MiR-122/Cyclin G1 Interaction Modulates p53 Activity and Affects Doxorubicin Sensitivity of Human Hepatocarcinoma Cells

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Abstract

The identification of target genes is a key step for assessing the role of aberrantly expressed microRNAs (miRNA) in human cancer and for the further development of miRNA-based gene therapy. MiR-122 is a liver-specific miRNA accounting for 70% of the total miRNA population. Its down-regulation is a common feature of both human and mouse hepatocellular carcinoma (HCC). We have previously shown that miR-122 can regulate the expression of cyclin G1, whose high levels have been reported in several human cancers. We evaluated the role of miR-122 and cyclin G1 expression in hepatocarcinogenesis and in response to treatment with doxorubicin and their relevance on survival and time to recurrence (TTR) of HCC patients. We proved that, by modulating cyclin G1, miR-122 influences p53 protein stability and transcriptional activity and reduces invasion capability of HCC-derived cell lines. In addition, in a therapeutic perspective, we assayed the effects of a restored miR-122 expression in triggering doxorubicin-induced apoptosis and we proved that miR-122, as well as cyclin G1 silencing, increases sensitivity to doxorubicin challenge. In patients resected for HCC, lower miR-122 levels were associated with a shorter TTR, whereas higher cyclin G1 expression was related to a lower survival, suggesting that miR-122 might represent an effective molecular target for HCC. Our findings establish a basis toward the development of combined chemotherapeutic and miRNA-based therapy for HCC treatment. [Cancer Res 2009;69(14):5761–7]

Introduction

Hepatocellular carcinoma (HCC) accounts for 80% to 90% of primary liver cancers, and it is the third cause of death from cancer worldwide. Nowadays, the curative treatments for HCC detected at an early stage include surgical resection, liver transplantation, and percutaneous ablation (1). Although the diffusion of surveillance programs of cirrhotic patients has led to an increased detection of HCC at an early stage, most patients are diagnosed at intermediate-advanced stages, when the only proven options available to improve survival are transarterial chemoembolization (TACE) and molecularly targeted therapy with the multikinase inhibitor, Sorafenib. In particular, TACE is an effective treatment increasing the survival rate of selected patients with intermediate stage HCC, and to improve its efficacy, new therapeutic approaches with enhanced antitumoral effect and reduced toxicity, such as doxorubicin-drug eluting beads and radiating agents, have been investigated. Doxorubicin is the most used traditional chemotherapeutic drug for HCC, either by systemic delivery or by means of TACE. In addition, surgical resection and percutaneous ablation display a recurrence rate approaching 70% at 5 years (1), opening the path to secondary prevention. Despite recent advancements, new more effective therapeutic approaches are urgently needed. Our improved understanding of the molecular basis of cancer has identified targets for molecular-based therapy. In this context, the discovery of the central role of microRNAs (miRNA) in human tumorigenesis has opened a new field that may be relevant not only for understanding cancer at the molecular level but also for the development of new diagnostic and therapeutic tools.

miRNAs are a class of endogenous phylogenetically conserved small RNAs (~22 nucleotides) responsible for the posttranscriptional regulation of mRNA translation and stability. miRNAs are involved in several biological processes, such as development, apoptosis, proliferation, and differentiation. Aberrant expression of numerous miRNAs has been associated with cancer development (2), and deregulated miRNAs have been linked to molecular pathways involved in neoplastic transformation (3).

Altered expression of miRNAs and the identification of their molecular target genes in HCC have previously been described by ours and other groups (4–6). Therefore, taking advantage of promising in vivo studies on miRNAs (7, 8), this class of molecules may represent a new kind of unconventional targeted treatment to be eventually associated with traditional approaches for HCC not amenable of curative therapies.

We previously reported that cyclin G1 is a gene target of the liver-specific miRNA miR-122. MiR-122 is down-regulated in ~70% of HCCs (4, 9), and an inverse correlation links miR-122 to cyclin G1 in HCC tissues and HCC-derived cell lines. Cyclin G1 was initially discovered as a novel member of cyclin family with homology to c-src, and successively, it was identified as a transcriptional target of p53 tumor suppressor gene (10). Cyclin G1 is the only known cyclin that is transcriptionally activated by p53 tumor suppressor gene, bearing two functional binding sites for this transcription factor (11). Moreover, cyclin G1 exerts a negative feedback regulation on p53 tumor suppressor protein by recruiting the B’ subunit of phosphatase 2A (PP2A) to dephosphorylate Mdm-2 (12).

