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TITLE OF THE Ph.D. THESIS

Identification of PRMT5 as a therapeutic target in cholangiocarcinoma

Scientific Disciplinary Sector(s)

MED-06

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APPENDIX

INTRODUCTION

Despite the discovery and introduction of new drugs in the treatment of cholangiocarcinoma (CCA) this disease still remains a very aggressive disease, with a lack of response to the standard treatment and with a poor prognosis. For this reasons it's strongly necessary the development of new strategies of treatment. Nowadays, with the development of personalized treatments and molecularly targeted drugs, it is necessary to know and discover the pathogenetic mechanism causing the development and progression of neoplastic diseases.

The rationale of this study lays in these assumptions and aim to increase the knowledge of genetic and epigenetic mechanism playing a possible role in the development of cholangiocarcinoma. As the PhD requires the involvement in molecular and translational research, other ambitious aim of this study is to try to identify a new possible therapeutic target in cholangiocarcinoma.

The study aims to analyze the implications, functions and interactions of Protein Arginine Methyltransferase 5 (PRMT5) in cholangiocarcinoma. We also wanted to understand how the inhibition of PRMT5 affects the replication and proliferation of cell of cholangiocarcinoma. The study was articulate to validate the biological assumptions and analyze the activity and efficacy of PRMT5 inhibitors.

1. CHOLANGIOCARCINOMA

The cholangiocarcinoma (CCA) is a malignant aggressive neoplasm originating from epitelial cells of biliary ducts of the liver. Even if the incidence of this tumor is globally low (less than 1%) it is still remain the second most common tumor of the liver with evidences of incidence increasing. Based on the site of origin, it is possible to divide the tumor in three groups: intrahepatic (iCCA) when the origin is into the hepatic parenchyma and it represents about 6-10 % of the cases, perihilar (pCCA) whit an origin on the perihilar area of liver and are about the 60% and distal (dCCA) when the tumor origin from the extrahepatic biliary duct[1].

1.1 Pathogenesis

The main model of pathogenesis of CCA identifies the origin and development of the tumor in a condition of cholestasis and chronic inflammation of biliary ducts[2]. A study of D. Sia et al identifies two main biological classification of intrahepatic cholangiocarcinoma[3]. The inflammatory group (38% of the iCCs) is characterized by an activation of a pro-inflammatory signal, expression of cytokines and activation of STAT3. The proliferative group (62% of iCCs) is characterized by an activation of pro-oncogenic signals (RAS, MAPK and MET between the others). In the process of cholangio-carcinogenesis influenced by inflammatory processes, inteleukin-6 (IL6) is the main cytokine involved. The IL6 through the link with its membrane receptor can activate some kinases (as Jak1 and Jak2) involved in the process of phosphorylation of transcription factors. Moreover the IL6 through the phosphorylation of ERK1/2 may regulate the synthesis of different transcription factors. SOCS3 results as a negative feedback regulation mechanism of IL6[4]. In cholangiocarcinoma the SOCS3 gene results often silencied by DNMT1[5]. Moreover the process of methylation described in cholangiocarcinoma mediated by DNMT1 seems to not be limited only to SOCS3 but also be involved even in the regulation of other tumor suppressor gene. DNA methylation seems another epigenetic mechanism relevant in the CCA development. DNA methylation silence tumor suppressor genes with consequent alteration of cell cycle[6]. Other studies tried to investigate the possible roles of

genetic factors involved in the process of development of CCA. Epigenetically, CCA tumors exhibit DNA hypermethylation with distinct DNA hypermethylation patterns[7]. IL6 decrease DNA methylation on promoter region of epidermal growth factor receptor (EGFR) gene with consequent increase of the EGFR protein[8].

1.2 Molecular classification

Nowadays are stronger the evidences about the different molecular profile between iCCA and eCCA[9] (fig. 1). As demonstrated in the clinical practice, the main targetable aberrations in patients with iCCA are the altered forms of IDH1 and FGFR2 while HER2 aberrations seem to be the most frequent in eCCA and gallbladder cancer (GBC)[9].

About iCCA the most frequent somatic mutations known result TP53 and KRAS which are genes involved in cell cycle regulation. Other single nucleotide variants of ARID1A, PBRM1, BAP1 have been reported with a significant incidence in iCCA. IDH1/2 mutations are known to be a therapeutic target in iCCA with an incidence of about 15% of the cases[10]. Are described other somatic mutations related to Akt pathway as PTEN and PI3K and other related to TGF-β pathway as SMAD4[11].

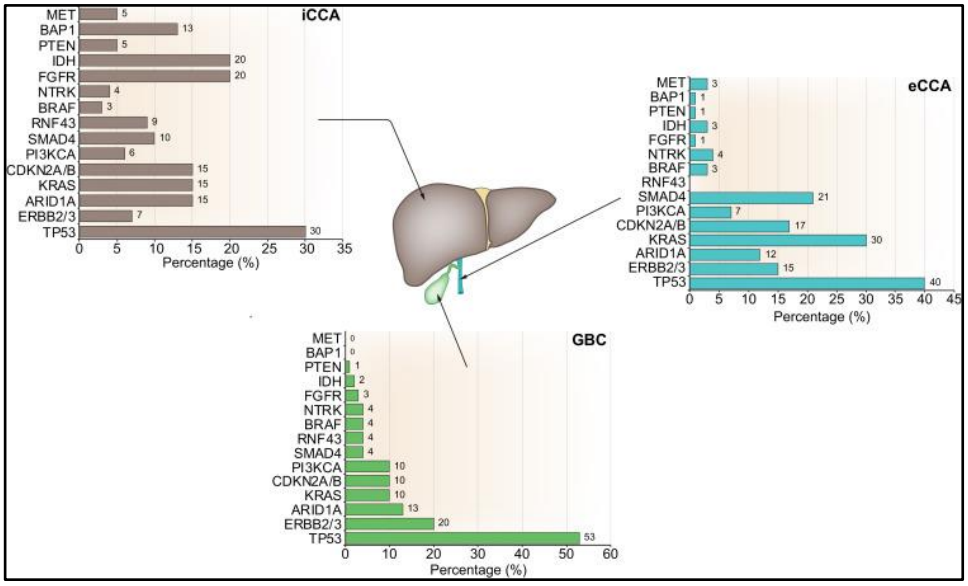


Fig. 1 Molecular profiling of biliary tract cancers. Percentage of main targetable and non-targetable genetic alterations are summarised for iCCA, eCCA, and GBC. Lamarca A, et al. J Hepatol. 2020;73:170–85

About the described mutations in iCCA it seems also there could be a correlation with neither clinical feature as the presence or absence of cirrhosis related hepatitis or etiology (as Opistorchis viverrini related CCA). Genetic alterations are also correlated with pathological features. Hotspot mutations in KRAS have been reported in periductal infiltrating type, but not in mass-forming type. Histopathologically, small duct type has been reported to have more frequent BAP1 and IDH1/2 hotspot mutations and FGFR2 fusion, and lower incidence of KRAS mutation than large duct type. On the contrary, large duct type is known to have frequent mutations in TP53, KRAS and some TGF-β pathway genes, including SMAD4, TGFBR2, FBXW7, and MYC[13].

2. METHYLATION AND CANCER

2.1 Role of methylation in malignancies

Despite the new knowledge of pathogenic mechanism involved in the development and progression of cholangiocarcinoma, still remain few the therapeutic options per the treatment of patients affected by CCA. For this reason it is mandatory to identify new therapeutic targets for the treatment of CCA. As described before, the methylation of DNA and protein is acquiring everyday a major interest in the investigation against cancer. DNA methylation and histone tail modifications are some of the epigenetic mechanisms crucial for the maintenance of heritable changes in gene expression. Epigenetic regulation of transcription allows cells to develop into different phenotypes.

The presence of an aberrant DNA methylation seems to be an often described phenomenon in cancer cells[14]. The hypothesis is that many cancer cells seem to originate or be dependent from methylation of few vital regions either as a process of cancer-genesis or maintenance of their proliferation. In a study of Heyn et al is evaluated the pattern of methylation of DNA methylation alteration in cancer cells of the distal regulatory sequences described as super-enhancers[15]. Based on the evidences available, it's possible to interpret the role of DNA methylation or as an expression of genetic mutation on mutated cells or as an epigenetic event with a consequent oncogenic transformation[16]. In fact the methylation is a post-transcriptional that can modify the structure and the functions of a protein but also change the activation status of a gene.

In the field of post-translational modification, arginine methylation seems to be one of the key events also due to is frequency. The nitrogen atoms of arginine within polypeptides can be modified to contain methyl groups, a process termed arginine methylation[17]. This process is mediated by the nine members of protein arginine methyltransferases (PRMTs) [18] although there are other proteins involved in the same process. PRMTs catalyze the transfer of a methyl group from S-adenosyl methionine (SAM) to the guanidino nitrogen atoms of arginine. The family of PRMTs include nine enzymes and it's divided in three classes: type I (PRMT1-2-3-4-6-8)

characterized by the asymmetric methylation of the substrates; type II (PRMT5 and PRMT9) characterized by a symmetric methylation activity; type III (PRMT7) characterized by a mono-methylation of arginine[19]. Between all the members PRMT5 is the main studied.

