MicroRNA-30d Promotes Tumor Invasion and Metastasis by Targeting Galphai2 in Hepatocellular Carcinoma

Jian Yao,1,2 Linhui Liang,2 Shenglin Huang,2 Jie Ding,2 Ning Tan,2 Yingjin Zhao,1,2 Mingxia Yan,3 Chao Ge,2 Zhenfeng Zhang,2 Taoyang Chen,4 Dafang Wan,2 Ming Yao,3 Jinjun Li,2 Jianren Gu,2 and Xianghuo He2

The pathological relevance and significance of microRNAs (miRNAs) in hepatocarcinogenesis have attracted much attention in recent years; however, little is known about the underlying molecular mechanisms through which miRNAs are involved in the development and progression of hepatocellular carcinoma (HCC). In this study, we demonstrate that miR-30d is frequently up-regulated in HCC and that its expression is highly associated with the intrahepatic metastasis of HCC. Furthermore, the enhanced expression of miR-30d could promote HCC cell migration and invasion in vitro and intrahepatic and distal pulmonary metastasis in vivo, while silencing its expression resulted in a reduced migration and invasion. Galphai2 (GNAI2) was identified as the direct and functional target of miR-30d with integrated bioinformatics analysis and messenger RNA array assay. This regulation was further confirmed by luciferase reporter assays. In addition, our results, for the first time, showed that GNAI2 was frequently suppressed in HCC by way of quantitative reverse-transcription polymerase chain reaction and immunohistochemical staining assays. The increase of the GNAI2 expression significantly inhibits, whereas knockdown of the GNAI2 expression remarkably enhances HCC cell migration and invasion, indicating that GNAI2 functions as a metastasis suppressor in HCC. The restoration of GNAI2 can inhibit miR-30d–induced HCC cell invasion and metastasis. Conclusion: The newly identified miR-30d/GNAI2 axis elucidates the molecular mechanism of HCC cell invasion and metastasis and represents a new potential therapeutic target for HCC treatment. (HEPATOLOGY2010;51:846-856.)

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and leading causes of death from cancer worldwide, especially in East Asia and South Africa.1 The pathogenesis of HCC is a multistage process that is usually associated with preneoplastic liver lesions, chronic inflammation, and/or cirrhosis. Despite great advances in the treatment of the disease, relapse or metastasis is frequently observed in clinics, and the 5-year survival rate is still quite low among patients with HCC.2 In past decades, studies have been performed to investigate the genes and proteins that underlie the development and progression of HCC. Several factors involved in the pathogenesis of this malignancy, including wnt/catenin, p53, Rb, and Ras/MAPK,3 have been identified. However, the roles and significances of non-protein coding genes with a particular focus on a class of endogenous tiny RNA molecules termed microRNA (miRNA) remain to be established in the pathogenic processes of HCC.

miRNAs are approximately 21- to 25-nucleotide non-coding RNA molecules that are highly conserved in a variety of eukaryotic organisms. Since their initial discovery in Caenorhabditis elegans,4 miRNAs have become widely accepted as posttranscriptional regulators of gene expression through directly degrading messenger RNA (mRNA) or indirectly repressing protein translation.5 Recent progress suggests that deregulation of miRNAs is involved in a wide range of human diseases, including...
cancer. Because miRNA genes are frequently located in the fragile sites of chromosomes from cancer patients, they have been characterized as a novel class of oncogene and/or tumor suppressor. Emerging evidence links the biological function of miRNAs to tumor metastasis, which is one of the most important hallmarks of cancer, and identifies miRNAs as metastasis regulators that target multiple important signaling proteins in various human cancers. Recent findings have shown that miRNAs are often deregulated in HCC and that some specific miRNA sets are associated with the clinicopathological features of HCC, such as cirrhosis, hepatitis B and C virus infection, metastasis, recurrence, and prognosis. More-
10% fetal bovine serum was injected into the lower chambers. After several hours (5 hours for SK-HEP-1 and Huh-7 in the migration assays, 16 hours for SMMC-7721 and HCCLM3 in the migration assays, 17 hours for SK-HEP-1 in the invasion assays, 24 hours for Huh-7 and SMMC-7721 in the invasion assays, or 64 hours for HCCLM3 in the invasion assays) of incubation at 37°C, any cells remaining in the top chambers or on the upper membrane of the inserts were carefully removed. After fixation and staining in a dye solution containing 0.1% crystal violet and 20% methanol, cells adhering to the lower membrane of the inserts were counted and imaged through an IX71 inverted microscope (Olympus Corp., Tokyo, Japan).

