do not express GPC-3. Cos7 cells seeded (0.3x10^6/ml) in 6-well plates were transfected with 4 μg of the recombinant (pCMV6-GPC) or empty pCMV6-XL-5 vector (Funakoshi Co., Ltd.) and 100 nM negative control miRNA (non-targeting sequence) or mimic miR-527 (Funakoshi Co., Ltd.) using 10 μl of COSFectin lipid reagent (Bio-Rad Laboratories, Inc.) at 37°C for 48 h. To confirm transfection efficiency, the cells were co-transfected with 4 μg of pcDNA3.1(+) plasmid containing the neomycin phosphotransferase II gene. After transfection, the cells were harvested and subjected to western blot analysis as aforementioned.

Bioinformatics. Target sites for miR-527 found in GPC-3 identified in GPC-3'UTR are aligned among human, mouse and rat using Target Scan software (http://www.targetscan.org/vert_72/).

Transfection of miRNA. Huh7 cells were transfected with miR-527 mimic (Funakoshi Co., Ltd.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. HCC cells were transfected with 100 nM miR-527 mimic, or 100 nM negative control #1 (Funakoshi Co., Ltd.). To study cell proliferation, the WST-8 assay [3-(4,5-di-methylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide] was conducted after 24 h of transfection (34).

Statistical analysis. The data were analyzed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference. For each miRNA probe of the microarray analyses, Student's t-test was performed on the two groups. False discovery rate (FDR) as determined using the Benjamini-Hochberg method (38) was used to control the

multiplicity of testing caused by the number of the miRNA probes used. All statistical analyses were performed in the GraphPad Prism 6 software (GraphPad Software).

Results

Dysregulation of miRNA expression in the human HCC tissues. To investigate the roles of miRNAs in HCCs, the miRNA expression profiles of four paired T tissues and their corresponding N liver tissues (cirrhotic liver tissues) using 985 miRNAs probes. The human T tissues and their corresponding N liver tissues exhibited differential miRNA expression profiles. The unsupervised hierarchical clustering analysis revealed that the T tissues clustered separately from the N tissues (Fig. 1). Among the tested 985 miRNAs, four miRNAs with P<0.01 and FDR <0.05 were differentially expressed (three upregulated and one downregulated) in the T compared with N cirrhotic tissues (Fig. 1). These results indicated that there are differences in microRNA expression between T and N cirrhotic tissues.

As shown in Fig. 2, miR-527 was significantly downregulated in the T tissues (P=0.0042). The data presented in Fig. 2 represent the average of the ratio of miR-527 expression in T compared with that in N tissues of the four specimens obtained from surgery. Previous studies have reported the target genes of miRNA-10a (39) miRNA-10b (40) and miRNA-379 (41). However, the target gene of miR-527 is not clear, to the best of our knowledge. Therefore, miRNA-527 was chosen for further investigation. In short, miR-527 was significantly downregulated in the T tissues compared with N tissues.