2.2 PRMT5: activity and role in cancer

The functional complex of PRMT5 result an octamer made by four molecules of PRMT5 and four molecules of its co-factor methylosome protein 50 (MEP50 or WDR77). The complex PRMT5-MEP50 showed a high affinity for SAM which is the molecule releasing the methyl group20. The methylation of protein PRMT5 mediated is a process involved in physiological processes of the cells but it seems to be involved even in pathological processes of tumor genesis through the regulation of tumor promotion or suppression (fig. 2). Some non-histone proteins with a tumor suppressor activity were identified as substrate of PRMT5. For example, STRAP is a member of p53 complex activated in the response to DNA damage. STRAP can recruit PRMT5 allowing the methylation of arginine with a consequent increase of nuclear p53 and expression of p21 and PUMA21. Another example is the methylation of E2F-1 which regulates the cell cycle acting as tumor suppressor. The methylation of E2F-1 mediated by PRMT5 reduces its half-life with an increase of cell life22. PRMT5 also methylate KLF4 with a reduction of the ubiquitination of VHL and consequent stabilization of KLF4. The increase of availability of KLF4 has been showed to contribute to the tumor genesis of mammal carcinoma[23]. PRMT5 methylate SREBP1 preventing the phosphorylation of GSK3β and the ubiquitination by FBXW7 with a consequent activation of lipogenesis and tumor growth in hepatocellular carcinoma (HCC)[24]. Other than regulate the activation of FGFR genes[25], PRMT5 can mutilate directly many proteins involved in the pathways of proliferation, differentiation and survival of cancer cells. The genetic of pharmacological inhibition of PRMT5 increase the amplitude of RAS/ERK activation[26]. Even platelet derived growth factor receptor (PDGFR) results positively regulated by PRMT5 that increase the availability of PDGFRα with activation of AKT/ERK necessary for cell proliferation[27]. NF-ƙB is known for the role in tumor genesis. PRMT5

activate NF-ƙB through the direct methylation of p65 subunit. Moreover, PRMT5 interacts even with TRAIL receptor with the TRAIL mediated activation of NF-ƙB[28].

It seems that PRMT5 could identify possible clinical application other than the evidences describe in vitro experiments.

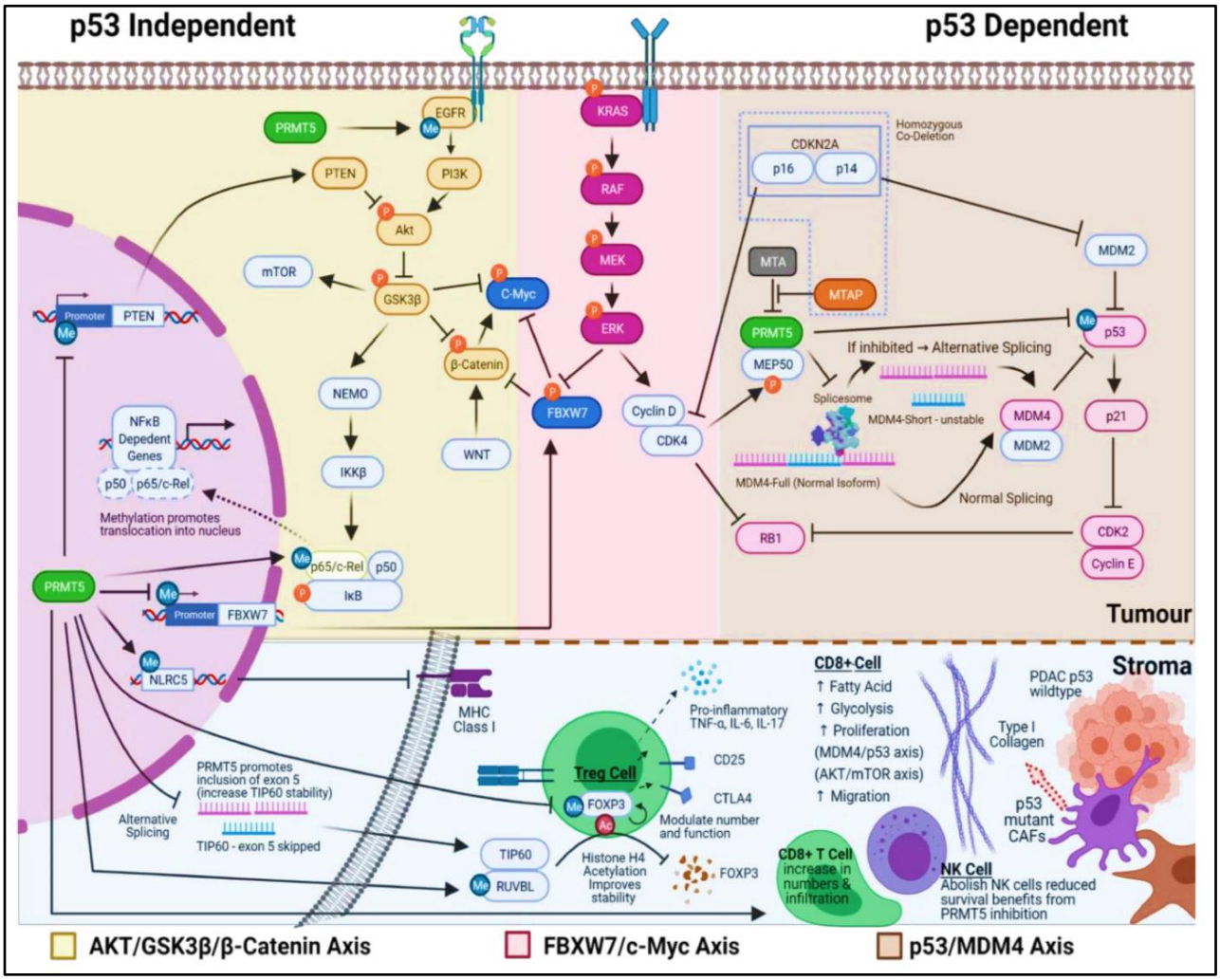


Fig. 2 The mechanism of action of PRMT5 is illustrated in the figure, which highlights three well-characterized pathways through which PRMT5 can promote cancer progression, potentially relevant to PDAC. These include the AKT/GSK3-β/β-Catenin axis, the FBXW7/c-Myc axis, and the p53/MDM4 axis. The intricate interactions between methylation, phosphorylation, acetylation, and ubiquitination are represented by blue (Me), orange (P), and brown (Ac) colors, respectively. The lower panel outlines some of the potential effects of PRMT5 inhibition on the tumor microenvironment, specifically how these effects may relate to the known characteristics of the PDAC stroma and its interaction with tumor cells. Michael K. C. Lee, Cancers 2021, 13, 5136

A Japanese study tries to identify a correlation between PRMT5 and the prognosis in patients with resected HCC[29]. It is reported that mRNA expression of PRMT5 resulted higher in HCC tissue compared to normal liver tissue, cirrhotic and chronic hepatitis tissue of liver (fig. 3).

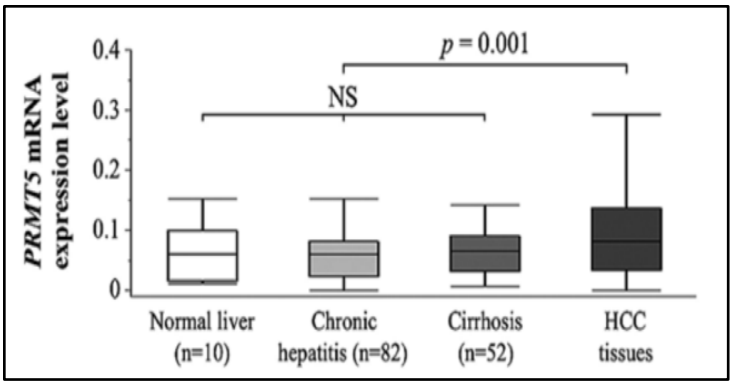


Fig. 3 Variations of mRNA levels of PRMT5 in different clinical hepatic conditions. Michael K. C. Lee, Cancers 2021, 13, 5136

In the same study the patients underwent surgical resection were followed and the authors correlate the overall survival (OS) with the levels of PRMT5 mRNA in the resected tissue. They showed how the patient with an high expression of PRMT5 in resected tissues showed a worst prognosis compared to patients with a low expression of PRMT5 mRNA (5y OS rate 53% vs 76%, p=0.021). PRTM5 seems to be a possible prognostic factor in terms of OS in hepatocellular carcinoma (fig. 4).

