C9orf100, a new member of the Dbl-family guanine nucleotide exchange factors, promotes cell proliferation and migration in hepatocellular carcinoma

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Received September 26, 2011; Accepted January 10, 2012

DOI: 10.3892/mmr.2012.783

Abstract. Dbl-family guanine nucleotide exchange factors (GEFs) are important activators of Rho GTPases, which are significantly associated with tumorigenesis and metastasis. The catalytic ability of the Dbl-family GEFs to activate Rho GTPases depends on their Dbl-homology domain followed by a pleckstrin-homology domain. In the present study, we showed that C9orf100, a new member of the Dbl-family GEFs with a minimal catalytic unit, may contribute to hepatocellular carcinoma (HCC). Quantitative real-time PCR results demonstrated that C9orf100 was highly and widely upregulated in 42/44 (95.5%, >2-fold) HCC specimens compared with adjacent non-cancerous livers, and this upregulation was correlated with intrahepatic metastasis and α-fetoprotein levels of HCC. Furthermore, the ectopic expression of C9orf100 promoted cell proliferation and colony formation in Huh-7 and YY-8103 cells, whereas silencing of C9orf100 resulted in the suppression of cell growth in MHCC-97H and PLC/PRF/5 cells. Flow cytometry confirmed this effect on MHCC-97H cell growth and indicated that C9orf100 may function in the G2/M phase. In addition, we showed that C9orf100 is involved in the positive regulation of HCC cell migration by a transwell chamber analysis. Our findings suggest that C9orf100 plays a potential oncogenic role in the development and metastasis of HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies worldwide, particularly in Asia and Africa. The main predisposing factors for its development include chronic infection with the hepatitis B and C virus, alcohol abuse and aflatoxin intake (1,2). Cirrhosis induced by a virus or alcohol poses the highest risk, as 80% of HCCs develop in cirrhotic livers (3). The primary treatments of HCC are surgical resection, liver transplantation, percutaneous ablation and conventional chemotherapy; however, prognosis of advanced HCC remains poor (4). Tumor recurrence and metastasis in HCC treatment are still the main problems. As with many other tumors, the development and progression of HCC is a complex multistep process and the molecular mechanisms involved in this process have yet to be fully clarified. Ongoing investigations to identify new and effective molecular targets controlling HCC progression are crucial for improving prognosis and treatment strategies.

Activation of oncogenes and inactivation of tumor suppressor genes are frequently associated with carcinogenesis and progression. Identifying the genes that are differentially expressed in tumor from paratumor tissue is currently a topic of much interest. In recent years, with various transcriptome approaches, numerous upregulated oncogenes or downregulated tumor suppressor genes in HCC have been reported, such as IGF2, FAT10, SCARA5, DLK1 and p53 (5-9).

The Rho family of small GTPases are key regulators of a variety of cellular events: cell cycle progression, cytoskeletal reorganization, membrane trafficking, cell migration and invasion (10,11). Previous studies have suggested that aberrant regulation of Rho family GTPases contributes to carcinogenesis and migration (12). Rho GTPases undergo a cycle between an active GTP-bound and inactive GDP-bound state, which is regulated mainly by the Dbl-family (diffuse B-cell lymphoma family) guanine nucleotide exchange factors
(GEFs). DbI-family GEFs are defined by the presence of a DbI homology domain (DH domain) and this domain is almost invariably followed by a pleckstrin homology domain (PH domain). The DH domain is responsible for catalyzing the exchange of GDP for GTP within Rho GTPases (13), while the PH domain acts as an anchor for targeting membrane (14). Previous studies have suggested that the PH domain is also involved in the protein-protein interactions (15). In the human genome, a total of 69 DbI-family GEFs were found, all of which share a core catalytic unit consisting of the DH domain and an adjacent PH domain (16). In addition to the DH/PH module, most of the DbI-family GEFs contain diverse structural domains such as serine/threonine kinase, RBD, PDZ, RGS, REM, RasGEF, Spectrin, CH, SH2 or SH3 (17). These domains are likely to integrate the upstream and downstream signals associated with GEFs. Several DbI-family GEFs with additional functional domains, such as DbI, Ect2, Vav3, were reported to be involved in tumor development and metastasis (18,19). However, little is known in tumor about the DbI-family GEFs that have no additional functional domains except for the DH and PH domains.

In this study, we identified a new member of the DbI-family GEFs, C9orf100, which only shares a minimal catalytic unit compared with other members, and may contribute to the tumorigenesis and progression of HCC. Quantitative real-time PCR results showed C9orf100 was significantly upregulated in HCC tissues compared with related adjacent non-cancerous tissues, and this upregulation of C9orf100 was correlated, to some extent, with clinicopathological features of HCC. Further cell biological evidence indicated that C9orf100 promoted proliferation and migration of HCC cells in vitro. Thus, we propose that C9orf100 is a potential candidate oncogene in HCC.