HCC cells exhibit enhanced expression of GPC-3. GPC-3 is an important carcinogenic factor that is highly sensitive for
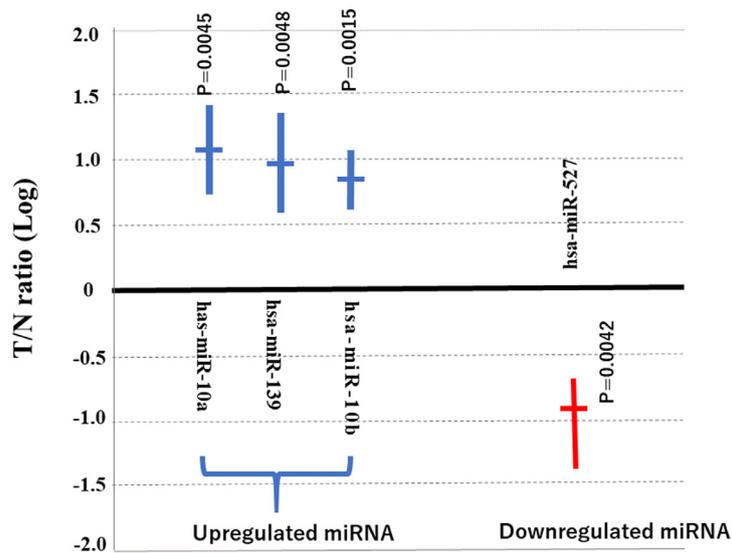
Figure 2. Identification of differentially expressed miRs in patients with hepatocellular carcinoma. Cancerous and non-cancerous tissues exhibited differential miR profiles. As compared with non-cancerous tissues, three miRs (miR-169, -10b and -10b) were upregulated (P<0.01, FDR <0.05) and one was significantly downregulated in the cancerous tissues (miR-527) (P<0.01, FDR <0.05]. miR, microRNA; FDR, false discovery rate.; T/N, cancerous tissue/ non-cancerous tissue.
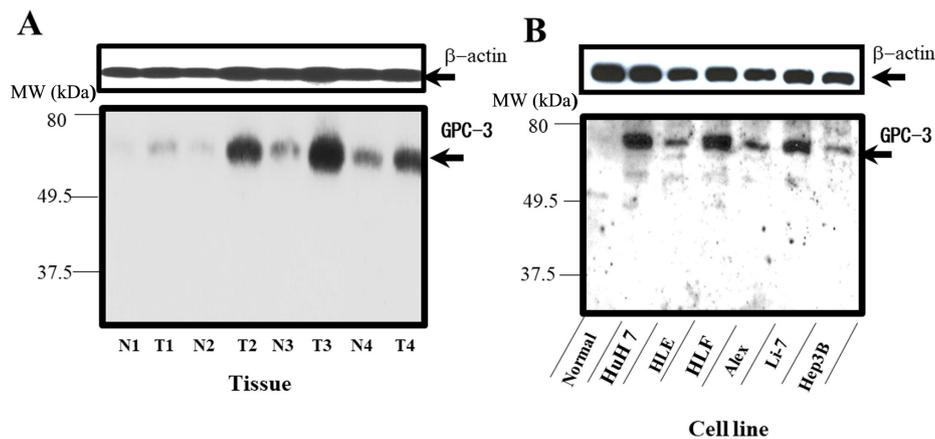
Figure 3. Expression levels of GPC-3 in HCC tissues and cell lines. (A) Western blotting revealed that HCC tissues exhibited higher GPC-3 expression compared with the corresponding N tissues. (B) Expression level of GPC-3 in the HCC cell lines and normal hepatocytes. HCC, hepatocellular carcinoma; GPC-3, glypican-3; N, non-cancerous tissues; T, HCC tissues; miR, microRNA; MW, molecular weight.

HCC (42). Western blotting revealed that the four HCC (T) tissues exhibited higher GPC-3 expression levels compared with the N tissues (Fig. 3A). Consistent with the observations in human tissues, the expression of GPC-3 was detected in various HCC cell lines (Huh7, HLE, HLF, Alexander, Li-7 and Hep3B) but not in normal hepatocyte line (Fig. 3B). These results suggested that the expression of GPC-3 in the N tissues was lower compared with that in the normal hepatocyte cell line.

miRNA-527 suppresses GPC-3 expression. To clarify if GPC-3 is the target gene of miRNA-527, transient gene transfection analysis was performed. The Cos7 cells transfected with the pCMV6-XL5 vector (control vector) did not exhibit GPC-3 protein expression (lane 1; Fig. 4A). The Cos7 cells transfected with the pCMV6-GPC recombinant vector exhibited strong GPC-3 protein expression (Fig. 4A, lane 2).

The transfection efficiency was evaluated by co-transfecting the Cos7 cells with pCDNA3.1(+) vector, which contains the neomycin phosphotransferase II gene, and pCMV6-GPC or pCMV6-XL5 vector. The expression of neomycin phosphotransferase II in the co-transfected cells was evaluated using western blotting (Fig. 4B, lane 1).

