https://doi.org/10.1016/j.hpb.2018.03.018 HPB

ORIGINAL ARTICLE

Enhancer of zeste homolog 2 (EZH2) regulates tumor angiogenesis and predicts recurrence and prognosis of intrahepatic cholangiocarcinoma

Shigeki Nakagawa, Hirohisa Okabe, Mayuko Ouchi, Ryuma Tokunaga, Naoki Umezaki, Takaaki Higashi, Takatoshi Kaida, Kota Arima, Yuki Kitano, Hideyuki Kuroki, Kosuke Mima, Hidetoshi Nitta, Katsunori Imai, Daisuke Hashimoto, Yo-ichi Yamashita, Akira Chikamoto & Hideo Baba

Kumamoto University - Gastroenterological Surgery, Kumamoto, Japan

Abstract

Background: Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the polycomb repressive complex 2 (PRC2) and regulates tumor malignancy by gene silencing via histone methylation. In this study we investigate the role of EZH2 in angiogenesis of intrahepatic cholangiocarcinoma (ICC).

Methods: The influence of EZH2 on tumor angiogenesis was examined by bioinformatics analysis of a public database. We also assessed the correlation between EZH2 and vasohibin 1 (VASH1) expression in 47 patients with ICC by immunohistochemical (IHC) staining and in vitro gene silencing assays. The prognostic significance of EZH2 and VASH1 expression by IHC was also examined in the ICC cohort.

Results: Bioinformatics analysis showed that EZH2 was associated with several angiogenesis gene sets in the public database. EZH2 suppressed VASH1 expression in in vitro assays and IHC studies. EZH2-high/VASH1-low status was independently associated with poor disease-free survival (P = 0.019) and poor overall survival (P = 0.0055).

Conclusion: The current study demonstrated that high EZH2 expression was associated with activation of tumor angiogenesis, and activation of the EZH2-mediated angiogenesis pathway predicted the prognosis of patients with ICC.

Received 2 October 2017; accepted 30 March 2018

Correspondence

Department of Gastroenterological Surgery, Graduate School of Life Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail: shigekn2@gmail.com

Introduction

Cholangiocarcinoma is a relatively rare neoplasm in humans that is further classified into intrahepatic, perihilar extrahepatic, or distal extrahepatic based on the location in the body where it develops. Intrahepatic cholangiocarcinoma (ICC) originates from the second segment of the bile duct.[1,2] ICC is generally considered incurable; it has a rapid progression and is lethal in most cases, with a 5-year survival rate of less than 5% for non-resectable cases.[3] Complete surgical resection has been considered the only curative treatment for patients with ICC, but the outcomes vary widely across affected patients. Few specific diagnostic or therapeutic tools are available because of limited information on the molecular pathogenesis of ICC, and new therapeutic targets are urgently needed. Currently, only two drugs are available for the treatment of cholangiocarcinoma: gemcitabine and cisplatin. There are no molecular-targeted drugs for ICC because the molecular biological characterization is not clear. It is therefore necessary to understand the molecular mechanism of ICC progression and discover new drugs for cholangiocarcinoma treatment, and to identify molecular subclasses or biomarkers for the selection of patients who would be likely to respond to molecular-targeted therapies.

Enhancer of zeste homolog 2 (EZH2) is one of several well-known oncogenes that accelerate malignancy by epigenetic mechanisms in several types of solid tumor such as breast, prostate, ovarian, and pancreatic cancer, and cholangiocarcinoma.[4–11] EZH2 has several roles in cancer progression and one of the important mechanisms by which EZH2

Please cite this article in press as: Nakagawa S, et al., Enhancer of zeste homolog 2 (EZH2) regulates tumor angiogenesis and predicts recurrence and prognosis of intrahepatic cholangiocarcinoma, HPB (2018), https://doi.org/10.1016/j.hpb.2018.03.018

promotes cancer is via the angiogenesis pathway. Lu et al. reported that EZH2 expression in cancer cells and tumor-associated endothelial cells accelerates tumor angiogenesis by silencing vasohibin1 (VASH1), resulting in ovarian cancer growth.[12] VASH1 is a newly discovered angiogenesis inhibitor that is normally expressed in human vascular endothelial cells in response to vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2).[13–15] Upregulation of VASH1 suppresses excessive angiogenesis activity directed by VEGFA and plays important roles in neo-angiogenesis of benign diseases such as retinal disease or diabetic nephropathy.[16,17] In addition, previous studies revealed that VASH1 is exclusively expressed in endothelial cells of several types of cancer such as breast, cervical, colorectal, lung, prostate, and liver cancer, and is related to the development and progression of these tumors.[18–28] It has also been reported that VASH1 is epigenetically regulated by EZH2 via the trimethylation of histone 3 lysine 27.[12] However, there is are few data on the association of VASH1 and tumor malignancy or the relationship between EZH2 and VASH1 in intrahepatic cholangiocarcinoma.