**In Vivo Metastasis Assays.** For in vivo metastasis assays, 2 × 10^6 SMMC-7721-LGS cells infected with miR-30d or mock vector, respectively, were suspended in 40 μL serum-free DMEM/matrix gel (1:1) for each mouse. Through an 8-mm transverse incision in the upper abdomen under anesthesia, each nude mouse (6 in each group, male BALB/c-nu/nu) was orthotopically inoculated in the left hepatic lobe with a microsyringe. After 6 weeks, mice were sacrificed, and their livers and lungs were dissected, fixed with phosphate-buffered neutral formalin and prepared for standard histological examination. Mice were manipulated and housed according to protocols approved by the Shanghai Medical Experimental Animal Care Commission.

**Luciferase Reporter Assay.** HEK 293T cells were seeded in 96-well plates and transfected with a mixture of 50 ng p-Luc-UTR, 100 ng pWPXL-miR-30d, and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 48 hours of incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

**Western Blot Analysis.** Cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked in phosphate-buffered saline/Tween-20 containing 5% nonfat milk. The membrane was incubated with antibodies for GNAI2 (Abnova, Taipei, China) or β-actin (Sigma, St. Louis, MO). The antigen–antibody complex was detected using enhanced chemiluminescence (Pierce, Rockford, IL).

**Immunohistochemical Staining.** All of the tissue samples were fixed in phosphate-buffered neutral formalin, embedded in paraffin, and then cut into 5-μm-thick sections. Tissue sections were deparaffinized, rehydrated, and microwave-heated in sodium citrate buffer (10 mM, pH 6.0) for antigen retrieval. The sections were then incubated with 0.3% hydrogen peroxide/phosphate-buffered saline for 30 minutes and blocked with SuperBlocking solution (Pierce). Slides were first incubated using the antibody for GNAI2 (Abnova) at 4°C overnight with optimal dilution, labeled by EnVision HRP (mouse) kits at room temperature for 30 minutes, incubated with DAB substrate liquid (DAKO, Glostrup, Denmark), and counterstained by Mayer’s hematoxylin (DAKO). All the sections were observed and photographed with an Axioskop 2 microscope (Carl Zeiss, Oberkochen, Germany). Scoring was conducted according to the ratio and intensity of positive-staining cells: <5% scored 0; 5-24% scored 1; 25-49% scored 2; 50-74% scored 3; more than 74% scored 4. These scores were determined independently by two senior pathologists.

**Statistical Analysis.** The results were presented as the mean ± standard error of the mean (SEM). The data were subjected to two-tailed Student t test (P < 0.05) unless otherwise specified (χ² test, linear regression).

**Results**

**miR-30d Is Frequently Up-regulated in HCC and Associated with Tumor Invasion and Metastasis.** In an attempt to explore the expression and significance of miR-30d in hepatic carcinogenesis, we first determined the expression of miR-30d in 50 cases of HCC and 10 normal liver tissues using quantitative reverse-transcription PCR. The expression of miR-30d is significantly up-regulated in HCC tissues when compared with normal liver tissues (Fig. 1A). Furthermore, the relationship between the expression of miR-30d and the metastatic status of HCC patients was analyzed, which showed that miR-30d expression is remarkably higher in HCC patients with tumor metastasis (n = 23) than in those without (n = 23) (Fig. 1B). These results suggested that up-regulation of miR-30d is closely related to the increase of HCC metastasis, and may play an important role in the pathological process. In addition, the expression of mature miR-30d in various HCC cell lines was determined (Supporting Fig. S1).