As shown in Fig. 4B, transfection with non-specific control miRNA did not suppress GPC-3 protein expression in the Cos7 cells at 48 h post-transfection (Fig. 4B, lane 3). Meanwhile, transfection with miRNA-527 suppressed GPC-3 protein expression in the Cos7 cells (Fig. 4B, lane 4). Cos-7 cells transfected with only pCMV6-GPC vector exhibited GPC-3 protein expression at 48 h post-transfection (Fig. 4B, lane 2), whereas those transfected with only pCMV6-XL5 control vector did not exhibit GPC-3 protein expression (Fig. 4B, lane 1). These results suggested that miRNA-527 suppresses protein expression of GPC-3.

miRNA-527 inhibits expression of intrinsic GPC-3 in the Huh-7 cells. Similar to the HCC tissues, several HCC cell lines are reported to express GPC-3 (43). The expression of GPC-3
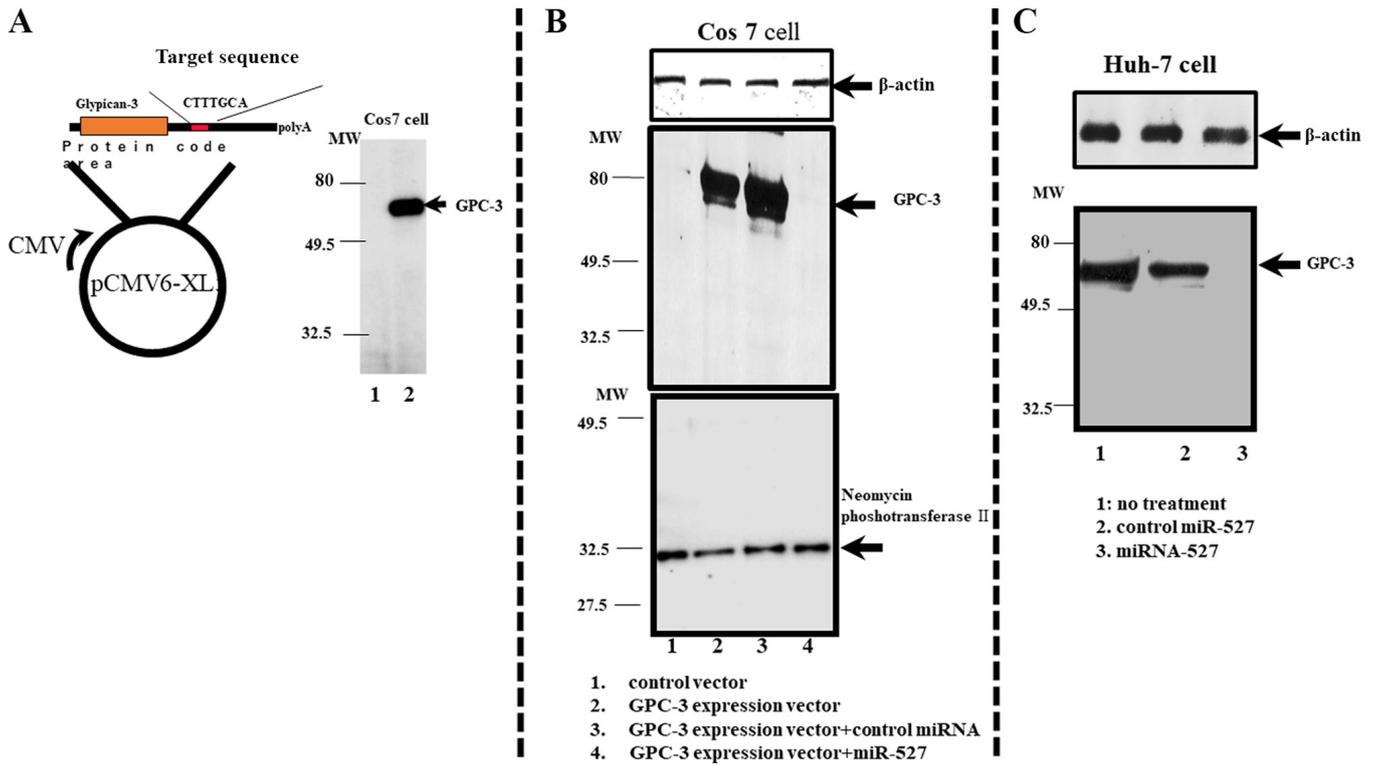
Figure 4. Expression of GPC-3 in the Cos7 and Huh-7 cells. (A) Construction of the pCMV6-GPC recombinant vector is shown. Expression of GPC-3 protein in the Cos7 cells after transient transfection with pCMV6-XL5 (lane 1) or pCMV6-GPC (lane 2). (B) Suppression of GPC-3 expression by miRNA-527 in the Cos 7 cells. (C) miRNA-527 inhibited intrinsic GPC-3 expression in the Huh-7 cells. GPC-3, glypican-3; MW, molecular weight; CMV, cytomegalovirus; miRNA, microRNA.
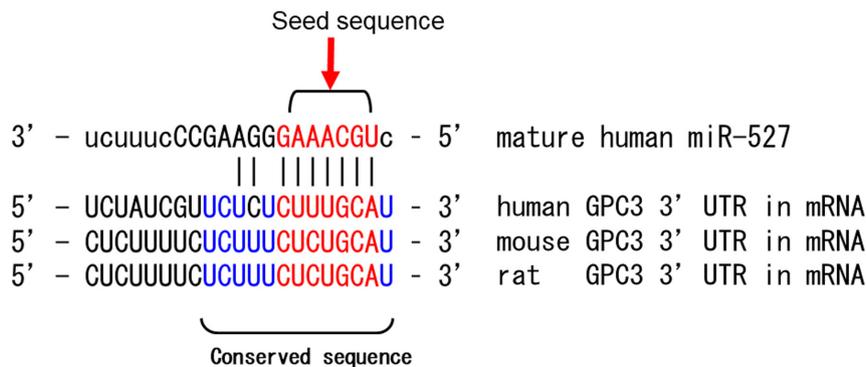
Figure 5. Target sites for miR-527 conserved among three mammals. Target sites for miR-527 were identified in the GPC-3 3'UTR and aligned among human, mouse and rat. UTR, untranslated region.