Materials and methods

Tissue specimens and cell culture. All 44 specimens were harvested from patients suffering from HCC, who underwent resection of the primary tumor at the department of Wuxi People's Hospital. Informed consent was obtained from all patients and the use of the tissue material for research was approved by the Ethics Committee of the Wuxi People’s Hospital and the Chinese National Human Genome Centre in Shanghai. The histopathological diagnosis was based on the criteria of the World Health Organization. Serum α-fetoprotein (AFP) ≥10 ng/ml by chemiluminescence was diagnosed as positive. The paired paratumor liver samples were from the same patients and 2 cm away from the edge of tumor regions. Nine of the HCC patients had one tumor and 35 had multiple tumors. In addition, 37 patients were diagnosed with liver cirrhosis and 7 were not. HCC cell lines were obtained from the same patients and 2 cm away from the edge of tumor regions.

Materials and methods

RNA interference. To suppress C9orf100 expression in HCC cells, C9orf100-specific small interference RNAs (siRNAs) were chemically synthesized (GenePharma). The siRNA sequences used were: si-693 (sense: 5'-UCCAUCAUUAUGUGGCAGAAACACGAdAdA-3' and antisense: 5'-UCCUGUUCUGACAAUAGUAUGdGdGdA-3'); si-1023 (sense: 5'-UCCCUCAUGAGAAGCUGCUUAdAdTdTdT-3' and antisense: 5'-AUAAGCAGUAGCUUCUAGAGGdGdGdA-3'). The irrelevant nucleotides not targeting any annotated human genes were used as negative control: si-NC (sense: 5'-UCCUGAACGCAGUCAGUdAdTdTdT-3' and antisense: 5'-ACGUGACGUGGAGAGAdTdTdT-3').

Cell transfection. Cell transfection with plasmids or siRNAs was conducted using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Cell proliferation and colony formation assay. For the cell proliferation assay, HCC cells (3-5x10^4 per well) were seeded into 96-well plates in 100 µl culture medium. Cell viability was measured daily at 450 nm using the Cell Counting Kit-8 (Dojindo Laboratories), according to the manufacturer's instructions. For colony formation, HCC cells (8x10^4 per 10 cm plate) transfected with empty vector or pcDNA3.1-C9orf100 were selected in an appropriate medium with the addition of 0.8 mg/ml G418 (Life Technologies). After 3 weeks, the colonies stained with crystal violet were photographed and counted. All experiments were performed in triplicate.

Western blot analysis. Cell lysates from HCC cells were prepared using cold lysis buffer [25 mmol/l Tris-Cl (pH 7.5), 1% SDS, 5 mmol/l EDTA, protease inhibitor cocktail (Sigma, St. Louis, MO, USA)]. Samples were boiled for 5 min, subjected to electrophoresis in SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% blocking solution for 60 min, incubated with the primary antibody in PBST (0.1% Tween-20 in PBS) overnight at 4°C. Incubation with the secondary antibody was performed for 1 h at room temperature. Anti-Flag (Sigma) and β-actin (Santa Cruz
Biotechnology, Santa Cruz, Ca, USA) antibodies were used in this study. Detection of proteins was achieved using the Odyssey Infrared Imaging System (Li-COR).

**Immunofluorescence assay.** HCC cells grown on coverslips were washed twice with PBS, fixed with 4% paraformaldehyde on ice for 30 min and blocked with 5% horse serum at room temperature. The cells were then stained with mouse anti-Flag antibody (1:50; Sigma) at 4˚C overnight, followed by incubation with Cy5-conjugated anti-mouse secondary antibody (1:200, red; Molecular Probes, Inc.) at room temperature for 30 min. After rinsing 3 times with PBST, the cells were stained for nuclei with 4,6-diamidino-2-phenylindole (DAPI) and analyzed by the Zeiss confocal microscope.

**Cell migration assay.** Cell migration assays were performed using 24-well transwells (8-µm pore size, BD Biosciences), according to the manufacturer's instructions. Briefly, HCC cells were trypsinized and washed 3 times in DMEM without FBS. A total of 5x10^4 cells was then suspended in 500 µl DMEM without FBS and added to the upper chamber, while 750 µl DMEM containing 10% FBS was placed in the lower chamber. The cells were incubated for 24-48 h at 37˚C in a 5% CO₂-humidified incubator. The non-migrating cells were removed with cotton swabs and the migrated cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in at least 5 random microscopic fields (magnification, x100) were counted and photographed. All experiments were performed in duplicate and repeated 3 times.