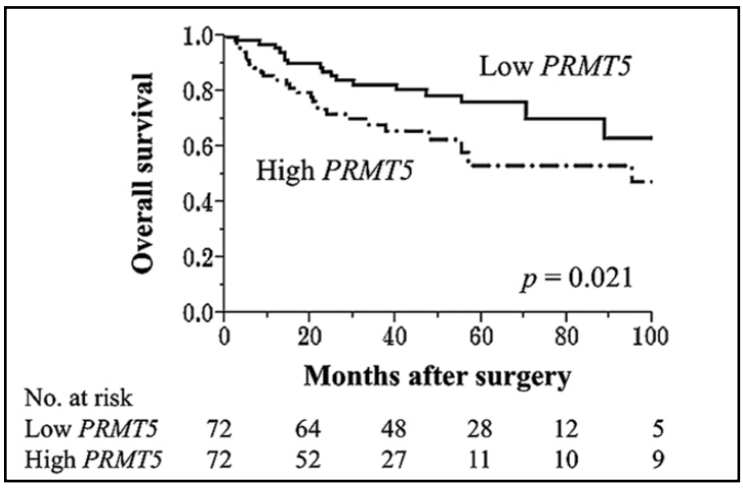


Fig. 4 Correlation between PRMT5 mRNA levels and Overall Survival in patients affected by HCC who underwent surgical resection.

Another study analyzed the possible prognostic role of PRMT5 in pancreatic carcinoma[31]. The authors used the clinical information available in TCGA-PAAD RNA-seq dataset. The analysis showed how the patients with pancreatic adenocarcinoma with high expression of PRMT5 mRNA showed a shorter OS (p=0.015) and a shorted Disease Free Survival (DFS) (p=0.015) (fig. 5).

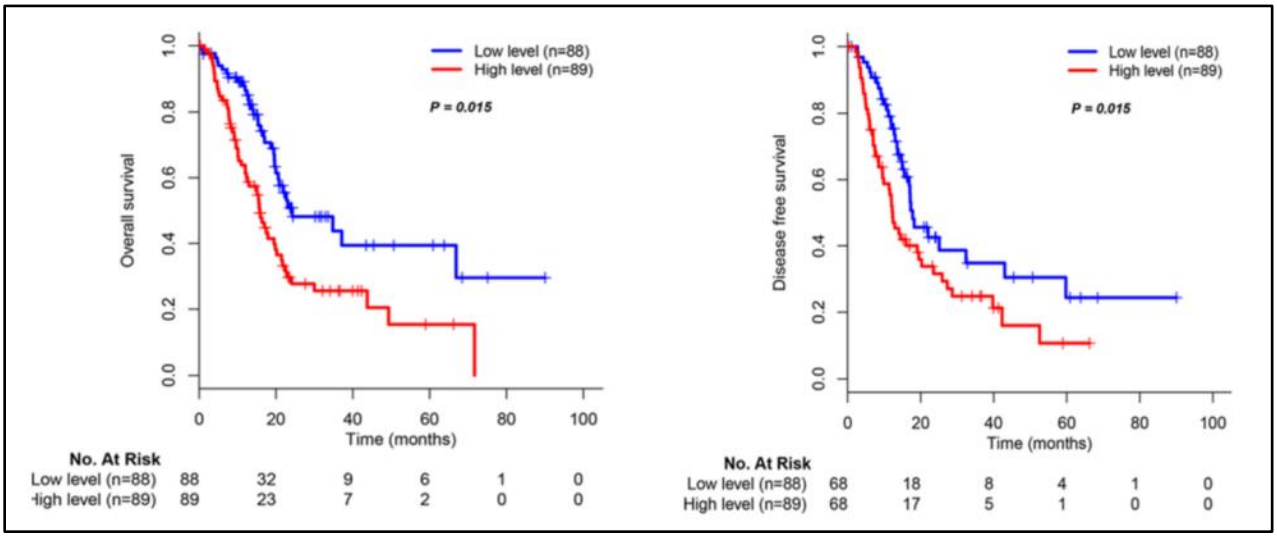


Fig. 5 PRMT5 expression in the TCGA-PAAD RNA-seq dataset was analyzed, and the results showed that higher PRMT5 expression correlates with a shorter overall survival (OS) time. Additionally, patients with high PRMT5 expression exhibited significantly shorter disease-free survival (DFS) times compared to those with low PRMT5 expression.

3. AIM OF THE STUDY

As already mentioned, PRMT5 is highly expressed in cancer cells and tissues, and its overexpression is directly linked to tumor progression in a broad range of solid malignancies. The aim of the study was to evaluate if PRMT5 could be a possible therapeutic target in cholangiocarcinoma. There are many observations and robust pre-clinical studies demonstrating the efficacy of PRMT5 inhibitors in tumors growth and reproduction. As PRMT5 was demonstrate to form a hetero-octameric complex together with methylosome protein 50 (MEP50) we extend our investigations also to MEP50. In this study we wanted to show how PRTM5 and MEP50 are overexpressed in cholangiocarcinoma. To study the function of PRMT5 in cholangiocarcinoma we defined experimental models in vitro to evaluated the role of PRMT5 and the effect of its inhibition under the activity of specific inhibitors of PRMT5.

We analyzed the expression of PRMT5 and MEP50 in transcriptomic datasets from human CCA tissues to confirm the hypothesis of and overexpression of PRMT5 in human CCAs. Based on this data, we wanted to confirm the expression of PRMT5 in human and mice tissue samples of cholangiocarcinoma. For this reason we performed immunohistochemistry (IHC) on malignant tissues of CCAs. Moreover we tried to identify a score of the expression of PRMT5 and MEP50 and demonstrate a possible prognostic value of the quantitative expression of PRMT5. To confirm the data of IHC on human and mice tissue samples of cholangiocarinoma, we wanted to quantify PRMT5 and MEP50 in different cell lines of CCA through Western Blot method. Once we confirmed the expression of PRMT5 and MEP50, we wanted to demonstrate the efficacy of pharmaceutical inhibition of PRMT5 in terms of reduction of the S-dimethyl-arginine (SDMA).

We performed in vitro experiments to confirm the activity of different molecules of inhibitors of PRMT5 in CCAs cell cultures. After demonstrating the efficacy of drugs inhibiting PRMT5 in CCAs, thinking about a possible application in clinical practice, we wanted to evaluate the synergic activity of PRMT5 inhibitors with the standard chemotherapy used in the treatment of cholangiocarcinoma (Cisplatin and Gemcitabine). Still in terms of possible clinical applications of

the drugs used to inhibit PRMT5, we also evaluated the effect of PRMT5 inhibition on the property of CCA cell to form and grown into colony. To further characterize the antineoplastic effects of PRMT5 inhibition in CCA cells, we performed complementary proteomic analyses. We also performed Western Blot analyses to understand the possible effect of PRMT5 inhibition on genes expression.

4. EXPERIMENTAL PROCEDURES

4.1 Cancer genome data analyses

We wanted confirm the significant hyper-expression of PRMT5 and MEP50 on genome data analysis available. We use the information available on TGCA Gene dataset on https://ualcan.path.uab.edu/index.html to confirm a higher expression of PRMT5 and MEP50 in cholangiocarcinoma tissue compared to normal biliary tract tissue. As represented in the image below the expression of PRMT5 and MEP50 in cholangiocarcinoma doesn't represent the higher expression. Analyzing the difference of expression compared to normal biliary duct tissue we observed a significant increase of PRMT5 and MEP50 expression (fig. 6).

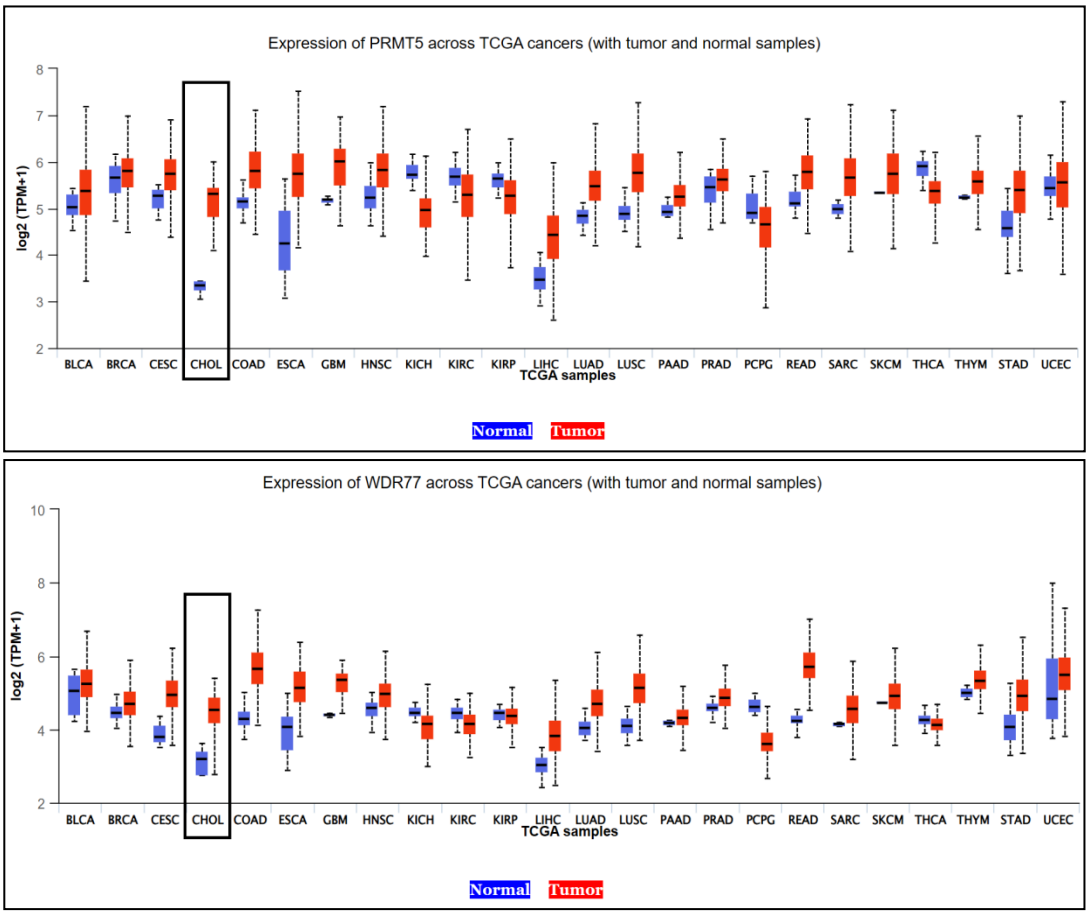


Fig. 6 PRMT5 and MEP50 gene expression in human cholangiocarcinoma (CCA): mRNA levels in intrahepatic CCA (iCCA) compared with normal bile ducts.