was confirmed in several HCC cell lines, including the Huh-7 cell line (Fig. 4C). The effect of miRNA-527 transfection on the intrinsic expression of GPC-3 in the Huh-7 cells was examined. As shown in Fig. 4C, GPC-3 is expressed in the Huh-7 cells (lane 1). Transfection with non-specific miRNAs did not suppress the expression of GPC-3 in the Huh-7 cells (lane 2). However, transfection with miRNA-527 completely inhibited the intrinsic expression of GPC-3 in the Huh-7 cells (lane 3). This indicated that miRNA-527 can also suppress the intrinsic expression of GPC-3 in the Huh-7 cell line. The expression of neomycin phosphotransferase II was similar in all groups, which suggested that the transfection efficiency was similar in all groups (Fig. 4B).

Target genes of miRNA-527. miRNA-527 is encoded on the 19th human chromosome using the data base of miRNA, miRbase (http://www.mirbase.org/) and its target genes were identified using Target Scan. The sequences at the 3'-UTR of the GPC-3 gene, which are potentially involved in the development of HCC, contained the target sites for miRNA-527. The GAAACGU sequences (from second to the eight base) at the 5' end of miRNA-527, which is referred to as seed sequence, was complementary to the sequences at the 3'-UTR in the GPC-3 gene among human, rat and mouse (Fig. 5).

Transfection of miRNA. To study cell proliferation, the WST-8 assay was conducted after 24 h of miR-527 transfection into
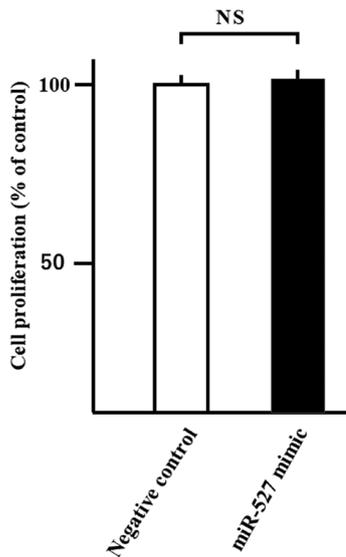
Figure 6. WST-8 assay was performed 24 h after the transfection of miR-527 mimic in HuH-7 cells. NS, not significant.

