Acta Histochemica xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

MiR-199-3p replacement affects E-cadherin expression through Notch1 targeting in hepatocellular carcinoma

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ARTICLE INFO

Keywords:
HCC
microRNA
Notch1
E-cadherin
miR-199a-3p

ABSTRACT

Hepatocellular carcinoma (HCC) represents the second cause of cancer-related mortality worldwide and is associated with poor prognosis, due to a high recurrence rate after curative treatments and a drug resistance phenotype. In this scenario, the identification of innovative and effective therapeutic strategies is an unmet clinical need. The safety and efficacy of microRNA (miRNA) mediated approaches in preclinical models and clinical trials have been widely described in cancer. MicroRNA-199a downregulation is a common feature of HCC where its reduced expression contributes to mTOR and c-Met pathways activation. Notch1 activation is also a common event in HCC, influencing epithelial-to-mesenchymal transition, tumor invasion and recurrence at least in part through E-cadherin regulation. Here we identified a negative correlation between miR-199a-3p and Notch1 or E-cadherin protein levels in HCC patients and demonstrated that miR-199a-3p regulates E-cadherin expression through Notch1 direct targeting in *in vitro* models. Moreover, we showed that a strong correlation exists between miR-199a-5p and miR-199a-3p in HCC specimens and that miR-199a-5p contributes to E-cadherin regulation as well, underlying the complex network of interaction carried out by miR-199a and its influence on tumor aggressiveness. In conclusion, our findings suggest the restoration of miR-199a-3p physiologic levels as a possible therapeutic strategy for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer and it represents the second leading cause of cancer-related death worldwide (Llovet et al., 2016). Surgery remains the most efficient treatment for patients with HCC; nonetheless, long-term prognosis after surgical resection remains unsatisfactory because of a high recurrence rate and the lack of effective adjuvant therapy (Kubo et al., 2013). Recurrence is frequently associated with the activation of a trans-differentiation process in cancer cells: the epithelial-to-mesenchymal transition (EMT), which contributes to tumor progression through the acquisition of motility properties and resistance to apoptosis (van Zijl et al., 2009). Different cellular pathways have been shown to trigger EMT including fibroblast growth factors receptors (FGFRs), platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Ahmad et al., 2012; van Zijl et al., 2009; Zheng et al., 2016). Recently, Notch signaling pathway has been found to be involved in EMT and to contribute to tumor aggressiveness (Wang et al., 2010b). Accordingly, we reported that Notch1 triggers EMT in HCC through E-cadherin upregulation, increasing cell migration and invasion (Giovannini et al., 2016). The EMT pathway is of increasing interest as a novel therapeutic opportunity in cancer treatment to prevent tumor cell dissemination in early stage or to kill existing metastatic cells in advanced stages (Davis et al., 2014). To this end, different EMT-reversing inhibitors have been tested in clinical trials, alone or in combination with other drugs, to promote MET (mesenchymal-to-epithelial transition) in metastatic cells or to target cancer cells that have undergone EMT and exhibit increased stem cell-like characteristics (Voon et al., 2017).

Non-coding RNAs (ncRNAs) are also recognized to be involved in

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https://doi.org/10.1016/j.acthis.2017.12.004
Received 26 October 2017; Received in revised form 23 November 2017; Accepted 12 December 2017

EMT and MET programs. Indeed, both long non coding-RNAs (lncRNAs) and microRNAs (miRNAs) have emerged as key regulators of EMT in different cancer types (Heery et al., 2017; Lee et al., 2014; Tseng et al., 2017) and recent evidences suggest that their crosstalk might represent an important mechanism in tumor metastasis (Cao et al., 2017). Regarding miRNA role in EMT in HCC, p53-transactivated miR-200 family members affects this process through ZEB1 and ZEB2 targeting, mediating cell plasticity (Kim et al., 2011). Moreover, others and our group reported the repressive role of miR-122 in tumor invasion and metastasis (Coulouarn et al., 2009; Fornari et al., 2009; Tsai et al., 2009) and, in line, Wang and coworkers demonstrated its contribution to MET through the inhibition of RhoA and Rac1, two molecules participating to cell motility and cytoskeletal reorganization (Wang et al., 2014). MiR-130b is upregulated in tumor cells and its expression levels are predictive of disease-free survival in HCC patients, it triggers proliferation and EMT-induced metastasis through the regulation of PTEN/AKT/HIF1α signaling pathway in HCC preclinical models (Chang et al., 2016).

In addition, it has been reported that several miRNA crosstalk with Notch pathway in different human diseases (Wang et al., 2010a). Specifically, miR-34c forms a regulatory loop with Notch1 to repress muscle development (Hou et al., 2017), miR-137 directly targets Notch1 in diabetic kidney disease (Han et al., 2018), miR-101 blocks Notch1expression and increases chemotherapeutic sensitivity of T-cell acute lymphoblastic leukemia (Qian et al., 2016) and, finally, miR-433 inhibits migration of cancer ovarian cells by targeting Notch1 (Liang et al., 2016).

We previously demonstrated that miR-199a-3p restoration blocks cell cycle progression and impairs invasion capability of HCC cells through mTOR and c-Met dual targeting (Fornari et al., 2010), mirroring the effect mediated by Notch1 silencing in HCC *in vitro* models. At the light of these experimental evidences, this study was undertaken to assay Notch1 as a possible target of miR-199a and to further characterize their contribution to HCC invasion.

