Research Article

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# MicroRNA-101 inhibits human hepatocellular carcinoma progression through *EZH2* downregulation and increased cytostatic drug sensitivity☆

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**Background & Aims**: Oncogene polycomb group protein enhancer of zeste homolog 2 (*EZH2*) has been proposed to be a target gene of putative tumor suppressor microRNA-101 (miR-101). The aim of our study was to investigate the functional role of both miR-101 and *EZH2* in human hepatocellular carcinoma (HCC).

**Methods**: MiR-101 and *EZH2* expressions were evaluated in tumor tissues of 99 HCC patients and 7 liver cancer cell lines by real-time PCR. Luciferase reporter assay was employed to validate whether *EZH2* represents a target gene of miR-101. The effect of miR-101 on HCC growth as well as programmed cell death was studied *in vitro* and *in vivo*.

**Results**: MiR-101 expression was significantly downregulated in most of HCC tissues and all cell lines, whereas *EZH2* was significantly overexpressed in most of HCC tissues and all cell lines. There was a negative correlation between expression levels of miR-101 and *EZH2*. Luciferase assay results confirmed *EZH2* as a direct target gene of miR-101, which negatively regulates *EZH2* expression in HCC. Ectopic overexpression of miR-101 dramatically repressed proliferation, invasion, colony formation as well as cell cycle progression *in vitro* and suppressed tumorigenicity *in vivo*. Furthermore, miR-101 inhibited autophagy and synergized with either doxorubicin or fluorouracil to induce apoptosis in tumor cells.

**Conclusion**: Tumor suppressor miR-101 represses HCC progression through directly targeting *EZH2* oncogene and sensitizes liver cancer cells to chemotherapeutic treatment. Our findings provide significant insights into molecular mechanisms of hepatocarcinogenesis and may have clinical relevance for the development of novel targeted therapies for HCC.



Keywords: Apoptosis; Autophagy; HCC; microRNA; Target gene.

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*Abbreviations: EZH2*, enhancer of zeste homolog 2; miR-101, microRNA-101; HCC, hepatocellular carcinoma; miRNA(s), microRNA(s); mRNA(s), messenger RNA(s); Mcl-1, myeloid cell leukemia sequence 1; siRNAs, small interfering RNAs; qPCR, quantitative real-time PCR; UTR, untranslated region; wt, wild type; mut, mutant type; Pre-miR-101, miR-101 precursor; miR-C, microRNA control; h, hour(s); DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; DPBS, Dulbecco's Phosphate-Buffered Saline; min, minute(s); L, length; W, width; V, volume; 5-FU, 5-fluorouracil; 7-AAD, 7-amino-actinomycin D; wk, week(s); LC3, microtubule-associated protein light chain 3; PARP, poly adenosine diphosphate ribose polymerase; CLSM, confocal laser scanning microscopy; LAMP-1, lysosome-associated membrane protein; DAPI, 4′,6-diamidino-2-phenylindole; pRb, phosphorylated retinoblastoma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SD, standard deviation; Anti-miR-101, miR-101 inhibitor; siR-C, siRNA control; shRNA, short hairpin RNA; PCD, programmed cell death; HH, human hepatocytes; N, non-tumor tissues; T, HCC tissues; APH, aphidicolin; Baf, bafilomycin; Doxo, doxorubicin; UICC/AJCC, International Union Against Cancer/American Joint Committee on Cancer; LV, lentiviral.

## Introduction

Hepatocellular carcinoma (HCC) represents the third leading cause of cancer-related death in the world [1]. Its incidence continues to increase worldwide and the long-term prognosis remains dismal [2]. An improved understanding of molecular pathogenesis of HCC has facilitated the development of new targeted therapies, which have shown a significant clinical impact [3].

**Table 1. Clinicopathologic characteristics of patients.**

| Characteristics | Cohort 1 | Cohort 2 | |
|---|---|---|---|
| | HCC (n = 93) | HCC (n = 6) | Normal (n = 6) |
| Age (mean ± standard deviation) | 59.2 ± 13.2 | 57.7 ± 11.5 | 52.1 ± 9.3 |
| HBsAg positive (yes/no) | 11/82 | 1/5 | / |
| HCV Ab positive (yes/no) | 20/73 | 2/4 | / |
| Tumor status (T1/T2/T3/T4) | 45/32/15/1 | 2/3/1/0 | / |
| Lymph node status (N0/N1) | 86/7 | 5/1 | / |
| Distant metastasis (M0/M1) | 91/2 | 6/0 | / |
| TNM stage (I/II/III/IV) | 42/31/13/7 | 2/3/1/0 | / |
| Histological grade (G1/G2/G3) | 14/68/11 | 2/2/2 | / |

Tumor stage and histological grade were classified in accordance with the criteria of International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) 7th Edition (2009).
HBsAg, Hepatitis B surface antigen; HCV Ab, Hepatitis C virus antibody.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs of 20 to 25 nucleotides in length that post-transcriptionally regulate the expression of complementary messenger RNAs (mRNAs) in eukaryotes, influencing various biological processes like cell development, infection, immunity, and carcinogenesis [4,5]. More than half of human miRNAs are associated with carcinogenesis, and this may provide the basis for the development of targeted therapies against cancer [6]. The putative tumor suppressor miR-101 counteracts tumor development and progression by downregulation of several oncogenes [7,8]. For instance, miR-101 has been shown to inhibit tumorigenicity via targeting myeloid cell leukemia sequence 1 (*Mcl*-1) in HCC [8].