Huh-7 cells. Transfection with miR-527 did not affect the proliferation of Huh-7 cells compared with negative control miRNA (Fig. 6).

Discussion

Globally, HCC is one of the most common cancer types (1). HCC is associated with complex and heterogenous carcinogenesis and molecular mechanisms (44). Hence, the pathophysiology of HCCs is not well understood. Previous studies have reported the molecular mechanisms of miRNA in the carcinogenic process (42,43). miRNAs regulate gene expression, and one miRNA is estimated to regulate >200 target genes (5,6). Therefore, miRNAs are potential therapeutic targets for various cancer types, including HCC (7,9-13). The present study analyzed the miRNA expression profiles of four paired HCCs and their corresponding non-cancerous liver tissues using 985 miRNA probes to identify the miRNAs that contribute to HCC pathogenesis.

The microarray analysis revealed one downregulated and three upregulated miRNAs in the HCC tissues when compared with the corresponding non-cancerous liver tissues. Generally, downregulated miRNAs are considered to regulate the carcinogenic or progressive factors of carcinomas (23-26). miRNA-527 was downregulated, which may function as a tumor suppressor miRNA in HCC. The association between miRNA-527 and HCC has not been previously reported, therefore miRNA-527 was selected for further investigation.

In addition, the current study identified GPC-3 as a target gene of miRNA-527 using the Human micro RNA Target database. The database analysis revealed that the GPC-3 gene had the target site for miRNA-527. The 5' end of miRNA-527 contains the seed sequence, which binds to the 3'-UTR of the GPC-3 gene. GPC-3 belongs to the heparin sulfate proteoglycan (HSPG) family, which are localized and bound to the cell membrane surface by glycosylphosphatidylinositol anchors (6,7). GPC-3 is overexpressed in the HCC tissues and is reported to be a therapeutic target for HCC (44).

GPC-3 expression was also observed in the fetal liver with levels of proliferation but not in the normal adult liver or cirrhotic liver (45). The results of the present study showed that non-cancerous tissues exhibited lower GPC-3 expression compared with HCC tissues. GPC-3 expression was not detected in the normal hepatocyte cell line; however, enhanced GPC-3 expression was observed in numerous hepatoma cell lines. The current data suggested that upregulated GPC-3 may contribute to HCC carcinogenesis.

Wang *et al* (46) reported that out of 111 HCC cases, GPC-3-overexpression was detected in the cytoplasm, cell membrane and canaliculus in 84 (75.7%) cases. Of these 84 cases, 61 (72.6%) cases exhibited diffuse immunoreactivity. Contrastingly, none of the 110 cases of hepatocellular adenoma, focal nodular hyperplasia and large regenerative nodule exhibited detectable GPC-3 expression. Additionally, the expression of GPC-3 in HCC tissue is not correlated with the size, differentiation or advanced stage based on Union for International Cancer Control Tumor Node Metastasis classification of the tumor (32). Moreover, patients with HCC exhibit higher serum GPC-3 levels compared with patients without HCC (46). Overall, these results and those of the present study indicate that GPC-3 can be a novel tumor marker for HCC.

GPC-3 is also reported to be a carcinogenic factor in HCC (47). GPC-3 may induce HCC tissue growth by stimulating the canonical Wnt/β-catenin pathway (46). Additionally, GPC-3 is closely associated with the function of sulfatase 2 (SULF2), an enzyme with 6-O-desulfatase activity on HSPGs. SULF2, which is upregulated in 60% of HCC cases, is closely associated with poor prognosis (32). SULF2 is also reported to upregulate FGF signaling in HCC cells via heparin sulfate-dependent and GPC-3-dependent mechanisms (47). Furthermore, overexpression of SULF2 increases the proliferation and migration of HCC cells (47). Knockdown of GPC-3 attenuates FGF2 binding in the SULF2-expressing HCC cells. The role of SULF2 in upregulating GPC-3 expression and promoting tumor growth was also confirmed in a nude mouse xenograft model (47). These studies indicate that GPC-3 may have an important role in the development of HCC.