The precise role of cyclin G1 on cellular growth control is still controversial. Some reports, based on the observation that cyclin
G1 overexpression enhances cell growth of cancer cells (13) and its silencing suppresses cell proliferation (14), indicate a promoting growth activity for cyclin G1. In line with these data, cyclin G1 overexpression has been found in human tumors, such as osteosarcoma, breast cancer, and leiomyoma (15–17). Conversely, the induction of cyclin G1 after DNA damage and its role in G2−M arrest suggest a possible growth inhibitory activity (18, 19). A recent study indicated that the growth inhibitory activity of cyclin G1 is dependent on the magnitude of its expression (20).

The aim of this study was to investigate the contribution of reduced miR-122 expression to hepatocarcinogenesis and the response to doxorubicin treatment. To this end, we proved that miR-122 enforced expression, as well as cyclin G1 silencing, leads to increased p53 protein stability and transcriptional activity and reduced invasion capability of HCC-derived cell lines. Finally, in a therapeutic perspective, we assayed the effects of a restored miR-122 expression in triggering apoptosis after doxorubicin challenge.

Materials and Methods

Patients. HCC and cirrhotic tissues were obtained from 57 consecutive patients (43 males and 14 females, median age 68 y, range 49–82 y) undergoing liver resection for HCC at University of Bologna Department of Surgery. Tissue samples were collected at surgery and stored as previously described (4). Informed consent was obtained from each patient. Histopathologic grading was scored according to Edmondson and Steiner’s criteria (21). Exclusion criteria were a previous history of local or systemic treatments for HCC and the presence of noncirrhotic tissues surrounding the HCC nodule/s. The characteristics of HCC patients included in this study are described in Supplementary Table S1.

Cell culture. HepG2 (ATCC no. HB-8065) cell line was cultured with MEM (Eagle), whereas Huh-7 cell line was cultured with RPMI 1640; both media were supplemented with 10% fetal bovine serum and antibiotics.

Cell transfection with RNA oligonucleotides. HCC-derived cell lines were transfected with 100 nmol/L of miR-122, Negative Control #1 precursor and inhibitor miRNAs (Ambion), anti–miR-122 (Dharmacon) or 40 nmol/L of cyclin G1 small interfering RNAs (siRNA, G 1/238 (5’TGGCCTCAGAATGACTGACTA−3’) and G1/832 (5’GCAAGAAGCTTTGT)
MiR-122 Sensibilizes Cells to Doxorubicin

MiR-122 regulates p53 protein expression through cyclin G1 in HCC-derived cells. Cyclin G1, a target of miR-122, negatively regulates p53 protein stability by acting on PP2A. Therefore, we decided to investigate the modulation of p53 protein after overexpression of miR-122 in HCC-derived cells. HepG2 cells were chosen for enforcing the expression of miR-122, because this cell line exhibits undetectable levels of constitutive miR-122 in the presence of wild-type p53 protein and high levels of cyclin G1 protein (Fig. 1A and B). At 48 h after transfection, miR-122 expression was assayed by real-time RT-PCR and compared with that of normal liver, cirrhotic, and HCC tissues (Fig. 1A).

Conversely, miR-122 silencing was performed in Huh-7 cells because this is the only HCC-derived cell line that exhibits miR-122 constitutive expression (23) associated with low cyclin G1 levels (Fig. 1A and B). Besides, Huh-7 cells harbor a mutant isoform of p53 protein, as it occurs in ~30% of HCCs (24). A reduction of miR-122 levels was observed in Huh-7 cells after anti–miR-122 transfection (Fig. 1A).

By WB analysis, miR-122–overexpressing HepG2 cells showed a decreased cyclin G1 expression, accompanied by an increase of p53 protein (Fig. 2A). Conversely, anti–miR-122 transfected Huh-7 cells showed an increase in cyclin G1 expression (Fig. 2B). p53 protein levels were not analyzed because Huh-7 cells harbor a p53 mutant isoform with nuclear accumulation.

To verify that cyclin G1 was responsible for p53 modulation after miR-122 transfection, cyclin G1 knockdown in HepG2 cells was obtained with two siRNAs (G1/238 and G1/832). Figure 2C shows a decrease in cyclin G1 mRNA after siRNAs transfection. Silencing of cyclin G1 by siRNAs ruled out an off-target effect. G1/832 siRNA was chosen because it proved to be the most effective. Immunoblotting analysis of cyclin G1–silenced HepG2 cells revealed a decrease of
cyclin G1 levels and an increase of p53 corresponding band (Fig. 2D). As a further confirmation, an increase of p53 protein was observed in HepG2 cells stably knocked down for cyclin G1 (Supplementary Fig. S1C). Taken together, these data suggested that miR-122 indirectly affects p53 expression through the modulation of cyclin G1.

**MiR-122 overexpression and cyclin G1 silencing increase p53 transcriptional activity.** Because the phosphorylation of p53 at Ser-20 modulates the interaction with Mdm-2 protein, increasing p53 half-life and its transcriptional activity (25), we assessed the effect of miR-122 and cyclin G1 on phosphorylated p53. WB analysis showed an increase of phosphorylated Ser levels in either miR-122–overexpressing or cyclin G1–silenced HepG2 cells (Fig. 3A).