In another transcriptomic dataset it is confirmed the increase in mRNA expression of PRMT5 and MEP50 in iCCA compared to normal tissue (fig. 7). We did find a positive correlation between the expression of both genes (PRMT5 and MEP50).

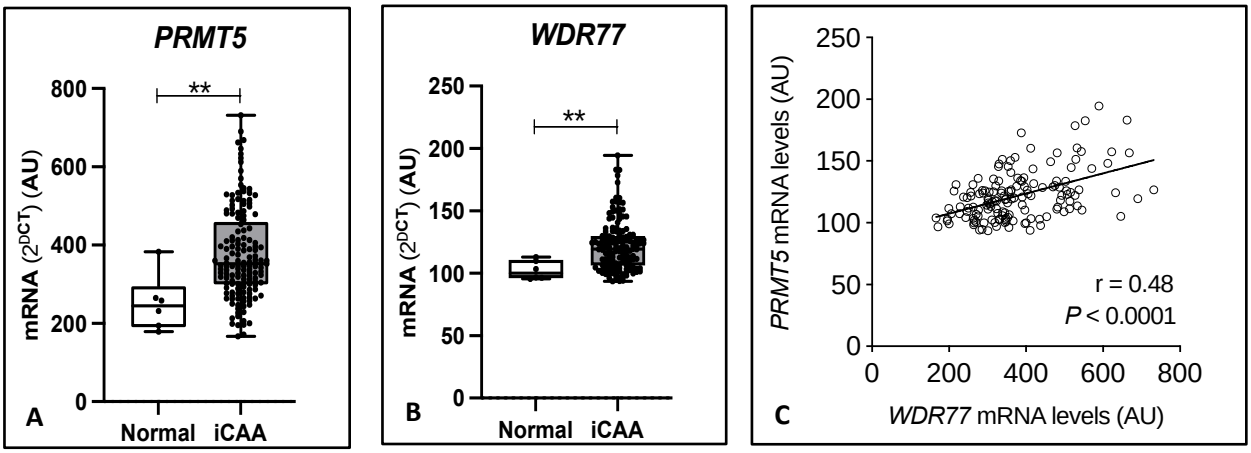


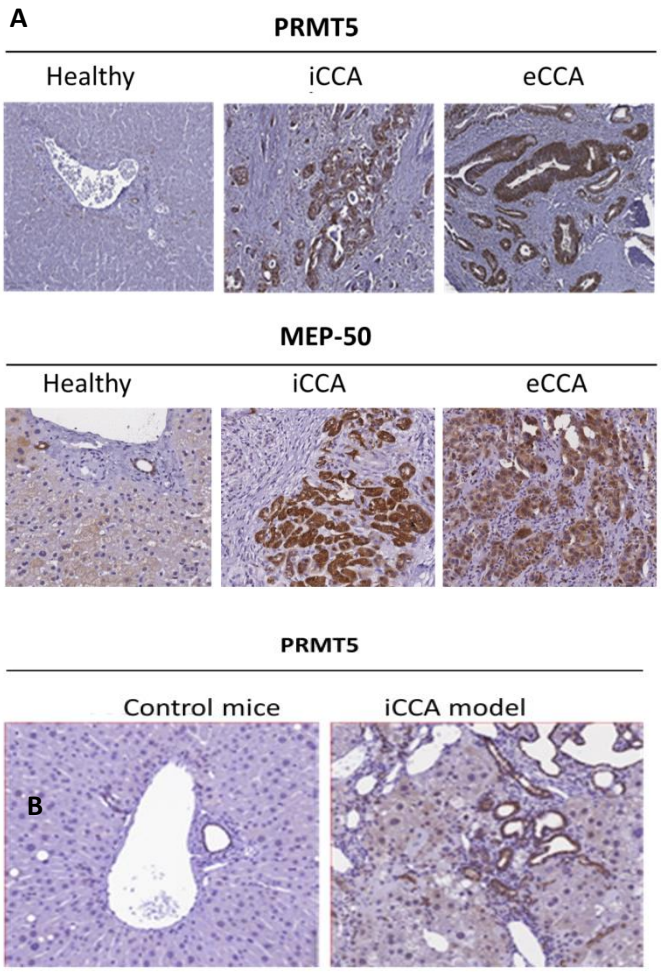
Fig. 7 PRMT5 and MEP50 gene expression in human cholangiocarcinoma (CCA) (transcriptomic dataset GSE32225): A-B) mRNA levels of PRMT5 in intrahepatic CCA (iCCA) compared with normal bile ducts. C) Correlation of mRNA expression of PRMT5 and MEP50 in iCCA.

4.2 Immunohistochemistry confirmation of PRMT5 and MEP 50

We wanted to confirm the overexpression of PRMT5 and MEP50 either than in genome dataset also in tissue samples of cholangiocarcinoma. CCA tissues were obtained from patients with iCCA (n=73; 43 surgical samples, 30 needle biopsies) or eCCA (n=23) that underwent biopsies or surgical resection for diagnostic or therapeutic purposes (fig. 8). Tumor grade was established on anatomopathological examination of tissue sections according to the WHO Classification of Tumors, Digestive System Tumors, fifth edition.

Consecutive sections (3 µm thick) cut from formalin-fixed paraffin-embedded human tumor tissues were deparaffined with xylene, dehydrated with ethanol, and incubated with 3% hydrogen peroxide to block endogenous peroxidase. Antigen retrieval was performed by heating in 10 mM Tris-EDTA buffer pH 9 before incubation with antibodies for PRMT5 (ab109451, Abcam, 1:200), MEP50 (HPA027271, Merck, 1:1000), MTAP (ab254265, Abcam, 1:1000). HRP-conjugated Envision secondary antibody (K4003, DAKO) followed by DAB reagent

(K3468, DAKO) were applied for the detection procedure. Tissue sections were counterstained with Hematoxylin (Sigma, St. Louis, MO, USA), dehydrated with ethanol and mounted in DPX (Sigma). Negative controls were included omitting primary antibodies. For mouse liver samples the immunohistochemistry protocol was the same used in human samples and with the same antibody for PRMT5 (1:500).



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	Intrahepatic (n=73)		Extrahepatic (n=23)	
	Number	Percentage (%)	Number	Percentage (%)
Age				
30-40	2	2.74	0	0.00
41-50	2	2.74	0	0.00
51-60	10	13.70	7	30.43
61-70	25	34.25	6	26.09
71-80	20	27.40	9	39.13
81-90	14	19.18	1	4.35
Gender				
Women	35	47.95	9	39.13
Men	38	52.05	14	60.87
Grade				
G1	17	23.29	3	13.04
G2	22	30.14	11	47.83
G3	34	46.58	9	39.13
Tumor				
T0	0	0	0	0.00
T1	10	13.70	7	30.43
T2	22	30.14	6	26.09
T3	24	32.88	9	39.13
T4	17	23.29	1	4.35
Tx	0	0	0	0.00
Metastasis				
M0	44	60.27	18	78.26
M1	21	28.77	4	17.39
Mx	8	10.96	1	4.35
Node				
N0	16	21.92	6	26.09
N1	24	32.88	13	56.52
N2	11	15.07	1	4.35
N3	0	0	1	4.35
N4	0	0	1	4.35
Nx	22	30.14	1	4.35
Resection margins				
R0	39	53.42	13	56.52
R1	21	28.77	8	34.78
R2	13	17.81	2	8.70
Vascular invasion*				
0	30	41.10	10	43.48
1	43	58.90	11	47.83
x	0	0	2	8.70
Neural invasion*				
0	35	47.95	8	34.78
1	38	52.05	14	60.87
x	0	0	1	4.35

* 0: No
1: Yes
x: Not determined

Fig. 8 A) IHC scanning with an anti-PRMT5 and anti-MEP50 antibody performed on histological sections of intrahepatic and extrahepatic cholangiocarcinoma, as well as normal tissue B) IHC analysis of PRMT5 in histological sections of intrahepatic cholangiocarcinoma (iCCA) from a murine cholangiocarcinoma model C) Demographic and clinical information of CCA samples included in the study

As reported in the figure above, we performed IHC of 13 samples of mice model of cholangiocarcinoma. As result PRMT5 was more expressed in cholangiocarcinoma compare to normal tissue.