Next, it was investigated whether miRNA-527 can suppress the expression of GPC-3. The pCMV6-GPC vector was constructed and transfected into the Cos7 cells to overexpress GPC-3, as Cos7 cells do not usually express GPC-3. miRNA-527 completely suppressed the expression of GPC-3 in the GPC3-overexpressing Cos7 cells. In addition, whether miRNA-527 could suppress intrinsically expressed GPC-3 in human HCC cells was investigated. miRNA-527 was transfected into the Huh-7 cells, which express intrinsic GPC-3. GPC-3 expression was inhibited in the miR-527-transfected Huh-7 cells. These data suggested that GPC-3 is a target gene of miRNA-527 is. To the best of our knowledge, the present study is the first to report the association between GPC-3 and miRNA-527 in HCC. Only one previous study has reported the correlation between miRNA-527 and cancer; Huo *et al* (48) demonstrated that lung tumor tissues and non-small cell lung cancer cells exhibit decreased miRNA-527 expression. Overall, the present results indicated that miR-527 has potential as a diagnostic biomarker for HCC in the future.

A previous study demonstrated that siRNA-mediated downregulation of GPC-3 expression in GPC-3-overexpressing

HCC cell lines markedly decreased the expression levels of growth signaling molecules, matrix metalloproteinases [(MMP)2 and MMP14], FGF receptor 1 and insulin-like growth factor 1 receptor in the cells (47). Thus, GPC-3 may be a potential therapeutic target for HCC (46).

To study the effect of miR-527 transfection on cancer cell proliferation, miR-527 mimic was transfected into the Huh-7 cells. Transfection with miR-527 did not affect the proliferation of Huh-7 cells (data not shown). These results suggested that gene transfection into HCC cells of with miR-527, which targets GPC-3, did not inhibit of cancer cells proliferation.

Further studies are needed to evaluate the suppression of HCC growth through GPC-3 expression modulation by miRNA-527. However, the results of the present study and previous studies indicate that miRNA-527 could have potential as a therapeutic target for HCCs through regulation of GPC-3 expression.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KN, AK, HI, JT, TN and MN designed the research and analyzed the data. KN, TM, JT, HY, HK, AM, KT and TH drafted this manuscript. KeO and YS collected the sample of patients. KN, KyO, TT, KF, SM analyzed western blotting and transfection data. KN, AK, HI, JT, TN, MN, KyO, TT, KF, SM, HY, HK, AM, KeO, YS, KT, TH and TM were involved in data interpretation and have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved by The Human Subjects Committee of Kagawa University (approval no. 22-163). Additionally, written informed consent was obtained from all patients to use the tissue for this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A and Roberts LR: A global view of hepatocellular carcinoma: Trends, risk, prevention and management. Nat Rev Gastroenterol Hepatol 16: 589-604, 2019.

2. Wang CH, Wey KC, Mo LR, Chang KK, Lin RC and Kuo JJ: Current trends and recent advances in diagnosis, therapy, and prevention of hepatocellular carcinoma. Asian Pac J Cancer Prev 16: 3595-3604, 2015.

3. Sherman M: Hepatocellular carcinoma: Epidemiology, surveillance, and diagnosis. Semin Liver Dis 30: 3-16, 2010.

4. Iwama H, Masaki T and Kuriyama S: Abundance of microRNA target motifs in the 3'-UTRs of 20527 human genes. FEBS Lett 581: 1805-1810, 2007.

5. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.

6. Krek A, Grün D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, et al: Combinatorial microRNA target predictions. Nat Genet 37: 495-500, 2005.

7. Bartel DP: Metazoan MicroRNAs. Cell 173: 20-51, 2018.

8. Tomasello L, Cluts L and Croce CM: Experimental validation of microRNA targets: Mutagenesis of binding regions. Methods Mol Biol 1970: 331-339, 2019.

9. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST and Patel T: MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 133: 647-658, 2007.

10. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, et al: Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res 64: 3753-3756, 2004.

11. Varnholt H, Drebber U, Schulze F, Wedemeyer I, Schirmacher P, Dienes HP and Odenthal M: MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma. Hepatology 47: 1223-1232, 2008.