To assess the role of miR-122 or cyclin G1 on transcriptional activity of p53, we measured the luciferase activity of a reporter vector containing a p53-responsive element (pp53- TA-luc) after transfection of miR-122 or cyclin G1 siRNAs in HepG2 cells. An increase of luciferase activity was observed in both miR-122–overexpressing and cyclin G1–silenced cells (Fig. 3B).

To confirm the increase of p53 transcriptional activity in miR-122–overexpressing and cyclin G1–silenced cells, we assayed cell distribution through the different phases of the cell cycle by FC analysis. We observed an increase in the G1-phase population and a corresponding decrease in the G2-M phase in both miR-122–overexpressing and cyclin G1–silenced cells (Fig. 4B). The decrease of cells in the G2-M phase was previously reported as a response to cyclin G1 knockdown (19).

To further define the effects of miR-122 and cyclin G1 in HCC-derived cells, the invasion capability was assayed after enforced miR-122 expression or cyclin G1 knockdown. Matrigel assay revealed a decreased number of invading cells in both conditions (Fig. 4C), suggesting a role of miR-122 both in cell cycle modulation and invasion. After miR-122 overexpression or cyclin G1 knockdown, increased E-cadherin (26) expression without any mRNA modulation was observed. Conversely, the analysis of matrix metalloproteinases (MMP) by zymography did not reveal any change in MMP-2 or MMP-9 activities in transfected cells (data not shown).

Short hairpin RNAs (shRNA) stable knockdown of cyclin G1 performed in HepG2 cells confirmed the findings obtained with transient transfection (Supplementary Fig. S1).
MiR-122 overexpression and cyclin G1 silencing increase doxorubicin sensitivity of HCC-derived cell lines. After restoration of miR-122 expression or cyclin G1 silencing in HepG2 cells, we did not observe any increase in cell death. However, because cyclin G1 is activated in response to DNA damage (19, 27), we assayed whether miR-122 overexpression or cyclin G1 silencing could affect cell sensitivity to doxorubicin. Doxorubicin is the most used drug for intermediate-advanced HCCs. Because doxorubicin induces apoptosis through both a p53-dependent and p53-independent manner (28), we assayed both HepG2 and Huh-7 cells, which differ in p53 status and miR-122 expression.

HepG2 cells were transfected with miR-122 or cyclin G1 siRNA and then treated with doxorubicin. At 24 h after treatment, FC analysis for Annexin V revealed an increase of apoptotic cells in miR-122–overexpressing and cyclin G1–silenced cells (Fig. 5A). As a further proof, an increase of the cleaved caspase-3 in miR-122 transfectedd cells and cyclin G1 knockdown cells was observed compared with doxorubicin only–treated HepG2 cells. Moreover, the p53 transcriptional target Puma increased in miR-122 and cyclin G1 siRNA-treated cells (Fig. 5B).

Because Huh-7 cells display detectable levels of miR-122, this cells were used for investigating the effect of anti-miR-122 on doxorubicin challenge. Doxorubicin treatment in miR-122–silenced Huh-7 cells determined a decrease of apoptotic cells. Whereas, similarly to what was observed in HepG2 cells, cyclin G1 silencing led to an increased susceptibility of Huh-7 cells to doxorubicin challenge (Fig. 5C). These data showed that miR-122 increases sensitivity of HCC cells to doxorubicin through both p53-dependent and p53-independent apoptotic pathways.

**Correlation of miR-122 and cyclin G1 with clinicopathologic variables.** We analyzed the expression of miR-122 by real-time RT-PCR in 57 surgically resected HCCs and matched nontumorous liver tissues (liver cirrhosis, LC). Due to tissue availability, the expression of cyclin G1 was investigated in 35 of 57 HCCs and matched LC by both WB and immunohistochemistry, with the latter technique adopted in few cases to confirm the cellular localization of cyclin G1. Low miR-122 expression was associated with a shorter TTR (Fig. 6A), whereas it did not achieve a statistical significance for the overall survival (Fig. 6B). Conversely, low levels of cyclin G1 turned out to be associated with a higher survival rate, but not with TTR (Fig. 6C and D). No correlations were found between miR-122 or cyclin G1 expression and α-fetoprotein (AFP), size, focality, etiology, and histopathologic grading of HCCs.