**Cell cycle analysis.** Flow cytometry was used to analyze the cell cycle. For DNA content analysis, MHCC-97H cells transfected with C9orf100-specific siRNA or control siRNA were harvested at different time-points, fixed in cold 70% ethanol, washed and rehydrated in PBS. DNA staining was achieved by treating the cells with RNase A (10 mg/ml) for 30 min and with propidium iodide (10 µg/ml; Sigma) for 5 min.

**Statistical analysis.** Statistical analyses were evaluated with Student's t-test using GraphPad Prism 5 software. P>0.05 was considered to indicate a statistically significant difference.

**Results**

C9orf100 is frequently upregulated in HCC and is associated with clinicopathological features. From a cDNA microarray of paired HCC tissue samples performed in our lab (unpublished data), we found and focused on a gene, C9orf100, which in all cases was significantly upregulated in HCC tissues compared with their non-cancerous counterparts. To confirm this result,
we detected C9orf100 expression in an additional 44 pairs of HCC tissues by quantitative real-time PCR. In accordance with microarray data, the mRNA level of C9orf100 in HCC samples was markedly higher than that of the corresponding adjacent non-cancerous tissues (Fig. 1B, P<0.001). Among the 44 pairs of clinical samples, 42/44 (95.5%) exhibited at least a 2-fold increase, 30/44 (68.2%) exhibited a >5-fold increase and none of the cases showed any decrease (Fig. 1A). More notably, C9orf100 mRNA levels were higher in samples with multiple tumors than those with a single tumor (Fig. 1C, P<0.05) and with a serum α-fetoprotein level (AFP) of ≥10 ng/ml than those <10 ng/ml (Fig. 1D, P<0.01). However, the upregulation of C9orf100 was not statistically correlated with gender, age, tumor size or other clinicopathological characteristics. Taken together, the expression pattern of C9orf100 in clinical samples suggested it is important in the pathogenesis of HCC.

C9orf100 is a member of the Dbl-family GEFs. Homo sapiens gene C9orf100 is located on 9p13.3 and encodes a predicted 38-kDa protein of 335 amino acids. The encoded protein belongs to the Dbl-family GEFs, as it shares a DH domain followed by a PH domain. In contrast to other members of this family, C9orf100 lacks additional functional domains in structure (Fig. 1E). We also examined its subcellular distribution of C9orf100 in Huh-7 and YY-8103 cells. As expected, exogenous Flag-tagged C9orf100 was mainly localized to the cell membrane, as indicated by the immunofluorescence assay (Fig. 1F), suggesting it functions as a GEF of Rho GTPases in or near the cell membrane.

Ectopic expression of C9orf100 promotes cell proliferation and colony formation. To determine whether C9orf100 affects HCC cell proliferation, recombinant pcDNA3.1-C9orf100 was transfected into Huh-7 and YY-8103 cells. Results of the western blot analysis showed that C9orf100 was successfully expressed in the two cell lines (Fig. 2A). Cell viability assays using Cell Counting Kit-8 demonstrated that cells transfected with pcDNA3.1-C9orf100 grew more rapidly than those transfected with empty vector in Huh-7 and YY-8103 cells, respectively (Fig. 2B, P<0.01). Moreover, the colony formation assay was also performed to determine the long term effects of C9orf100 on cell growth. Following transfection and selection with G418 for 3 weeks, more colonies were formed in Huh-7 and YY-8103 cells transfected with exogenous C9orf100 than the controls (Fig. 2C, P<0.05), indicating that C9orf100 overexpression promoted cell proliferation of HCC cells.

Silencing of C9orf100 inhibits cell growth and induces cell cycle delay. To further elucidate the function of C9orf100, we then examined whether a reduction of endogenous C9orf100 expression suppresses cell growth in contrast with promotion by overexpression. To knock down C9orf100 expression, two different small interference RNAs (siRNAs) were chemically synthesized. Quantitative real-time PCR was performed to evaluate their interference efficiency. Compared with the control, si-NC, si-693 and si-1023 strongly decreased the mRNA level of endogenous C9orf100 in MHCC-97H and PLC/PRF/5 cells (Fig. 3A). Cell viability assays demonstrated that effective silencing of C9orf100 significantly inhibited the cell growth of MHCC-97H and PLC/PRF/5 cells (Fig. 3B), suggesting that C9orf100 knockdown induced the suppression of cell growth.

To understand the mechanism by which C9orf100 affects cell growth, we performed flow cytometry to detect the cell cycle distribution of MHCC-97H cells at different time-points following transfection. As shown in Fig. 3C, silencing of C9orf100 with si-693 and si-1023 led to a G2/M phase arrest at
The cells transfected with si-NC were used as the control. This result suggested that C9orf100 was involved in the cell cycle control of the G2/M phase.