**miR-30d Promotes HCC Cell Invasion and Metastasis In Vitro and In Vivo.** To better understand the biological functions of miR-30d in the development of HCC, we first constructed a lentivirus vector expressing miR-30d and established three stable cell lines, which were named SK-HEP-1-30d, Huh-7-30d, and SMMC-7721-30d, after lentivirus transduction (Supporting Fig. 2A). In cell proliferation and colony formation assays, ectopic expression of miR-30d showed no obvious impacts on in vitro HCC cell proliferation (Supporting Fig. 3A,B). Given that expression of miR-30d is highly associated with the metastatic property of HCC, we won-
dered whether miR-30d could play an important part in HCC cell invasion and metastasis. Transwell assays without Matrigel demonstrated that miR-30d could significantly promote migration of Huh-7 and SMMC-7721 cells when compared with vector groups (Fig. 2A). Transwell assays with Matrigel showed that the invasive capacities were dramatically enhanced in these two stable cell lines when compared with the control cells (Fig. 2B). However, the migration and invasiveness of HCCLM3 cells decreased when endogenous miR-30d was silenced.

Fig. 1. miR-30d is frequently up-regulated in HCC and is associated with tumor invasion and metastasis. (A) The expression level of mature miR-30d in HCC or normal liver tissues using quantitative PCR. Box-plot lines represent medians and interquartile ranges of the normalized threshold values; whiskers and spots indicate 10-90 percentiles and the remaining data points. (B) The relative expression of mature miR-30d in 23 HCC tissue samples with and 23 HCC tissue samples without metastasis. The expression level of mature miR-30d is normalized by U6 small nuclear RNA.

Fig. 2. miR-30d promotes HCC cell invasion and metastasis in vitro and in vivo. (A) Transwell migration assays of Huh-7 and SMMC-7721 cells expressing miR-30d or mock control. Representative images are shown on the left, and the quantification of five randomly selected fields is shown on the right. The values shown are expressed as the mean ± SEM; asterisks indicate significance. (B) Transwell Matrigel invasion assay of Huh-7 and SMMC-7721 cells expressing miR-30d or mock control. (C) Transwell migration and invasion assays of HCCLM3 cells transfected with miR-30d inhibitor or negative control. (D) Hematoxylin-eosin-stained sections of orthotopic primary liver tumors, intrahepatic metastatic nodules, and distal metastatic nodules in the lung formed by SMMC-7721-LGS-30d or mock, at the sixth week after intraperitoneal transplantation. White arrows indicate the orthotopic primary liver tumor; black arrows indicate the metastatic foci in the liver or in the lung. Magnification: a, b, e, and f, ×50; c, d, g, and h, ×100. The numbers of metastatic nodules in the livers or in the lungs of each mouse are counted; the statistical significance is labeled using the $\chi^2$ test.
with a miRNA inhibitor (Fig. 2C). These results indicate that miR-30d can significantly enhance HCC cell migration and invasion in vitro. To further explore the role of miR-30d in tumor invasion and metastasis in vivo, SMMC-7721 cells stably expressing miR-30d were transplanted into the livers of nude mice. The HCC cell line SMMC-7721 has relatively strong in vitro invasive property, and has been employed in in vivo metastasis assays in nude mice, including subcutaneous transplantation for lung metastasis, in vivo injection under spleen capsule for liver metastasis, and orthotopic liver implantation for intrahepatic metastasis.

Interestingly, the number and size of the metastatic nodules in the liver and lung were dramatically increased in miR-30d groups when compared with vector controls after in situ growth for 6 weeks (Fig. 2D). Taken together, these observations suggest that miR-30d is a positive metastatic regulator for HCC.

