Research Article

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Regulation of placenta growth factor by microRNA-125b in hepatocellular cancer

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Background & Aims: microRNAs (miRNAs) are a class of small noncoding RNAs that can regulate gene expression by translation repression or mRNA degradation. Our aim was to evaluate the role of aberrantly expressed miRNAs in hepatocellular cancer (HCC).

Methods: miRNA expression in HCC tissues and cells was evaluated by qPCR array and Taqman miRNA assay. Cell proliferation, motility, invasion, and the angiogenesis index were quantitated using commercial assays. DNA methylation status, matrix metalloproteinases (MMPs) mRNA expression was quantitated by real-time PCR analysis.

Results: miRNA profiling identified a decrease in *miR-125b* expression in HCC tumor tissues and cell lines. The expression of *miR-125b* was significantly increased by the methylation inhibitor 5-aza-2′-deoxycytidine in HCC cells but not in normal controls, suggesting that the expression of *miR-125b* could be epigenetically modulated. Methylation-specific PCR revealed hypermethylation status of *miR-125b* in HCC cells compared to non-malignant controls. Cell proliferation, anchorage-independent growth, cell migration, invasion, and angiogenesis were significantly decreased by the introduction of *miR-125b* precursor in HCC cell lines. Placenta growth factor was identified as a target of *miR-125b* by bioinformatics analysis and experimentally verified using luciferase reporter constructs. Overexpression of *miR-125b* in HCC cells decreased PIGF expression, and altered the angiogenesis index. Furthermore, modulation of *miR-125b* also distorted expression of MMP-2 and -9, the mediators of enzymatic degradation of the extracellular matrix.

Conclusions: Our studies showing epigenetic silencing of *miR-125b* contributes to an invasive phenotype provide novel mechanistic insights and identify a potential target mechanism that could be manipulated for therapeutic benefit in HCC.

Keywords: microRNA; Liver cancer; PIGF; Invasion; Angiogenesis; Methylation.
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Abbreviations: 5-aza-CdR, 5-aza-2′deoxycytidine; PIGF, placenta growth factor; HCC, hepatocellular cancer; miRNA, microRNA; MMP, matrix metalloproteinase.

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy arising within the liver. Worldwide, HCC is the fifth most common primary malignancy. HCC is the leading cause of death in patients with cirrhosis in Europe and the United States. Although the incidence of HCC in the United States has been low, both the incidence and the mortality from HCC have been increasing. Clinical outcomes for HCC reflect the potential for invasion, which results in intrahepatic spread and a high rate of recurrence following surgical resection. Thus, an understanding of the molecular mechanisms involved in tumor cell invasion and spread is important, and may lead to more effective therapeutic approaches for HCC.

microRNAs (miRNAs) are a group of non-coding RNA that are being increasingly recognized as important players in human cancers [3]. Aberrant expression of specific microRNAs, including *miR-21, miR-122, miR-221, miR-21, miR-122, miR-221, miR-222*, and *miR-125b*, has been discovered in human HCC cells [22,27]. Aberrant expression of miRNAs such as *miR-21* and *miR-122* alters cellular expression of PTEN and cyclin G1 [10,22]. However, the contribution of the majority of aberrantly expressed miRNAs in tumor cell behavior in HCC is unknown.

The regulation of the tumor suppressor p53 by *miR-125b* suggests a potential role for *miR-125b* in tumor cell behavior [19]. E2F3, a transcription factor involved in cell cycle progression, has been also identified as a target of *miR-125b* [14]. Although de-repression of E2F3 promotes neoplastic growth in tumors in which *miR-125b* is reduced, such as bladder and breast cancer, the silencing of *miR-125b* in human HCC indicates the presence of additional mechanisms by which *miR-125b* may contribute

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to cancer metastasis [2,20,21]. Decreased expression of *miR-125b* has also been reported in other gastrointestinal and breast cancers [28]. In this study, we investigated the epigenetic regulation of *miR-125b* in hepatocarcinogenesis with respect to the regulation of the specific target gene which is involved in HCC tumor growth, metastasis, and angiogenesis.

Materials and methods

Human liver tissues and cell lines

HCC cell lines HepG2 and PLC/PRF-5 were obtained from ATCC (Manassas, VA) and cultured as recommended by the supplier. HCC tumor and normal control tissues were obtained from BioChain Institute, Inc. (Haywood, CA) and used for microRNA real-time PCR array analysis (Supplementary Table 1). Additional 19 paired HCC patients and adjacent non-tumoral liver tissues were obtained from the Bank of Tumor Resources, Cancer Center, Sun Yat-sen University in Guangzhou, China (Supplementary Table 2).

Luciferase reporter assay

Intact putative *miR-125b* recognition sequence from the 3′-UTR of placental growth factor (PlGF) (pMIR-PlGF-wt-3′-UTR) or with random mutations (pMIR-PlGF-mut-3′-UTR) were cloned downstream of the firefly luciferase reporter gene. Luciferase assays were performed 72 h after transfection using the Dual Luciferase Reporter Assay system (Promega, Madison, WI).