4.3 Immunohistochemistry score

Intensity of the signal (0 = no expression; 1 = weak expression; 2 = intermediate expression; 3 = high expression and percentage of positive cells were considered. Scores according to percentage of cells stained were as follows: 0 (no cell staining), 1 (≤30%), 2 (31–60%) and 3 (61–100%) (fig. 9). The final score was obtained by the multiplication of the intensity score by the percentage score. From the analysis of the final score of PRMT5 results in the iCCA a 53.3% of the samples with a high score (between 6 and 9) while the 24% of the sample showed and intermediate score (between 3 and 6). In the eCCA the 69.2% of the samples showed a high score. About the expression of MEP50, 49% of the iCCA samples showed a high score and 29% an intermediate score. About the eCCA, 70% of the sample showed a high score and 20% an intermediate score (fig. 10).

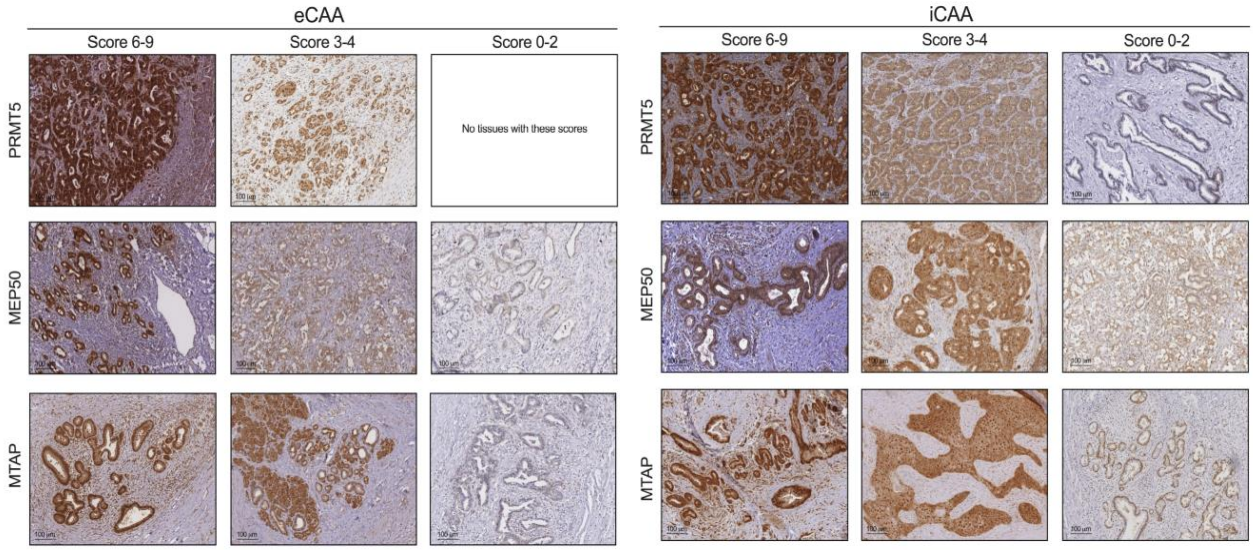

Fig. 9 Representative images of iCCA and eCCA tumours with 0-2, 3-4 and 6-9 PRMT5, MEP50 and MTAP immunostaining scores.

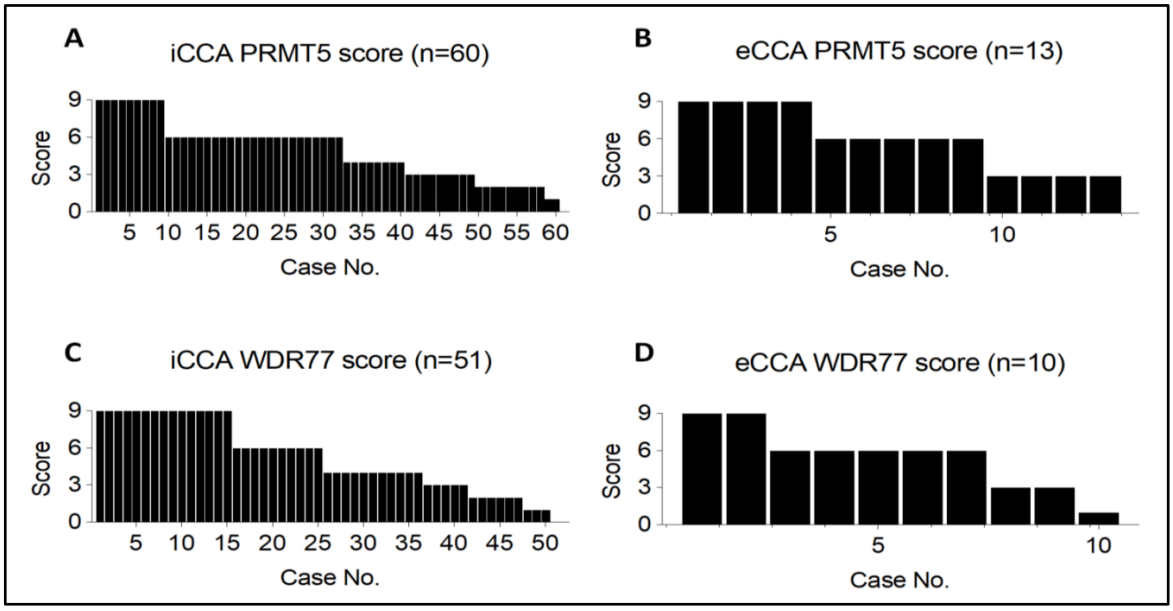

Fig. 10 Distribution of histological samples processed for IHC according to the documented score. A) IHC of PRMT5 in intrahepatic cholangiocarcinoma tissue samples B) IHC of PRMT5 in extrahepatic cholangiocarcinoma tissue samples C) IHC of WDR77 (MEP50) in intrahepatic cholangiocarcinoma tissue samples D) IHC of WDR77 (MEP50) in extrahepatic cholangiocarcinoma tissue samples.

4.4 Immunoblot analysis

An analysis of data about the expression of PRMT5 in CCA cell lines (https://score.depmap.sanger.uk/) supported the hypothesis of PRMT5 as a therapeutic target. We found that 83% of the CCA cell lines showed a significant reduction of the growth with a genetic inactivation of PRMT5 and MEP50 (fig. 11).

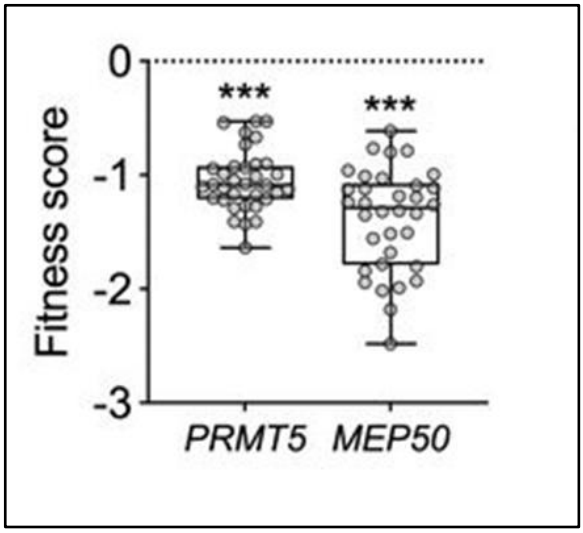

Fig. 11 CCA cell lines viability (fitness score) on CRISPR/Cas9 drop- out screen for PRMT5 and MEP50. Negative values indicate reduced survival on gene knockout. Data were retrieved from https://score.depmap. sanger.uk/.

Based on this information we wanted to confirm the expression of PRMT5 and MEP50 in different CCA cell lines through immunoblot analysis. We selected six cell lines of CCA: KMCH, HuH28, EGI-1, TFK, HUCCT, KMBC and Mz-ChA-1. Cells were seeded in sterile plates and incubated for three days in culture medium. Subsequently, they were washed with DPBS (Dulbecco's Phosphate Buffered Saline), centrifuged at 4500 rpm for 5 minutes at 4°C, and the supernatant was discarded. Cell pellets were lysed using RIPA lysis buffer, followed by sonication and centrifugation at 14,500 rpm for 15 minutes at 4°C. The resulting supernatant was collected for further analysis. Protein concentrations from each cell line were determined using the Bradford assay. The antibodies used were: anti-Symmetric DiMethyl Arginine Motif (SDMA) (13222, Cell Signaling Technology), anti-MTAP (4158, Cell Signaling Technology). Blots were probed with anti-α-TUBULIN (2144, Cell Signaling Technology), anti-glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) (2118S, Cell Signaling Technology) or anti-β-ACTIN (ab6276, Abcam) to demonstrate equal loading. As reported in image below the expression of PRMT5 and MEP50 was confirmed in all the cell lines (fig. 12).