12. Visone R and Croce CM: miRNAs and cancer. Am J Pathol 174: 1131-1138, 2009.

13. Morishita A and Masaki T: miRNA in hepatocellular carcinoma. Hepatol Res 45: 128-141, 2015.

14. Calin GA, Liu CG, Sevignani C, Ferracin M, Felli N, Dumitru CD, Shimizu M, Cimmino A, Zupo S, Dono M, et al: MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci USA 101: 11755-11760, 2004.

15. Sevignani C, Calin GA, Siracusa LD and Croce CM: Mammalian microRNAs: A small world for fine-tuning gene expression. Mamm Genome 17: 189-202, 2006.

16. Metzler M, Wilda M, Busch K, Viehmann S and Borkhardt A: High expression of precursor microRNA-155/BIC RNA in children with Burkitt lymphoma. Genes Chromosomes Cancer 39: 167-169, 2004.

17. Michael MZ, O' Connor SM, van Holst Pellekaan NG, Young GP and James RJ: Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 1: 882-891, 2003.

18. Tokarz P and Blasiak J: The role of microRNA in metastatic colorectal cancer and its significance in cancer prognosis and treatment. Acta Biochim Pol 59: 467-474, 2012.

19. Del Vescovo V and Denti MA: microRNA and lung cancer. Adv Exp Med Biol 889: 153-177, 2015.

20. Yang X, Liang L, Zhang XF, Jia HL, Qin Y, Zhu XC, Gao XM, Qiao P, Zheng Y, Sheng YY, et al: MicroRNA-26a suppresses tumor growth and metastasis of human hepatocellular carcinoma by targeting interleukin-6-Stat3 pathway. Hepatology 58: 158-170, 2013.

21. Zhu Y, Lu Y, Zhang Q, Liu JJ, Li TJ, Yang JR, Zeng C and Zhuang SM: MicroRNA-26a/b and their host genes cooperate to inhibit the G1/S transition by activating the pRb protein. Nucleic Acids Res 40: 4615-4625, 2012.

22. Wang Y, Lu Y, Toh ST, Sung WK, Tan P, Chow P, Chung AYF, Jooi LLP and Lee CG: Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3. J Hepatol 53: 57-66, 2010.

23. Wang Z, Lin S, Li JJ, Xu Z, Yao H, Zhu X, Xie D, Shen Z, Sze J, Li K, et al: MYC protein inhibits transcription of the microRNA cluster MC-let-7a-1~let-7d via noncanonical E-box. J Biol Chem 286: 39703-39714, 2011.

24. Tsang WP and Kwok TT: Let-7a microRNA suppresses therapeutics-induced cancer cell death by targeting caspase-3. Apoptosis 13: 1215-1222, 2008.

25. Di Fazio P, Montalbano R, Neureiter D, Alinger B, Schmidt A, Merkel AL, Quint K and Ocker M: Downregulation of HMGA2 by the pan-deacetylase inhibitor panobinostat is dependent on hsa-let-7b expression in liver cancer cell lines. Exp Cell Res 318: 1832-1843, 2012.