Polycomb group protein enhancer of zeste homolog 2 (*EZH2*) plays an important role in regulation of cell proliferation, determination of stem cell fate, and carcinogenesis [9,10]. Overexpression of *EZH2* has been reported for a variety of malignancies including HCC [11–14]. Sudo *et al.* [15] were the first to report that *EZH2* is upregulated in human HCC and overexpression of *EZH2* is associated with a more aggressive biological behavior [16] and worse prognosis [17]. Moreover, detection of *EZH2* protein by immunohistochemistry in liver needle biospies may represent a diagnostic tool for discrimination between dysplastic nodules and early HCC [18]. *EZH2* has previously been proposed to be a target gene of miR-101 in prostate and bladder cancer [19,20]. The precise regulatory mechanisms of *EZH2* expression and its relationship with miR-101 in the process of hepatocarcinogenesis are still not fully elucidated. Therefore, we sought to investigate the functional role of both miR-101 and *EZH2* in the pathogenesis of HCC.

## Materials and methods

*Tissue specimens*

Paired HCC tissues and adjacent non-tumor liver tissues were collected from patients who underwent surgery between November 2000 and January 2011 at the University Hospital Essen (Essen, Germany) and had not received local or systemic treatment. Additionally, normal liver tissues were collected from patients receiving liver resection for benign tumors (hepatic hemangioma, n = 4; focal nodular hyperplasia, n = 2). Tumor tissues and adjacent non-tumor liver tissues were confirmed histologically. All patients provided written informed consent and the project was in accordance with the Helsinki Declaration of 1975. Clinicopathologic characteristics are presented in Table 1.

*Cell lines and transfection*

Human hepatoma/hepatoblastoma cell lines HepG2, Hep3B, and SNU-182 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). BEL-7402 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HuH7 was a kind gift from Dr. Brigitte Pützer (University of Rostock, Rostock, Germany). PLC/PRF5 and HepaRG were a kind gift from Dr. Mengji Lu. Primary human hepatocytes were obtained from ScienCell (San Diego, CA, USA). Plasmids, miRNAs, and small interfering RNAs (siRNAs) were transfected into cells at indicated concentrations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (See Supplementary data for details).

*RNA preparation and quantitative real-time PCR (qPCR) assay*

See detailed description in Supplementary data and Supplementary Table 2.

*Vector construction and luciferase reporter assay*

Standard molecular biology techniques were used for generation of the luciferase reporter vectors pMIR-*EZH2*-3′-untranslated region (UTR)-wild type (wt) and mutant type (mut). The 3′-UTRs of *EZH2* (wt and mut) were inserted between SpeI and HindIII restriction sites into pMIR-REPORT luciferase vector. Luciferase reporter plasmids were cotransfected with miR-101 precursor (Pre-miR-101) or miR-control (miR-C) into tumor cells to determine whether *EZH2* is a target gene of miR-101 (described in detail in Supplementary data).

*Cell proliferation assay, colony formation assay and cell cycle analysis*

Cell proliferation was determined using the Cell Proliferation Reagent WST-1 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's protocol. For colony formation assay, 24 h after transfection, 200 cells were seeded into a six-well plate and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) for 10 d–14 d. Colonies were fixed, stained, and the numbers of colonies were counted and pictures were captured. For cell cycle analysis, cells were transfected as indicated before and treated with or without 5 μg/ml of aphidicolin (Sigma-Aldrich, Munich, Germany) for additional 24 h. After fixation, cells were incubated in Dulbecco's Phosphate-Buffered Saline (DPBS) containing 20 μg/ml of propidium iodide (Sigma-Aldrich), 200 μg/ml of RNase A, and 0.1% Triton X-100, and analyzed for cell cycle distribution by flow cytometry (FACSCalibur flow cytometer; BD Biosciences, Franklin Lakes, NJ, USA).

*Cell invasion assay*

Cell invasion assay was conducted using BD BioCoat Matrigel invasion chambers (BD Biosciences, Heidelberg, Germany) following the manufacturer's protocol. In brief, 24 h after transfection, $5 \times 10^4$ cells in 0.5 ml serum-free medium were seeded into the top chamber with a Matrigel coated filter and 0.75 ml DMEM