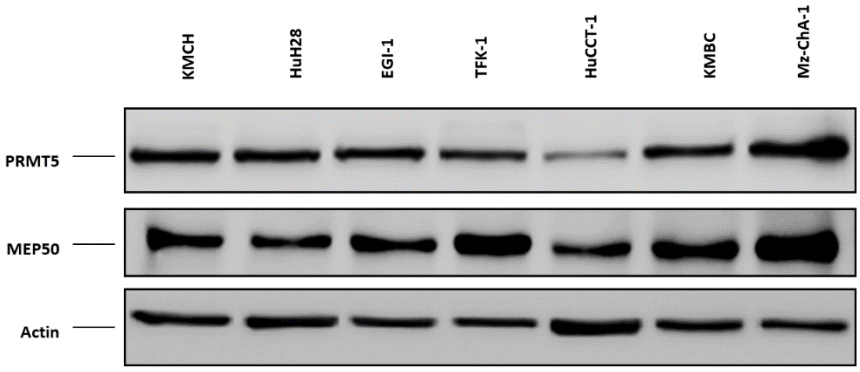

Fig. 12 Immunoblot assay of PRMT5 and MEP50 in different cell lines of CCA

4.5 PRMT5 inhibitors

After confirming the concrete expression of PRMT5 in different CCA cell lines, we wanted to confirm the efficacy of inhibition of PRMT5 in CCA cell lines. For this, we tested the effect of two clinically approved inhibitors of PRMT5: GSK3326595 and JNJ64619178[30].

GI_{50} calculation

We determined the GI_{50} of GSK3326595 and JNJ64619178 for each cell line and in all cases it was in the range of low nanomolar. Cells were grown in DMEM (EGI-1, KMCH) or DMEM-F12 (HuCCT1, TFK-1), supplemented with 10% FBS and antibiotics, all from Gibco Thermo-Fisher (Waltham, MA, USA). The concentration of drug inhibiting cell growth by 50% relative to the untreated control (GI_{50}) was calculated after curve fitting with GraphPad-Prism-v5 software. The same procedure was used for the calculation of GI_{50} of cisplatin and gemcitabine, the chemotherapies representing the standard first line of treatment in cholangiocarcinoma (fig.13).

Cell line	GI50 GSK	GI50 JNJ64619178	GI50 Cisplatin	GI50 Gem
HUCCT	0.25 µM	0.25 µM	3.6 µg/ml	12 µM
TFK	0.5 µM	0.5 µM	1.8 µg/ml	15 µM
KMCH	0.2 µM	0.2 µM	4.5 µg/ml	0.01 µM
SB1	1.5 µM	1.5 µM	1.5 µg/ml	~ 0.001 µM

Fig. 13 IC50 values for the PRMT5 inhibitors (GSK3326595 and JNJ64619178) and the chemotherapeutic agents Cisplatin and Gemcitabine determined through the described experiment

Synergistic Activity of PRTM5 inhibitors and chemotherapy

Combination strategies were explored to understand if the association of PRMT5 with cisplatin gemcitabine based chemotherapy could reveal a synergistic activity. With this purpose, we tested the combination of GSK3326595 and JNJ64619178 in combination with Cisplatin or Gemcitabine. For the calculation of combination index (CI) values, growth inhibition was determined at different combined concentrations of the PRMT5 inhibitor and the antitumoral drugs cisplatin and gemcitabine from Sigma (Sigma Aldrich, St. Louis, MO, USA). Briefly, 1000 cells were seeded in a 96-well plate in triplicates, after overnight culture PRMT5 inhibitors were added and four days later treatment with cisplatin or gemcitabine started. Three days later viability was measured using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. Data were analyzed using the Compusyn software (https://compusyn.software.informer.com/1.0/), and the combination index (CI) determined whether the effects of drug combinations were additive (CI=1) or synergistic (CI<1). In all cell lines and in the majority of combinations, a synergistic effect was observed in the combination of PRMT5 inhibitors and chemotherapy (fig. 14).

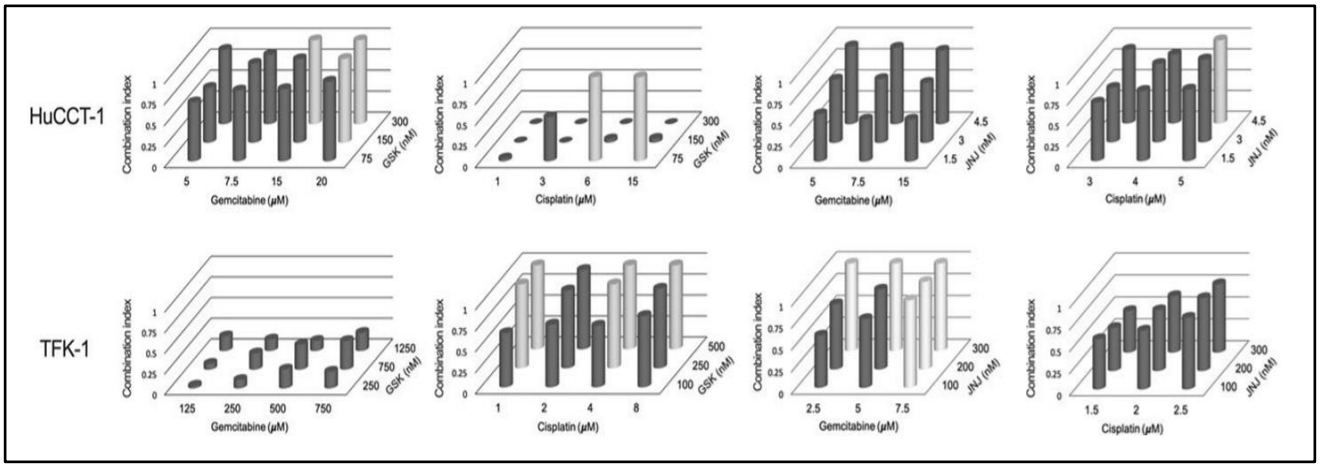

Fig. 14 Combination studies of the growth inhibitory effects of GSK3326595 or JNJ64619178 with cisplatin or gemcitabine in HuCCT- 1 and TFK- 1 cells. Dark grey bars denote the existence of synergism at the indicated doses (combination index, CI, <1)

4.6 Efficacy of PRMT5 inhibitors in CCA cells

After demonstrating a synergistic activity of PRMT5 inhibitors in combination with standard chemotherapy in the treatment of CCA, we aimed to assess the efficacy of GSK3326595 and JNJ64619178 in CCA cell lines.

First we performed an immunoblot analysis of PRMT5-dependent symmetric dimethyl arginine (SMDA) protein marks in control and GSK3326595 or JNJ64619178 treated HuCCT-1 and TFK-1 cells at the indicated doses for 3 and 5 days. Cells were cultured under standard in vitro conditions and treated, in biological duplicates, with escalating concentrations of the PRMT5-selective inhibitor GSK3326595. Treatment durations of 3, 5 or 7 days were applied, with vehicle-treated cells serving as controls. At the end of each exposure period, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested and lysed to obtain total protein extracts for downstream biochemical and molecular analyses. The antibodies used were: anti-Symmetric Dimethyl Arginine Motif (SDMA) (13222, Cell Signaling Technology) PRMT5 (ab109451, Abcam, 1:200), MEP50 (HPA027271, Merck, 1:1000), MTAP (ab254265, Abcam, 1:1000. Blots were probed with anti-α-TUBULIN (2144, Cell Signaling Technology), anti-

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2118S, Cell Signaling Technology) or anti-β-ACTIN (ab6276, Abcam) to demonstrate equal loading (fig. 15).