We found that Notch1 direct targeting by miR-199a-3p mediates E-cadherin downregulation in HCC cell lines. These *in vitro* findings were confirmed in a HCC patient cohort, suggesting miR-199a-3p replacement as a promising therapeutic option for the treatment of HCC reducing cancer progression and recurrence.

2. Patients and methods

2.1. Patients

Tumor and cirrhotic tissues were obtained from 39 consecutive patients undergoing liver resection for HCC at St. Orsola-Malpighi Hospital after obtaining an informed consent. Tissues were collected at surgery and samples were snap frozen in liquid nitrogen and stored at −80 °C. No patient received anticancer treatment prior to surgery. The study protocol was conform to the ethical guidelines of the 1975 Declaration of Helsinki. Local ethics committee of St. Orsola-Malpighi University Hospital approved the study and all patients signed an informed consent. Patient characteristics are summarized in Table S1.

2.2. HCC cells culture and transfection

HepG2 cells derived from a 15-year-old Caucasian male (HB-8065; ATCC, Manassas, Virginia, USA) and Hep3B (HB-8064; ATCC) cells derived from an 8-year-old Black male were cultured with MEM (Eagle). Huh-7 cells derived from a 57-year-old Japanese male (kindly provided by Prof. Alberti, Padua University), SNU398 (CRL-2233; ATCC) cells derived from a 42-year-old Asian male, SNU449 (CRL-2234; ATCC) cells derived from a 52-year-old Asian male, SNU182 (CRL-2235; ATCC) cells derived from a 24-year-old Asian male and SNU475 (CRL-2236; ATCC) cells derived from a 43-year-old Asian male were cultured with RPMI 1640; both media were supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were cultured at 37 °C in a 5% CO2 incubator. All the cell lines used were between passage 5 and 10. Mycoplasma test, performed on all the cell lines accordingly to the manufacture instruction, resulted negative (Universal Mycoplasma Detection Kit).

The choice of these cell lines was linked to the fact that, coming from different ethnic groups, they are representative of the genetic variability of HCC. Moreover, regarding the presence of hepatitis virus, Hep3B, SNU398, SNU449, SNU182 and SNU475 cells are positive for Hepatitis B virus, Huh-7 cells are permissive to Hepatitis C virus replication, whereas HepG2 cells are negative for both viruses, depicting the heterogeneity of HCC etiology. Finally, these cell lines possess different epithelial and mesenchymal characteristics. Specifically, HepG2, Hep3B and Huh-7 cells exhibit a more epithelial phenotype, whereas the other cell lines mainly show mesenchymal characteristics (Fuchs et al., 2008).

Oligonucleotide transfection of pre-miR-199a-3p, pre-miR-199a-5p, anti-miR-199a-3p, or negative controls (75 nM, Thermo Fisher Scientific, Whaltam, USA) was obtained by using TransIT-X2 dynamic delivery system (Mirus Bio, Madison, USA) according to the manufacturer instructions.

Notch1 stable silencing in HepG2 cells was obtained by retroviral infection as previously described by our group (Giovannini et al., 2009). Regarding miRNA plasmid construction, it was obtained by cloning the DNA sequence of mature miR-199a-3p into BamH1 and HindIII sites of pGFP-V-RS retroviral vector (OriGene Technologies, Rockville, Maryland, USA). HepG2 cells were infected with retroviral particles and miR-199a overexpressing cell clone was selected as previously described (Fornari et al., 2014). A scramble short hairpin (shRNA) bearing vector was used as control (pGFP-V-RS-shRNA).

2.3. Luciferase activity assay

The 3′UTR regions of human Notch1 gene was amplified by PCR using primers and conditions reported in Table S2. The DNA fragment was cloned into the pMIR-REPORT Luciferase vector (Promega, Madison, USA) by using SacI and HindIII restriction enzymes. Dual-luciferase reporter assay (Promega) was used to assess the direct interaction between miR-199a-3p and Notch1 3′UTR. In particular, the reporter assay was performed in HepG2 and SNU398 cells co-transfected with 3′UTR-containing vector or control vector and miR-199a-3p or anti-miR-199a-3p or negative controls after 24 h. QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) was used to perform the mutagenesis of miR-199a-3p seed sequence in Notch1 3′ UTR-bearing vector.

2.4. Quantitative PCR and semi-quantitative RT-PCR

MiRNA-199a-3p (ID:499) and miR-199a-5p (ID:498) expression was quantified by using TaqMan MicroRNA assays (Thermo Fisher Scientific), as previously described (Gramantieri et al., 2007). RNU6B (ID:001093) was used as housekeeping gene. $2^{-\Delta\Delta Ct}$ method was used to quantify miRNA expression, using a pool of four non-neoplastic livers as reference sample. Each sample was run in triplicate. For semi-quantitative PCR, one microgram of total RNA was retrotranscribed by using High Capacity RNA-to-cDNA kit (Life Technologies) following manufacturer instruction. β-actin was used as housekeeping gene. Products were resolved on 2% agarose gels stained with ethidium bromide and quantified by densitometry (Quantity-One, Bio-Rad, Hercules, USA). Primers and conditions for RT-PCR are detailed in Table S2.

2.5. Western blot

Protein extraction from frozen HCC tissue samples (N = 18) and cell