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Original contribution

The histone methyltransferase G9a: a new therapeutic target in biliary tract cancer☆,☆☆

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Summary The histone methyltransferase G9a (EHMT2) is a key enzyme for dimethylation of lysine 9 at histone 3 (H3K9me2), a suppressive epigenetic mark. G9a is over-expressed in tumor cells and contributes to cancer aggressiveness. Biliary tract cancer (BTC) is a rare cancer with dismal prognosis due to a lack of effective therapies. Currently, there are no data on the role of G9a in BTC carcinogenesis. We analyzed G9a expression in n = 68 BTC patient specimens and correlated the data with clinicopathological and survival data. Moreover, we measured G9a expression in a panel of BTC cell lines and evaluated the cytotoxic effect of G9a inhibition in BTC cells using established small-molecule G9a inhibitors. G9a was considerably

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expressed in about half of BTC cases and was significantly associated with grading and tumor size. Additionally, we observed significant differences of G9a expression between growth type and tumor localization groups. G9a expression diametrically correlated with Vimentin (positive) and E-Cadherin (negative) expression. Importantly, survival analysis revealed G9a as a significant prognostic factor of poor survival in patients with BTC. In BTC cells, G9a and H3K9me2 were detectable in a cell line–dependent manner on mRNA and/or protein level, respectively. Treatment of BTC cells with established small-molecule G9a inhibitors resulted in reduction of cell viability as well as reduced G9a and H3K9me2 protein levels. The present study strongly suggests that G9a contributes to BTC carcinogenesis and may represent a potential prognostic factor as well as a therapeutic target.

1. Introduction

Epigenetic regulation is essential for cellular function and diversity. Major mechanisms include DNA methylation and histone protein modifications. Histone methylation is an epigenetic mark leading to either transcriptional activation or repression [1].

G9a (EHMT2) is a histone methyltransferase that dimethylates lysine 9 at histone 3 (H3K9me2), thus reducing transcription. G9a consists of a catalytic active SET domain, ankyrin repeats for protein–protein interactions and a nuclear localization signal [2]. Physiologically, G9a is required for correct differentiation of embryonic stem cells and immune cells [2]. G9a depletion resulted in global methylation loss specifically at euchromatin—a unique feature suggesting that G9a controls active promoter regions [2]. Overly active G9a contributes to development and progression of various cancers and is directly involved in cancer metabolism, metastasis, cell survival and response to hypoxia [2-5]. Currently, only a few studies have investigated G9a in human tumors demonstrating higher expression of G9a in cancer tissues compared to healthy controls [3,5-10]. Moreover, high G9a expression was associated with unfavorable clinicopathological parameters and poor survival in lung, ovarian, endometrial cancer as well as in esophageal squamous cell and hepatocellular carcinoma (HCC) [3,7-10].

Biliary tract cancers (BTC) are categorized into intrahepatic, perihilar, extrahepatic BTC (or cholangiocarcinoma) as well as gallbladder cancer (GBC). Although the incidence of BTC is low in developed countries (3% of gastrointestinal cancers), BTC is the second most common primary liver tumor after HCC [11]. Current therapies include surgery (applicable in only 30%), chemotherapy (cisplatin, gemcitabine), radiation, and photodynamic therapy (PDT) [11-13]. However, due to the heterogeneous and aggressive nature as well as high therapeutic resistance, prognosis remains poor with a 5-year survival rate of only 5% to 10% [11]. A better understanding of the molecular oncogenesis of BTC and identification of new therapeutic targets is therefore of utmost importance.

As currently no studies describe the role of G9a in BTC, this study aims at initial evaluation of G9a as a potential prognostic marker and therapeutic target in BTC and to examine the effect of G9a inhibition in an in vitro BTC cell model.

2. Materials and methods

2.1. Clinical BTC samples and immunostaining

Sixty-eight (68) cases of formalin-fixed, paraffin-embedded (FFPE) BTC samples archived between 1997 and 2017 at the Institute of Pathology (Paracelsus Medical University, Salzburg, Austria) were included and comprise intrahepatic, perihilar and extrahepatic cases. Immunohistochemical analyses (IHC) of human BTC samples were carried out on anonymized specimens according to the local ethics committee (Reference No. 415-EP/73/37-2011). IHC for G9a (ab134062, Abcam, Cambridge, UK) was performed with a dilution of 1:200 (no pre-treatment) as previously described for E-Cadherin and Vimentin [14] using the "quickscore" method (multiplication of the intensity [0–3] and extensity [0%-100%]) [15]. For double IHC stainings, the first (G9a) and second (E-Cadherin and Vimentin) antibodies were sequentially detected with the DAB (brown color) and Fast-Red (red) chromogen detection kit (Ventana, Tucson, AZ).

2.2. Substances and cell culture

G9a inhibitors BIX01294 and BRD4770 were purchased from Selleckchem (Houston, TX) and dissolved in cell culture−grade water (for BIX01294) or dimethyl sulfoxide (DMSO, Sigma Aldrich, Vienna, Austria; for BRD4770). UNC0642 was purchased from MedChem Express (Monmouth Junction, NJ) and dissolved in DMSO. Inhibitors were stored in aliquot stocks of 10 mmol/L at −20 °C. Resazurin was purchased at Sigma Aldrich and dissolved in Dulbecco's Phosphate Buffered Saline (DPBS, Sigma Aldrich).

A panel of nine cell lines was used in the experiments: BDC, CCSW-1, EGi-1, HuCCT1 [16], SkChA-1, TFK-1 as bile duct carcinoma cell lines and GBC, MzChA-1, MzChA-2 as gallbladder cancer cell lines (see [17] for references). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Vienna, Austria) as described previously [17,18]. See Supplementary Table 1 for cell seeding concentrations.