RNA isolation, real-time PCR, and Western blots

Total RNA was extracted from cells and tissues using Trizol (Invitrogen, Carlsbad, CA). The miRNome MicroRNA PCR Array Kit was purchased from SBI (System Biosciences, Mountain View, CA) and used for miRNA profiling. Real-time PCR analysis of mRNA and miRNAs was performed as described [22]. Western blot analysis was performed as described previously [22]; the membranes were blotted with antibodies for PlGF and β-actin (both from Santa Cruz Biotechnology, Santa Cruz, CA).

In vitro proliferation, migration, invasion, and angiogenesis assay

Commercial available kits were used for proliferation, migration, invasion [1], and angiogenesis assay in normal and malignant hepatic cells. Cell migration and invasion index was further normalized with proliferation index under the same conditions to rule out the impact factor of altered cell growth rates.

Statistics

A double-sided Student *t*-test was performed to compare two groups (p <0.05 was considered significant) unless otherwise indicated.

Please see Supplementary data for more detailed information of this section.

Results

miR-125b is aberrantly expressed in HCC tissues and cell lines

Aberrant expression of selected miRNAs has been observed in HCC. To identify miRNAs that are differentially decreased in expression in tumor tissues, we analyzed miRNA expression in three pairs of HCC tumor and normal liver tissues using SBI miRNome MicroRNA Profiling PCR Array. Among 318 of the 379 human miRNAs detected by this PCR array, the expression of 37 miRNAs was significantly altered compared to normal tissues. Of these, the majority of aberrantly expressed miRNAs were increased in expression. However, the expression of two miRNAs, *miR-125b* and *miR-122* was markedly decreased (<4-fold) in tumor tissues compared to normal tissues (Fig. 1). The expression of *miR-125b* was decreased in malignant hepatocytes (HepG2 and PLC/PRF-5) compared to that of normal human hepatocytes (Fig. 2). By the real time-PCR analysis, *miR-125b* expression was decreased by 3-fold or more in all three samples compared with normal liver tissues (Fig. 2D). Furthermore, among additional 19 paired HCC patients and adjacent non-tumor liver tissues, reduced *miR-125b* expression was observed in 16 out of 19 HCC tumors and positively correlated with HCC patients' survival time after surgery (Fig. 2E and F, and Supplementary Table 2; p <0.01). These results show that the aberrant expression of *miR-125b* is a frequent event in human primary HCCs.

Modulation of miR-125b alters cell migration and invasion in HCC cell lines

The ability of cells to migrate into adjacent tissues and invade ECM is a key determinant of tumor progression, spread, and metastases. We began by first verifying the efficacy of transfection and target effects by assessing the expression of mature *miR-125b* by real-time PCR in HepG2 cells (transfected with *miR-125b* precursor) as well as normal human hepatocytes with anti-*miR-125b* inhibitor (Fig. 3A). Next, we assessed vertical cell migration and cell invasion. Pre-*miR-125b* decreased cell migration as well as invasion in HepG2 and PLC/PRF-5 HCC cell lines, as well as in human liver cancer derived endothelial cells (T-LECs) when compared to relative controls (Fig. 3B). These results support a functional role for *miR-125b* in mediating cell migration and invasion in malignant hepatocytes and hepatic tumor endothelial cells, and provide a mechanism by which down-regulation of *miR-125b* may contribute to tumor spread.

Inhibition of miR-125b increases HCC growth in vitro

We then assessed cellular proliferation in HepG2, PLC/PRF-5, and T-LEC cell lines. In cells transfected with Pre-*miR-125b*, there was a reduction in proliferation compared to cells transfected with control Pre-miRNA (Fig. 4A). Moreover, there was a significant change in anchorage-independent growth following the modulation of Pre-miR-125b (Fig. 4B). Overexpression of *miR-125b* in PLC/PRF-5 and T-LECs also significantly increased the fractions of early and late apoptotic cell populations (Fig. 4C). These observations indicate a role for *miR-125b* in growth regulation of malignant human hepatic cells.

Identification of PlGF as a target for miR-125b

The target prediction program miRNA Viewer database (http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl) indicated the presence of a highly conserved binding site for *miR-125b* that is present in the 3′-UTR region of PlGF, an angiogenic and survival cytokine in cancer biology. To demonstrate PlGF expression pattern in human HCC, 24 human HCC and matched noncancerous liver tissues were analyzed by immunohistochemistry (Fig. 5A). The human liver cancer tissue array showed the signal intensity was strong (+++) or positive (++) in 18 out of 24 HCC tissues; whereas the weak (+) or negative (++) signals were seen in only 4 out of 24 samples of HCC tissues. Therefore, the PlGF protein expression was much higher in the HCC tissues as compared with the non-cancerous tissues (p <0.01). We have also checked *miR-*