**Discussion**

MiR-122 is a liver-specific miRNA representing ~70% of the liver miRNA population, and it has been characterized for its multiple roles in liver physiology, metabolism (7, 29), and modulation of hepatitis C virus (HCV) replication (23). Notably, its loss or down-regulation has been associated with human and rodent HCC development and progression (4, 9). In this study, we investigated a mechanism through which the down-regulation of miR-122 might contribute to hepatocarcinogenesis and doxorubicin challenge. Trying to uncover the pro-oncogenic events after the reduction of miR-122 physiologic levels, we showed that both miR-122 overexpression or siRNA-mediated silencing of its target cyclin G1 determined an increase of p53 transcriptional activity and stability, indicating that miR-122 could modulate p53 through the inhibition of cyclin G1.

The oncogenic role of cyclin G1 is well documented, and its overexpression has been reported in different human tumors (14–16, 30). Moreover, it has been previously shown that cyclin G1–null mice treated with a potent hepatocarcinogen, N-diethylnitrosamine, display a lower tumor incidence, likely due to an increased p53 activity (31). Here, we observed that shRNA-mediated silencing of cyclin G1 modifies the cell phenotype, resulting in morphologic changes, inhibition of cell growth, decreased cell invasion capability, and reduced AFP secretion (Supplementary Fig. S1F).

Interestingly, the extent of p53 increase after miR-122 restored expression was slightly greater than that caused by direct cyclin G1 silencing. This suggests that miR-122 transfection affects the expression of more than one molecular target belonging to the p53-dependent pathway. Indeed, a bioinformatic analysis (by MiRanda algorithm) indicated the regulatory subunit β′, β-isofom (PPP2R5B), of PP2A as a hypothetical target of miR-122. It is known that cyclin G1 associates with the β′-subunit of PP2A, promoting the dephosphorylation of p53 repressor protein Mdm-2.
(12, 32); therefore, a direct regulation of PP2A regulatory subunit by miR-122 might increase cyclin G1–mediated effect. Supplementary Fig. S2 showed the increase of p53 levels after both siRNA-mediated PPP2R5B silencing or miR-122–enhanced expression. These findings confirm that both miR-122–mediated inhibition of cyclin G1 and PPP2R5B silencing contribute to the modulation of p53 expression. Further studies are warranted to elucidate molecular mechanisms through which miR-122 might regulate PPP2R5B expression. Moreover, PDGF1A and Met are both putative targets of miR-122, as predicted by MiRANDA algorithm, suggesting that miR-122 might affect multiple invasion pathways.

The analysis of primary HCC samples did not show any relationship between miR-122 or cyclin G1 expression and size, focality, grading, etiology (Supplementary Fig. S3), and AFP serum levels of HCC patients. Indeed previous studies reported heterogeneous miR-122 expression patterns in different HCC series. Beside reports showing a down-regulation of miR-122 in HCC tissue regardless of chronic liver disease etiology (9, 33, 34), other studies reported an up-regulation of miR-122 in HCV-related HCC (35) or an up-regulation of miR-122 restricted to HBV-related HCCs and a down-regulation of miR-122 in metastatic HCCs (36). This variable miR-122 behavior is not unexpected due to the well-known molecular heterogeneity of HCC in relation with different liver carcinogens, viruses, and genetic background.

Interestingly, TTR was shorter in patients with lower miR-122 expression, whereas lower cyclin G1 levels turned out to be associated with a higher survival rate, suggesting that miR-122 and its target gene cyclin G1 might influence the clinical course of HCC.

The multiple key cancer-associated functions affected by the down-regulation of miR-122 in liver cancer cells may also suggest a potential use of miR-122 in miRNA-based anti-HCC gene therapy. To start shedding light on the possible advantages offered by miR-122 combination treatments, we observed that after doxorubicin challenge, enforced miR-122 expression or cyclin G1 silencing increased doxorubicin sensitivity through enhanced apoptotic cell death. Human HCC covers a heterogeneous group displaying low miR-122 expression levels and p53 mutations in a considerable percentage of cases. Therefore, miR-122 silencing was performed in Huh-7 cells, constitutionally expressing miR-122 and harboring a mutant p53 isoform. As observed in HepG2 cells, a protection against doxorubicin-induced cell death was reproduced in this different molecular setting, thus confirming that miR-122 sensitizes cells to doxorubicin-induced apoptosis either through a p53-dependent and p53-independent mechanism.

Figure 6. Recurrence rate and overall survival of HCC patients. A and B, association between miR-122 levels and TTR or overall survival of surgically resected HCC patients. High and low miR-122 expressions were categorized according to the mean value. C and D, association between cyclin G1 levels and TTR or overall survival of HCC patients. High and low cyclin G1 expressions were categorized according to the mean value. Among the 57 patients tested for miR-122 expression, data on survival and TTR were missing in 12 cases. Among the 35 patients tested for cyclin G1 expression, data on survival and TTR were missing in seven cases. Log-rank P values are from Kaplan-Meier analysis.
Disclosed Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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