This study aimed to elucidate the role of EZH2 in the regulation of angiogenesis and as a predictive prognostic factor after curative surgery in patients with ICC.

Materials and methods

Patients and tissue specimens

A total of 62 patients diagnosed with primary ICC underwent surgical resection from January 1993 to January 2009 at Kumamoto University Hospital (Kumamoto, Japan) and were pathologically confirmed to have ICC. Patients with combined hepatocellular carcinoma and cholangiocarcinoma (one patient) or R2 resection (seven patients) were excluded from the analysis. An additional seven patients were excluded because there was insufficient FFPE tissue for analysis. Finally, 47 patients were enrolled in this study (the flow diagram is shown in Supplementary figure 1). Patients who underwent R1 resection and were lymph node (LN)-positive received postoperative 5-fluorouracil (5-FU), cisplatin, and gemcitabine–based chemotherapy. The median follow-up period after surgery was 30.2 months (95% confidence interval [CI]: 32.5–56.5 months). This study protocol was approved by the Human Ethics Review Committee of the Graduate School of Medicine, Kumamoto University. Also written informed consent were obtained from these patients.

Immunohistochemical staining and scoring

Immunohistochemical staining was performed on 3-μm formalin-fixed, paraffin-embedded sections as described previously.[9,11,29,30] In brief, endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 5 min. The sections were incubated with primary mouse monoclonal anti-EZH2 antibody (1:50 dilution; Dako, Tokyo, Japan) or primary mouse monoclonal anti-VASH1 antibody (1:200 dilution; Abcam, Tokyo, Japan) overnight at 4 °C, followed by incubation with a biotin-free horseradish peroxidase-labeled polymer of the Envision Plus detection system (Dako, Tokyo, Japan). A positive reaction was visualized with diaminobenzidine solution, followed by counterstaining with Mayer's hematoxylin. EZH2 and VASH1 staining was scored by counting the percentage of positively stained cells in five high-magnification fields. The cut-off value defined as the median percentage of stained cells (10% for EZH2, 35% for VASH1) was used to classify low expression and high expression.

Depletion of EZH2 by synthetic small interfering RNAs (siRNAs)

The cholangiocarcinoma RBE cell line was purchased from RIKEN Bioresource Center (Ibaraki, Japan), cultured in RPMI-1640 (Invitrogen, Tokyo, Japan) containing 10% fetal bovine serum (FBS), and maintained in a 5% CO_2 air-humidified atmosphere at 37 °C. As previously described, two individual EZH2-specific small interfering siRNAs with the sequences 5′—UUUCCUUGGAGGAGUAUCCACAUCC-3′ (#1) and 5′-GGAUGUGGAUACUCCUCCAAGGAAA-3′ (#2) were chemically synthesized (Invitrogen, Tokyo, Japan).[9,11,29,30] Stealth RNAi Negative Control (Invitrogen, Tokyo, Japan) was used as a negative control. The dose of siRNA was set at 100 nM to cause <20% inhibition of EZH2. At 24 h after plating, the cells were transfected with 100 nM EZH2-siRNA or control siRNA using Lipofectamine Transfection Reagent RNAiMAX (Invitrogen, Tokyo, Japan) in accordance with the manufacturer's instructions. At 48 h after transfection, cells were harvested and subjected to western blotting analysis and quantitative real-time polymerase chain reaction (qRT-PCR). We finally used siRNA#2 for subsequent experiments. All transfections were performed in triplicate and repeated three times.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA extraction and qRT-PCR were performed as described previously.[9,11,29,30] The transcript levels were measured in a duplicate set of reactions and calculated as fold change relative to control siRNA-transfected cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were used for normalization. The sequences of primers used in this study were as follows: GAPDH: F 5′-AGCCACATCGCTCAGACAC-3′, 5′-GCCCAATACGACCAAATCC-3′; VASH1: F 5′-AAGCACTCGGTGCTGGAC-3′, R 5′-GGTCCTTGGTGGGAGAGG-3′.

Western blotting

Western blotting was performed as described previously.[31] Cells were lysed in cell lysis buffer containing 25 mM Tris (pH7.4), 100 mM NaCl, and 1% Tween 20. Equal amounts of protein were loaded onto 10% gels and separated by SDS-PAGE. Resolved proteins were electrophoretically transferred to polyvinylidene

Please cite this article in press as: Nakagawa S, et al., Enhancer of zeste homolog 2 (EZH2) regulates tumor angiogenesis and predicts recurrence and prognosis of intrahepatic cholangiocarcinoma, HPB (2018), https://doi.org/10.1016/j.hpb.2018.03.018