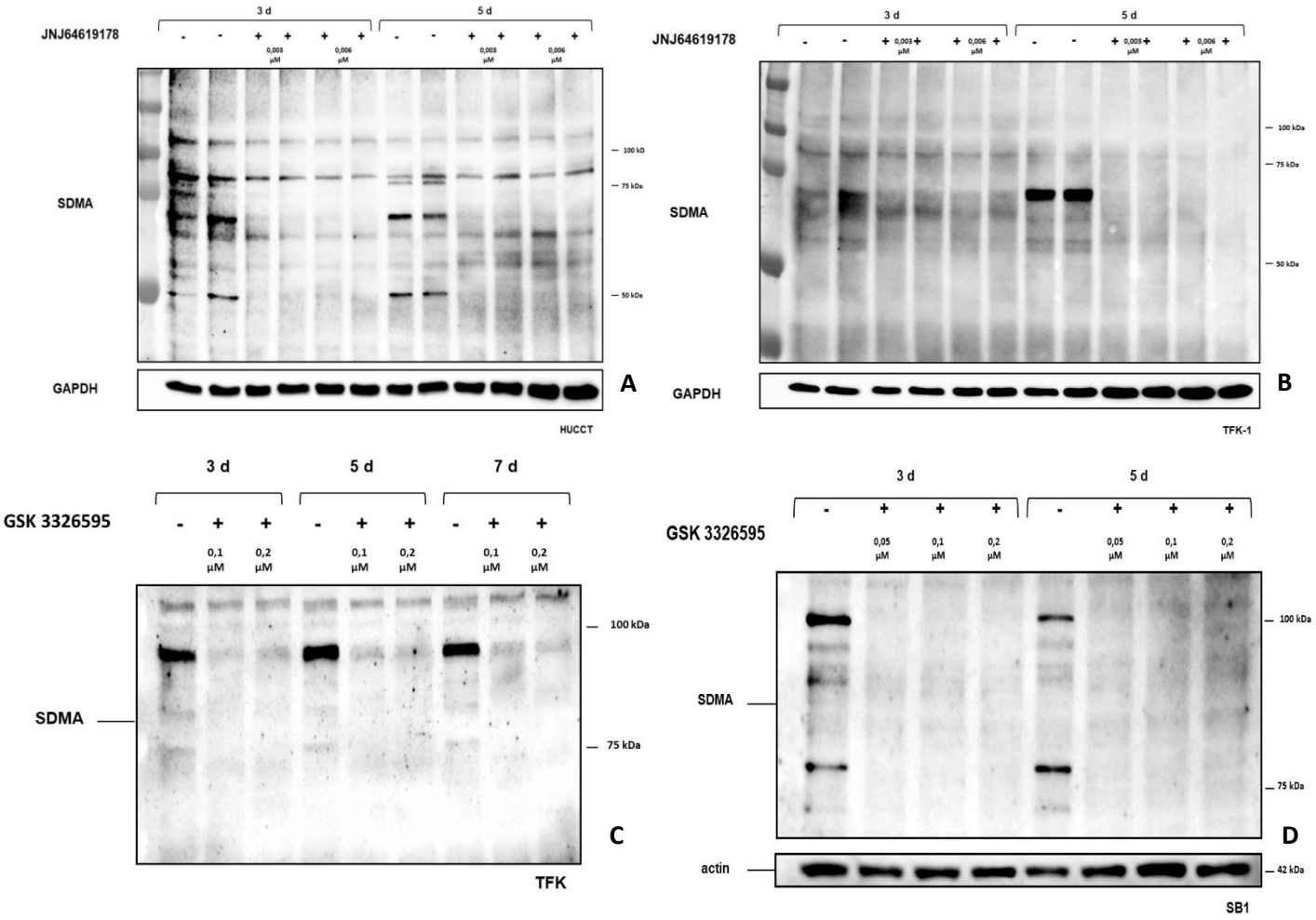

Fig. 15 Western Blot of SDMA A) immunoblot of SDMA in HUCCT cell treated in vitro with JNJ64619178 at a concentration of 0.008 µM and 0.006 µM for 3 days and 5 days compared to control B) immunoblot of SDMA in TFK1 cell treated in vitro with JNJ64619178 at a concentration of 0.008 µM and 0.006 µM for 3 days and 5 days compared to control. In A) and B) GAPDH as to demonstrate equal loading. C) immunoblot of SDMA in TFK cell treated in vitro with GSK3326595 at a concentration of 0.1 µM and 0.2 µM for 3, 5 and 7 days compared to control D) immunoblot of SDMA in SB1 cell treated in vitro with GSK3326595 at a concentration of 0.05 µM, 0.1 µM and 0.2 µM for 3 and 5 days compared to control.

The immunoblot confirmed a significant reduction of SDMA in cell treated with GSK3326595 and JNJ64619178 compared to cell not treated. The reduction of SDMA is already evident at the lowest dose in the shorter time and seems to be continuous in the time still after 7 days of

treatment. Based on these data, GSK3326595 and JNJ64619178 demonstrate their efficacy in terms of inhibition of PRMT5 activity in CCA.

To further validate the reduction of SDMA observed upon pharmacological inhibition of PRMT5, selective gene silencing of PRMT5 was performed to assess its effect on SDMA levels. The KMCH cell line, which basally expresses both PRMT5 and MEP50, was selected for this purpose. Gene silencing was achieved using RNA interference (RNAi). siRNA (small interfering RNA) is a class of double-stranded RNA molecules, 19–21 nucleotides in length, capable of specifically interfering with gene expression by binding complementary mRNA sequences, leading to mRNA degradation and preventing translation. Two RNAi-based approaches were employed: transient siRNA transfection and shRNA-mediated knockdown. Transient siRNA requires delivery via a lipid-based transfection reagent and typically induces temporary gene silencing. In contrast, shRNA (short hairpin RNA) is encoded by plasmid vectors, often lentiviral, and can achieve either transient or stable gene knockdown. Each shRNA plasmid expresses a specific hairpin RNA targeting the gene of interest.

KMCH cells were selected to PRMT5 knockdown using shRNA plasmids. Silenced cells were cultured in vitro, washed, and processed for protein extraction. Western blot analysis was initially performed to confirm effective suppression of PRMT5 translation. Once PRMT5 silencing was verified, the effects on MEP50 expression and SDMA levels were subsequently assessed by Western blot (fig. 16).

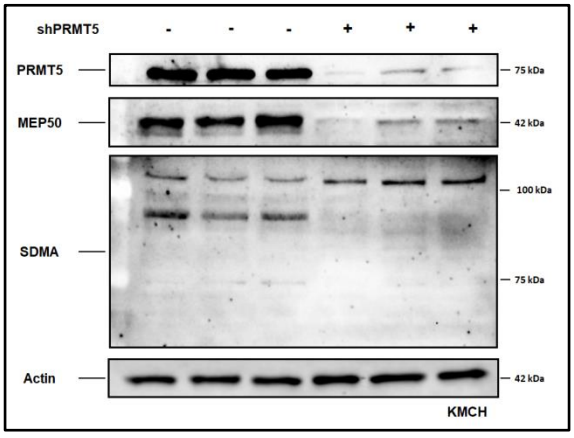

Fig. 16 KMCH cells were subjected to PRMT5 knockdown using shRNA. Western blot analysis was performed on triplicate samples of control and shPRMT5 cells to assess the expression levels of PRMT5, MEP50, and SDMA. β-Actin was used as a loading control.

4.7 PRMT5 inhibition and colony formation

After confirmed the efficacy of the inhibitors of PRMT5 in CCA cell lines, we aimed to evaluate the in the same celli lines the impairment in clonogenic activity in in-vitro model of CCA under the effect of GSK3326595 and JNJ64619178. Cells subjected to shPRMT5-mediated knockdown were used. Six-well cell culture plates were prepared, and cells were counted prior to seeding. A total of 100,000 cells per well were plated in triplicate. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for the duration required to allow cellular aggregation and colony formation. Following incubation, cells were washed with DPBS, fixed with 5% formaldehyde for 15 minutes, and subjected to two additional washes with DPBS. Colonies were then stained with 5% Crystal Violet for 15 minutes, followed by two washes with DPBS (Figure 22A). Plates were protected from light and left at room temperature for 24 hours. Subsequently, 10% acetic acid was added to each well, and the intensity of the staining was quantified spectrophotometrically at 460 nm (fig. 17).

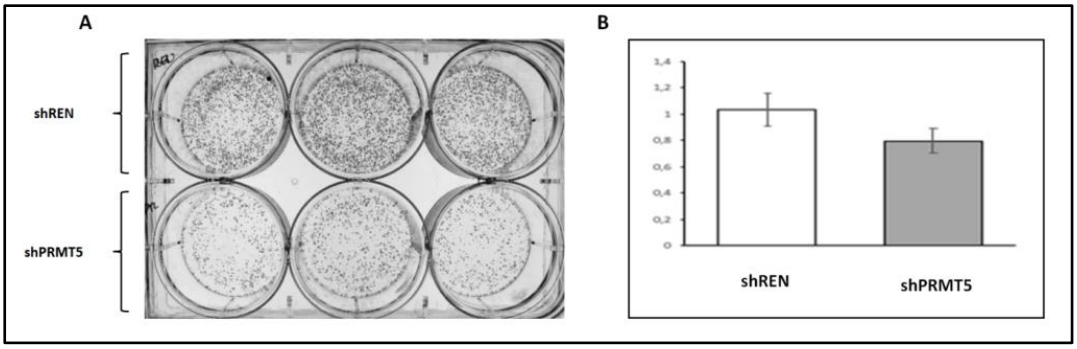

Fig. 17 A) Colony formation assay scan of KMCH cells silenced with shPRMT5 and control.B) Graph of colony formation quantification using acetic acid and spectrometr.

Based on this evidence, we wanted to verify if under the pharmaceutical inhibition of PRTMS there would be a reduction in the clonogenic activity of CCA cells. For colony formation assays, cells were seeded in 6-wells plates (5000 cells/well) and after 24 h JNJ64619178 or the same volume of DMSO (vehicle) was added to the cultures. For quantification of colony formation, at the end of treatments (7-10 days) plates were washed once with PBS, fixed with 3.7 %

formaldehyde and stained with Crysral violet. Stained wells were photographed and de-stained with 10% acetic acid and absorbances were read in a spectrophotometer at 570 nm wavelength (fig. 18).