26. Zhu XM, Wu LJ, Xu J, Yang R and Wu FS: Let-7c microRNA expression and clinical significance in hepatocellular carcinoma. J Int Med Res 39: 2323-2329, 2011.
27. Lan FF, Wang H, Chen YC, Chan CY, Ng SS, Li K, Xie D, He ML, Lin MC and Kung HF: Hsa-let-7g inhibits proliferation of hepatocellular carcinoma cells by downregulation of c-Myc and upregulation of p16(INK4A). Int J Cancer 128: 319-331, 2011.
28. Traister A, Shi W and Filmus J: Mammalian Notum induces the release of glypicans and other GPI-anchored proteins from the cell surface. Biochem J 410: 503-511, 2008.
29. Li N, Gao W, Zhang YF and Ho M: Glypicans as Cancer Therapeutic Targets. Trends Cancer 4: 741-754, 2018.
30. Capurro MI, Xu P, Shi W, Li F, Jia A and Filmus J: Glypican-3 inhibits Hedgehog signaling during development by competing with patched for Hedgehog binding. Dev Cell 14: 700-711, 2008.
31. Shirakawa H, Suzuki H, Shimomura M, Kojima M, Gotohda N, Takahashi S, Nakagohri T, Konishi M, Kobayashi N, Kinoshita T, *et al*: Glypican-3 expression is correlated with poor prognosis in hepatocellular carcinoma. Cancer Sci 100: 1403-1407, 2009.
32. Zhang J, Zhang M, Ma H, Song X, He L, Ye X and Li X: Overexpression of glypican-3 is a predictor of poor prognosis in hepatocellular carcinoma: An updated meta-analysis. Medicine (Baltimore) 97: e11130, 2018.
33. Fujihara S, Kato K, Morishita A, Iwama H, Nishioka T, Chiyo T, Nishiyama N, Miyoshi H, Kobayashi M, Kobara H, *et al*: Antidiabetic drug metformin inhibits esophageal adenocarcinoma cell proliferation *in vitro* and *in vivo*. Int J Oncol 46: 2172-2180, 2015.
34. Fujita K, Iwama H, Sakamoto T, Okura R, Kobayashi K, Takano J, Katsura A, Tatsuta M, Maeda E, Mimura S, *et al*: Galectin-9 suppresses the growth of hepatocellular carcinoma via apoptosis *in vitro* and *in vivo*. Int J Oncol 46: 2419-2430, 2015.
35. Bolstad BM, Irizarry RA, Astrand M and Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19: 185-193, 2003.
36. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685, 1970.
37. Towbin H, Staehelin T and Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354, 1979.
38. Benjamini Y and Hochberg Y: Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc B 57: 289-300, 1995.
39. Chen Y-H, Song Y, Yu YL, Cheng W and Tong X: miRNA-10a promotes cancer cell proliferation in oral squamous cell carcinoma by upregulating GLUT1 and promoting glucose metabolism. Oncol Lett 17: 5441-5446, 2019.
40. Pal R and Greene S: microRNA-10b is overexpressed and critical for cell survival and proliferation in medulloblastoma. PLoS One 10: e0137845, 2015.
41. Werk AN, Bruckmueller H, Haenisch S and Cascorbi I: Genetic variants may play an important role in mRNA-miRNA interaction: Evidence for haplotype-dependent downregulation of ABCC2 (MRP2) by miRNA-379. Pharmacogenet Genomics 24: 283-291, 2014.
42. Nishida T and Kataoka H: Glypican 3-targeted therapy in hepatocellular carcinoma. Cancers (Basel) 11: E1339, 2019.
43. Vongchan P and Linhardt RJ: Characterization of a new monoclonal anti-glypican-3 antibody specific to the hepatocellular carcinoma cell line, HepG2. World J Hepatol 9: 368-384, 2017.
44. Singh AK, Kumar R and Pandey AK: Hepatocellular Carcinoma: Causes, Mechanism of Progression and Biomarkers. Curr Chem Genomics Transl Med 12: 9-26, 2018.
45. Zhou F, Shang W, Yu X and Tian J: Glypican-3: A promising biomarker for hepatocellular carcinoma diagnosis and treatment. Med Res Rev 38: 741-767, 2018.
46. Wang L, Yao M, Pan LH, Qian Q and Yao DF: Glypican-3 is a biomarker and a therapeutic target of hepatocellular carcinoma. Hepatobiliary Pancreat Dis Int 14: 361-366, 2015.
47. Lai JP, Sandhu DS, Yu C, Han T, Moser CD, Jackson KK, Guerrero RB, Aderca I, Isomoto H, Garrity-Park MM, *et al*: Sulfatase 2 up-regulates glypican 3, promotes fibroblast growth factor signaling, and decreases survival in hepatocellular carcinoma. Hepatology 47: 1211-1222, 2008.
48. Huo W, Zhu XM, Pan XY, Du M, Sun Z and Li ZM: MicroRNA-527 inhibits TGF-β/SMAD induced epithelial-mesenchymal transition via downregulating SULF2 expression in non-small-cell lung cancer. Math Biosci Eng 16: 4607-4621, 2019.