**miR-30d Posttranscriptionally Down-regulates GNAI2 Expression by Directly Targeting Its 3’-UTR.**

In order to explore the underlying molecular mechanism through which miR-30d initiates HCC cell invasion and metastasis, we first established several SMMC-7721 stable cell lines that can overexpress miR-30d (Supporting Fig. 2B). The gene expression profile in two of the selected stable clones was then analyzed by way of mRNA microarray. The results showed that the expression of 69 genes is suppressed by miR-30d (Supporting Fig. 4A,B). In addition, potential targets were analyzed using four prediction algorithms PicTar, TargetScan, and miRanda (microrna.org and miRBase). Forty-three candidate genes were commonly predicted to be the possible targets of
miR-30d by all of the four algorithms (Fig. 3A). Interestingly, only GNAI2 was shown to be down-regulated by miR-30d out of these 43 predicted candidate genes. Western blot analysis further demonstrated that the high expression of miR-30d dramatically suppressed the endogenous protein level of GNAI2, whereas the silencing of miR-30d could significantly increase the expression of GNAI2 (Fig. 3B). Next, analysis of the 3′-UTR sequence of GNAI2 using PicTar and TargetScan revealed two possible binding sites for miR-30d, both of which are highly conserved in human, chimpanzee, mouse, rat, and dog (Fig. 3C). In an effort to determine whether GNAI2 is regulated by miR-30d through direct binding to its 3′-UTR, a series of 3′-UTR fragments—including full-length 3′-UTR, binding site 1 (wild-type and mutant), and binding site 2 (wild-type and mutant) (Fig. 3D)—were constructed and inserted into the region immediately downstream of the luciferase reporter gene. For luciferase activity assays, miR-30d was cotransfected with different luciferase–3′-UTR constructs into HEK 293T cells. The relative luciferase activity was remarkably reduced, by almost 80%, by miR-30d when the full-length wild-type 3′-UTR of GNAI2 was present (Fig. 3E). This reduction was sequence-specific, because the relative lu-

Fig. 4. GNAI2 is often down-regulated in HCC at the mRNA and protein levels. (A) Relative expression levels of GNAI2 in HCC tissues or matched noncancerous tissues using quantitative reverse-transcription PCR assays. (B) Correlation between expression level of GNAI2 and that of mature miR-30d in 50 HCC tissues. Linear regression coefficient and statistical significance is indicated. (C) Immunohistochemical staining of GNAI2 in HCC, matched noncancerous tissues (b, d), or normal liver tissues (e, f). The staining intensities are scored and represented as follows: a, score 0; b, score 3; c, score 2; d, score 4; e, score 3; f, score 4. (D) Statistical analysis of groups of cases according to the scoring (χ² test).
ciferase activity did not drop as sharply in UTRs that contained mutant binding sites as in those that contained wild-type binding sites. In addition, our results indicate that both of the binding sites could be involved in miR-30d regulation, as mutation of the two sites separately could restore luciferase activity when this construct was cotransfected with miR-30d (Fig. 3E).

GNAI2 Is Often Down-regulated in HCC and Inhibits HCC Cell Migration and Invasion Previous reports indicated that GNAI2, as a member of the G alpha protein family, plays an important part in cell differentiation and proliferation.23-26 Activation of GNAI2 could suppress lymph cell invasion.27 However, the biopathological significance of GNAI2 in human malignancies, especially HCC, is still unknown. To better understand the potential role of GNAI2 in miR-30d–mediated tumor invasion and metastasis, the mRNA level of GNAI2 in 50 HCC patient samples and corresponding noncancerous liver tissues was measured. The level of GNAI2 mRNA is significantly down-regulated in HCC tissues when compared with those in adjacent noncancerous liver tissues (Fig. 4A). This down-regulation was strongly correlated with the up-regulated expression of mature miR-30d in 50 cases of HCC tissues (Fig. 4B). Analysis of GNAI2 protein expression in HCC and normal liver tissues using immunohistochemical staining revealed strong staining of GNAI2 in normal liver tissue as well as in adjacent noncancerous liver tissue (Fig. 4C). By contrast, GNAI2 expression was very low in more than half (56%) of the HCC tissues. Only six out of 50 HCC cases had relatively high GNAI2 expression (Fig. 4D). Taken together, these clinical observations suggest that GNAI2 expression is reduced at both the mRNA and protein levels in HCC tissues and that this reduction is closely related to the up-regulation of miR-30d expression. In addition, the expression of GNAI2 in various HCC cell lines was determined (Supporting Fig. 5).

