Digestive and Liver Disease 48 (2016) 953–960

Contents lists available at ScienceDirect

Digestive and Liver Disease

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

Oncology

Methylenetetrahydrofolate dehydrogenase 2 overexpression is associated with tumor aggressiveness and poor prognosis in hepatocellular carcinoma

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ARTICLE INFO

Article history:
Received 30 August 2015
Accepted 22 April 2016
Available online 16 May 2016

Keywords:
EMT
Hepatocellular carcinoma
Invasion
MTHFD2
Neoplasm metastasis

ABSTRACT

Background: Methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) is the key enzyme in the transformation of folic acid metabolites. MTHFD2 overexpression plays a key role in the progression of human cancers, and depletion of MTHFD2 has shown potential antitumor activities in several types of cancer. However, the role of MTHFD2 in hepatocellular carcinoma (HCC) has not been investigated.
Aims: To investigate the expression of MTHFD2 in HCC patients, and its associated clinical implications and possible functions in HCC.
Methods: Reverse transcription-polymerase chain reaction and immunohistochemical staining were used to detect MTHFD2 expression in liver tissues from HCC patients, then associations of MTHFD2 expression with demographic and clinicopathologic features were analysed. The effects of siRNA interference of MTHFD2 on cell proliferation, cell cycle, apoptosis, and migration were investigated in HCC cell lines.
Results: Significant overexpression of MTHFD2 was observed in HCC tissues, and overexpression of MTHFD2 was correlated with TNM stage, tumor microembolus, tumor metastasis, recurrence and the time of recurrence ($P < 0.05$) in HCC patients. siRNA-mediated silencing of *MTHFD2* inhibited migration, invasion and epithelial–mesenchymal transition progression in HCC cell lines, but no obvious effects on cell proliferation, apoptosis or cell cycle distribution were detected.
Conclusions: MTHFD2 is overexpressed in HCC, and is associated with poor prognosis and cellular features connected to metastatic disease.

1. Introduction

Hepatocellular carcinoma (HCC) is currently one of the most common malignant tumors worldwide. An annual report on the status of cancer in China showed that HCC was the fourth common cancer with estimated 355,595 new cases and the second cause of death with estimated 322,417 deaths in 2011 [1]. Radical tumor resection is the preferred and most effective treatment; however, in most operation cases, tumor relapse occurs within 5 years [2], and neoplasm metastasis and recurrence are the main causes for poor prognosis for HCC patients [3]. The advances in molecular biology techniques during the past decades have provided new ways to diagnose and treat tumors, and have led to the identification of several biomarkers implicated in HCC cell migration and invasion that can be used in the disease diagnosis [4–6]. However, because of individual differences and the complexity of tumorigenesis, none of these methods are satisfactory, thus it is imperative to find out new functional biomarkers for detection of HCC and to annotate their potential molecular mechanisms in HCC.

Tumorigenesis is a multi-step process involving multiple factors, genes, and mutations, and HCC is no exception. A recent study showed that methylenetetrahydrofolate dehydrogenase 2 (MTHFD2), the key enzyme in the transformation of folic acid metabolites, is highly expressed in a variety of human tumors and negatively correlates with survival in breast cancer patients [7]. Further, elevated expression of MTHFD2 has been associated with an increased risk of bladder cancer [8] and MTHFD2 was significantly up-regulated in lung fibrosis and hepatic inflammation [9,10]. However, the role of MTHFD2 in HCC is not clear.

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http://dx.doi.org/10.1016/j.dld.2016.04.015
1590-8658/

NAD-dependent MTHFD2 is a 34 kDa bifunctional mitochondrial enzyme with methylenetetrahydrofolate dehydrogenase and cyclohydrolase activities, and is encoded by the nuclear *MTHFD2* gene located on chromosome 2 in humans [11–13]. MTHFD2 was first identified by Scrimgeour and Huennekens in Ehrlich ascites tumor cells in 1960 [14]. MTHFD2 is expressed in the developing embryo, and in transformed and non-differentiated cells, but it is absent in most healthy adult tissues [15]. Emerging evidence shows that MTHFD2 overexpression promote cancer cell proliferation and invasion in several types of tumors, and since increased MTHFD2 expression is associated with poor prognosis of patients with breast cancer, it has potential as a new drug target [7,16–18]. In the current study, for the first time, we explored the expression of MTHFD2 in HCC tissue specimens ex vivo, as well as the associated clinical implications. In addition, for the sake of future disease management, we investigated the effects of MTHFD2 silencing on cell viability, cell cycle, apoptosis, migration, and invasion in HCC cell lines.