C9orf100 is involved in the positive regulation of HCC cell migration. Intrahepatic cancer metastasis is a critical aspect of HCC recurrence following hepatectomy. We have demonstrated that C9orf100 expression was significantly higher in the group with multiple tumors than in the group with a single tumor in clinical samples, suggesting it plays a role in intrahepatic metastasis. To verify this hypothesis, we employed a transwell chamber assay to access the effect of C9orf100 on cell migration. After suspending the cells in the upper chamber for 24 h, we found that Hep3B cells transiently transfected with pcDNA3.1-C9orf100 migrated to the lower chamber more easily than those transiently transfected with the empty vector. There was a 3-fold increase in Hep3B/C9orf100 compared to Hep3B/vector cells (Fig. 4A, P<0.001). A similar result was also observed in Focus cells (Fig. 4B, P<0.001). Of note, the ectopic expression of C9orf100 in Hep3B and Focus cells had no impact on cell proliferation within 48 h (data not shown), suggesting that the increased migration rate induced by C9orf100 was independent of cell proliferation. By contrast, when C9orf100 was knocked down by specific siRNAs in MHCC-LM6 cells, the migrated cells from the upper chamber were obviously reduced relative to those treated with si-NC (Fig. 4C, P<0.001). Taken together, these data demonstrated that an increase or decrease in the C9orf100 level had a strong impact on cell migration, indicating that C9orf100 may contribute to the metastasis of HCC.

Discussion

C9orf100 is a new gene and its identification has not previously been reported. Little is known about C9orf100 except that it is supposed to be a member of the Dbl-Family GEFs in structure. In the present study, we provide compelling evidence, for the first time, that C9orf100 serves as a potential oncogene in HCC. C9orf100 overexpression was found in 95.5% of HCC samples used in our investigation and was correlated with HCC clinico pathological features. Furthermore, the enhanced expression of C9orf100 promoted cell proliferation and the
knockdown of endogenous C9orf100 suppressed cell growth by inducing cell cycle arrest in the G2/M phase. Additionally, we demonstrated that C9orf100 was involved in the positive regulation of HCC cell migration. These data strongly suggest that C9orf100 mediates the development and progression of HCC.

To date, only a few biomarkers have been applied to diagnose HCC. α-fetoprotein (AFP), for its best specificity and sensitivity, has been extensively used in clinics; however it remains insufficient for identifying HCC at the early stage of tumor formation (20). Furthermore, tumor multiplicity is an adverse prognostic factor in the AJCC staging systems. Patients with multinodular HCCs have a poorer 5-year disease-free outcome and overall survival rate than those with large solitary tumors who have undergone liver resection (21). Given that C9orf100 was highly and widely upregulated in the HCC samples, particularly closely associated with the AFP level and tumor multiplicity (Fig. 1C and D), it is likely that C9orf100 may be applied as a novel biomarker for HCC early diagnosis or prognosis outcome. Nevertheless, further experiments still need to be conducted.

Sequence analysis of C9orf100 predicted it possesses a DH-PH module, which is commonly shared in the Dbl-family GEFs. We have shown the difference between C9orf100 and other known members of this family (Fig. 1E). Despite lacking additional functional domains, C9orf100 still exhibited a marked oncogenic role as other members do, suggesting the minimal catalytic unit of the Dbl-family GEFs is sufficient to maintain the active GTP-bound states of Rho GTPases. RhoA, Rac1, and Cdc42 are the main members of Rho GTPases that have previously been reported as key regulators of cell migration (12). GEFs such as Dbs and Dbl are able to activate both Cdc42 and RhoA (22), whereas Vav3 catalyzes exchange only on RhoA and Rac1 (23), indicating GEFs have their substrate specificity. Further research is required to determine which Rho GTPase serves as the substrate of C9orf100. Moreover, the mechanism by which C9orf100 promotes cell proliferation and migration via Rho GTPases should also be explored in the future.

In summary, we identified C9orf100, the new member of the Dbl-family GEFs, as a candidate oncogene in HCC. Our findings may provide new insight into identifying a novel diagnostic biomarker and developing a new therapeutic strategy against HCC. Furthermore, our study has also allowed us to gain a better understanding of the Dbl-family GEFs in tumor development.

Acknowledgements

We thank our laboratory members, Qing Deng, Ruifang Liu, Xiao Xu and Fei Chen for their helpful discussion and generous technical assistance. This study was supported by grants from the Academician Workstation of Wuxi People’s Hospital (CYE00917), the Chinese National Key Program on Basic Research (2010CB529204) and the China National Key Projects for Infectious Disease (2008ZX10002-021).

References