![Fig. 5. GNAI2 can significantly inhibit HCC cell migration and invasion. (A) Western blotting of GNAI2 protein levels in Huh-7 cells infected with lenti-GNAI2 or lenti-mock control. (B) Western blotting of GNAI2 expression in SK-HEP-1 cells transfected with GNAI2-siRNAs or negative control-siRNA. (C) Transwell migration and invasion assays of Huh-7 cells stably expressing GNAI2 or mock control were performed to investigate the effects of GNAI2 on HCC cell migration and invasion. Representative images are shown, along with the quantification of five randomly selected fields. The values shown are expressed as the mean ± SEM; asterisks indicate significance. (D) Transwell migration and invasion assays of SK-HEP-1 cells transfected with GNAI2-siRNA or negative control small interfering RNA. Transwell assays were performed in triplicate, and representative images are displayed.](image-url)
In order to explore the biological functions of GNAI2 in HCC cells, Huh-7 cells were infected with a lentivirus construct containing the complete ORF of the GNAI2 gene or vector alone (Fig. 5A). Remarkably, overexpression of GNAI2 can strongly inhibit HCC cell migration and invasiveness (Fig. 5C). In addition, knockdown of endogenous GNAI2 expression by siRNA in SK-HEP-1 cells (Fig. 5B) resulted in a dramatic increase in HCC cell migration and invasiveness (Fig. 5D). This phenotype was similar to the one that was induced by the overexpression of miR-30d.

**Restoration of GNAI2 Inhibits miR-30d–Mediated HCC Cell Migration and Invasion.** Because GNAI2 is frequently down-regulated in HCC and inhibits HCC cell migration and invasion, and because miR-30d can posttranscriptionally regulate the expression of GNAI2 at the mRNA and protein levels by directly binding to its 3′-UTR, we hypothesized that down-regulation of GNAI2 directly mediates miR-30d–initiated liver tumor invasion and metastasis. To further address this critical issue, we reintroduced GNAI2 without its 3′-UTR, which resulted in the constitutive expression of GNAI2 without the potential for miR-30d–mediated inhibition, and infected the Huh-7-30d stable cell lines with this construct. The restoration of GNAI2 in this stable cell line was confirmed through western blot analysis (Fig. 6A). Importantly, transwell assays indicated that the restoration of GNAI2 significantly reduced the HCC cell migration and invasiveness that can be initiated by miR-30d (Fig. 6B,C), suggesting that GNAI2 is a direct and functional target for miR-30d.

**Discussion**

In the present study, we demonstrated that miR-30d is commonly up-regulated in HCC and can promote HCC cell migration, invasion, and metastasis both in vitro and in vivo. A direct and functional target of miR-30d was also identified. The target gene, GNAI2, is frequently repressed and functions as a metastasis suppressor in HCC. Taken together, these findings indicate that miR-30d plays a fundamental role in hepatic carcinogenesis, especially in the process of HCC metastasis.
Invasion and metastasis, two of the most important hallmarks of cancer, are the leading lethal factors for malignant cancer, especially for HCC. The long-term survival of HCC patients after curative resection is still confronted by the major obstacle of a high recurrence rate, which is mainly due to the spread of intrahepatic metastases. Therefore, the identification of metastatic factors and an understanding of the underlying molecular pathways that are involved in the progression of metastasis become critical issues. Recent studies have shown that miRNAs play a fundamental role in the invasion and metastasis of HCC, thereby opening a novel avenue to investigate the molecular mechanism of HCC progression and to develop potential therapeutics against HCC. Meng et al. indicated that aberrant expression of miR-21 could not only contribute to HCC growth, but also mediate cell migration and invasion through the PTEN-dependent pathway. Tsai et al. suggested that miR-122 could affect HCC intrahepatic metastasis partially via the regulation of ADAM17. In this study, miR-30d, a new metastatic regulator for HCC, was shown to dramatically promote HCC cell invasion and metastasis both in vitro and in vivo. Noticeably, miR-30d is localized to chromosomal region 8q24, which is often gained or amplified in HCC, and is related to the metastatic properties of HCC, suggesting that the up-regulation of miR-30d in HCC tissues might be due to chromosomal gain of this locus, which needs further exploration in the next. Additionally, another member of miR-30 family miR-30b, which is located approximately 4 kb upstream of miR-30d locus, has no significant change in HCC tissues compared with the noncancerous liver tissues.