2. Materials and methods

2.1. Patients and tissue specimens

Ninety-eight patients with pathologically confirmed HCC who underwent surgical resection between October 2010 and April 2014 were enrolled in this study. In addition, eight hepatic specimens diagnosed as hepatic hemangioma or intrahepatic bile duct stone were used as control tissues. Surgeries were conducted at the Department of Hepatobiliary Surgery, Nanfang Hospital, Southern Medical University (Guangzhou, China). The inclusion criteria were as follows: (a) no other treatment before surgery; (b) curative surgical resection was performed; (c) resected specimens were available for pathological examination and examined for metastasis after the operation; (d) the cut edge was confirmed without residual carcinoma; (e) no hepatitis C diagnosis; and (f) a complete medical record. All samples were flash-frozen in liquid nitrogen and stored at −80 °C. Cancerous tissue was defined as tissue within 1 cm from the tumor edge without necrosis, and noncancerous tissue as tissue exceeding the edge of the tumor by more than 2 cm. The specimens were subjected to immunohistochemical staining, and the patients were monitored for prognostic analysis via outpatient examinations or telephone follow-up. We defined tumor relapse as occurring in patients with two imaging examinations showing characteristic liver cancer lesions or a radiological examination showing characteristic liver lesions, accompanied by AFP $\geq$ 400 μg/L (excluding pregnancy, active hepatitis, etc.). Metastasis was defined in patients whose recurrence time was less than 6 months or patients with cancer embolus (included portal vein tumor thrombus, biliary tract tumor thrombus, and hepatic vein tumor thrombus). In addition, mRNA expression was measured in cancerous and adjacent noncancerous tissue specimens of 60 cases by using reverse transcription-polymerase chain reaction (RT-PCR). The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) and was approved by the Ethical Committee of Nanfang Hospital in Southern Medical University. All subjects gave written, informed consent.

2.2. RNA extraction, reverse transcription, and RT-PCR

Total RNA of HCC tissues and adjacent noncancerous counterparts was extracted using Trizol reagent (TaKaRa, Dalian, China). Five hundred nanograms of total RNA was used for reverse transcriptase (RT) reactions that were carried out using the PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) according to the manufacturer's instructions. PCR was carried out using the SYBR® Premix Ex Taq™ kit (TaKaRa, Dalian, China). The sequences of forward and reverse primers (synthesized by Invitrogen, Shanghai, China) were as follows:

MTHFD2: 5′-CTGCGACTTCTCTAATGTCTGC-3′ and 5′-CTCGCCAACCAGGATCACA-3′, GAPDH: 5′-CAGGAGGCATTGCTGATGAT-3′ and 5′-GAAGGCTGGGGCTCATTT-3′. The data were processed using the $2^{-\Delta\Delta CT}$ method with GAPDH serving as a reference gene for normalization.

2.3. Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized in 0.2% Triton X-100 for 10 min, and blocked with 5% BSA for 1 h. The cells were incubated overnight with mouse anti-MTHFD2 antibody (1 μg/10^6 cells, Abcam, USA) in 5% BSA at 4 °C, and detected using anti-mouse antibodies conjugated to Alexa Fluor 488. Slides were mounted using ProLong® Gold antifade reagent with DAPI (Cell Signaling Technology, USA). Images were acquired using a confocal laser scanning microscope (FV10i-w, Olympus, Japan) and accompanying FV10i-ASW 3.0 software.

2.4. Immunohistochemistry

The tumor tissue specimens were fixed with 10% neutral formalin for 24–48 h, routinely processed for paraffin embedding, and cut into 4-μm-thick slices. The sections were roasted for 2 h (60 °C), dewaxed with dimethylbenzene, and gradually hydrated in descending grades of methanol. Microwave antigen retrieval for 10 min, followed by incubation in 3% H_2O_2 for 15 min to eliminate endogenous peroxidase, The sections were blocked in 1% bovine serum albumin (BSA) for 30 min and incubated overnight (more than 16 h) with anti-MTHFD2 antibody (1:200, Abcam, USA) at 4 °C. Then the sections were washed with phosphate-buffered saline (PBS), and incubated with secondary antibody (EliVision, China) for 30 min. After extensive washing, 3,3-diaminobenzidine (DAB) was used to detect immunoreactivity and the sections were counterstained with hematoxylin, dehydrated in ascending grades of methanol, cleared in dimethylbenzene, and mounted with a coverslip. Each specimen was incubated with diluent instead of antibody as a negative control, and immunoreactivity was independently evaluated by two professional pathologists. We defined hepatocytes with light brown- to dark brown-stained cytoplasm as positive cells, and expression was classified according to the following staining intensity criteria: 0, no staining; 1, light-colored staining; and 2, deep-colored staining. Additionally, we scored the percentage of positive cells as follows: 1, 1–25% positive cells; 2, 26–50% positive cells; 3, >50% positive cells. Finally, expression was evaluated semi-quantitatively based on the product of the color intensity and positive cell percentage scores: 0, negative expression; ≥1, positive expression; and ≥4, strong positive expression [7].

2.5. Cell lines

Human HepG2 and Huh7 HCC cells (Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences, China) were cultured in high-glucose Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2. Cells in the exponential phase were harvested at approximately 80% confluence.

2.6. Gene knockdown

HepG2 and Huh7 cells were transfected with siRNAs (GenePharma, Shanghai, China) using Lipofectamine 3000