To date, five members of the hsa-miR-30 family have been identified in humans: hsa-miR-30a, b, c, d, and e. Recent findings have shown that miR-30 is involved in various biological and pathological processes. For example, miR-30a is required for biliary morphogenesis, whereas miR-30c is involved in polycythemia vera, the drug resistance of cancer cells, and myocardial matrix remodeling. Wu et al. reported that a reduced expression of miR-30e contributes to the up-regulation of Ubc9 in cancer cells. Tang et al. reported that miR-30d might increase glucose-induced insulin gene transcription but not insulin secretion. In addition, the deregulation of miR-30d in chronic lymphocytic leukemia cells and anaplastic thyroid carcinoma has been observed. However, little is known about the role and underlying molecular mechanism of miR-30d in human cancer, especially in HCC. In this report, we have, for the first time, established the role of miR-30d in tumor invasion and metastasis. Moreover, we identified GNAI2, which is an inhibitory G protein, as a direct and functional target of miR-30d. Although the expression of miR-30d is often up-regulated in HCC, it is significantly down-regulated in chronic lymphocytic leukemia and anaplastic thyroid carcinoma, which seems to conflict with our observations in HCC. We postulate that this might be a consequence of different cancer contexts. However, the exact function and underlying mechanism of miR-30d in chronic lymphocytic leukemia and anaplastic thyroid carcinoma need to be addressed in future studies.

G proteins are composed of α, β, and γ subunits. The α subunit primarily defines the function of G proteins. GNAI2 is the second member of the inhibitory G alpha family, which mediates a wide variety of receptor-coupled signal transmissions. To date, GNAI2 has been well-established as playing critical parts in growth, cell movement, differentiation, and development. Defects in GNAI2 are involved in a wide variety of human diseases, including hypertension, insulin resistance, ulcerative colitis, and cancer. We report for the first time that GNAI2 is often down-regulated in HCC. Moreover, the enhanced expression of GNAI2 could inhibit HCC cell migration and invasion. Previous findings have indicated that GNAI2 is usually regulated by mutation at the genomic level or by transcription at the mRNA level. In this report, we demonstrated that miR-30d can bind to two sites of the 3′-UTR of GNAI2 and dramatically decrease the level of GNAI2 protein expression, which provides the first line of evidence in support of a mechanism for GNAI2 regulation at the posttranscriptional level. It is noteworthy that all miR-30 (a-e) have predicted binding sites within the 3′-UTR of GNAI2 according to the three online prediction programs TargetScan, PicTar, and Microcosm Targets (Supporting Fig. 6A). In addition, miR-30d can significantly inhibit GNAI2 expression (Fig. 3B), and miR-30e can dramatically decrease the protein level of GNAI2, whereas miR-30a-c have no obvious effects on GNAI2 expression (Supporting Fig. 6B). Importantly, the previous microarray assays indicated that the expression of miR-30e is not up-regulated in HCC tissues. These findings suggest that the reduced levels of GNAI2 in HCC are mostly, if not completely, due to the increased abundance of miR-30d in HCC.

In conclusion, we demonstrated that miR-30d can significantly promote HCC cell invasion and metastasis and that up-regulation of GNAI2, which is a bona fide functional target of miR-30d, can conversely inhibit HCC cell migration and invasion. The newly identified miR-30d/GNAI2 axis provides a new insight into the pathogenesis of HCC, especially with respect to invasion and metastasis, and represents a new, potential therapeutic target for the treatment of HCC.
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