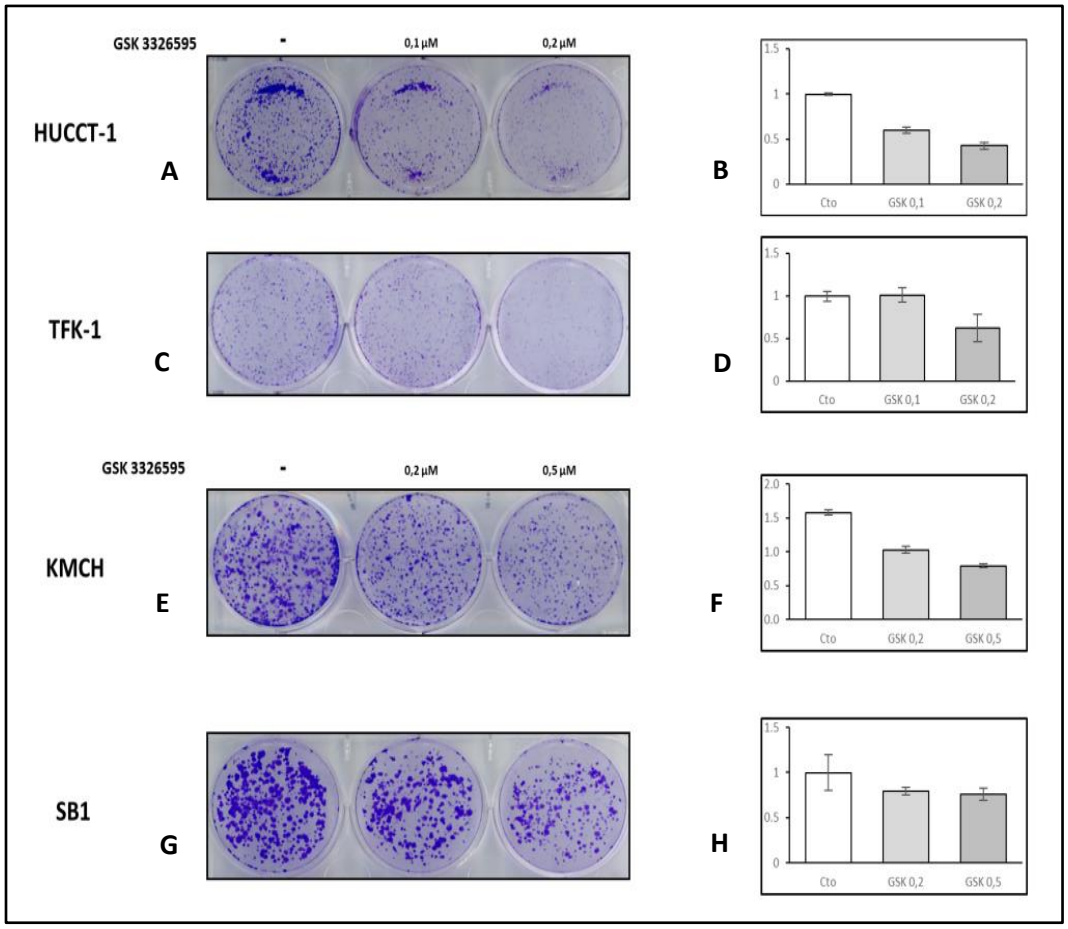

Fig. 18 Scanning of 6-well plates stained with crystal violet of cholangiocarcinoma cells treated with GSK3326595 and corresponding quantifications. A) Colony formation of HUCCT-1; B) Quantification of colony formation in HUCCT-1; C) Colony formation of TFK-1; D) Quantification of colony formation in TFK-1; E) Colony formation of KMCH; F) Quantification of colony formation in KMCH; G) Colony formation of SB-1; H) Quantification of colony formation in SB-1.

Literature reports indicate that JNJ64619178 exerts a more potent inhibitory effect on PRMT5 activity than GSK3326595. This observation is consistent with IC50 determinations, which demonstrate lower IC50 values for JNJ64619178 relative to GSK3326595. In colony formation assays, KMCH cells treated with JNJ64619178 at IC50 concentrations showed a pronounced reduction in colony aggregation capacity. Notably, unlike GSK3326595, JNJ64619178-treated

cells exhibited a statistically significant decrease in colony formation compared to controls, while no additional differences were observed between the two administered concentrations of JNJ64619178 (fig. 19).

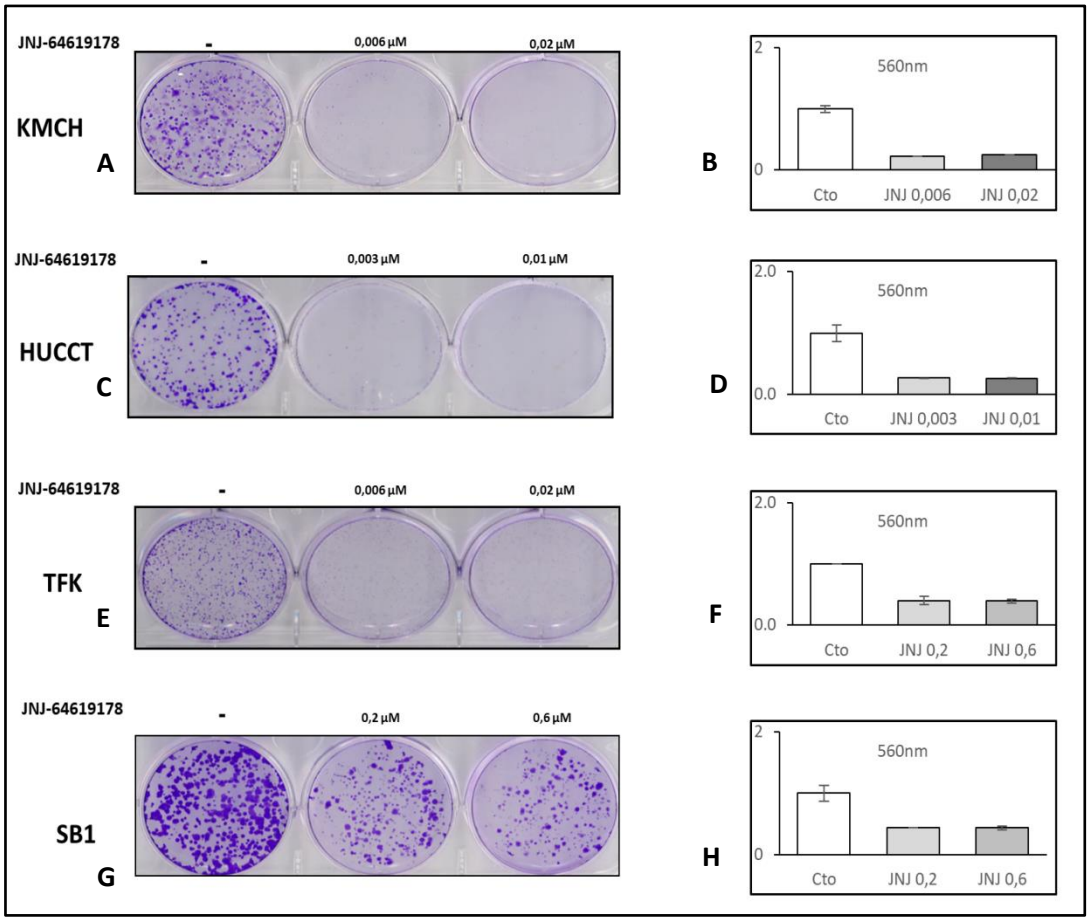

Fig. 19 Scanning of 6-well plates stained with crystal violet of cholangiocarcinoma cells treated with JNJ-64619178 and corresponding quantifications. A) Colony formation of KMCH B) Quantification of colony formation in KMCH C) Colony formation of HUCCT D) Quantification of colony formation in HUCCT E) Colony formation of TFK F) Quantification of colony formation in TFK G) Colony formation of SB-1; H) Quantification of colony formation in SB-1.

5. CONCLUSION

Numerous scientific studies have described the role of PRMT5 in tumorigenesis and in the regulation of cancer cell biology. Several investigations have evaluated PRMT5 function in solid tumors, including breast, lung, ovarian, colon, pancreatic carcinomas, and hepatocellular carcinoma. However, no studies have addressed the role of PRMT5 in cholangiocarcinoma, nor have they analyzed the effects of PRMT5 inhibition in this tumor type in either in vitro or in vivo models.

The aim of this study was to elucidate and explore the role of PRMT5 in cholangiocarcinoma. Immunohistochemistry (IHC) analysis demonstrated that PRMT5 is significantly overexpressed in cholangiocarcinoma cells compared to the surrounding normal hepatic tissue. This observation was consistent across histological samples from surgical liver resections, liver biopsy specimens, and murine models of cholangiocarcinoma.

To corroborate these findings, PRMT5 expression was further evaluated by immunoblotting using a specific anti-PRMT5 antibody. Human cholangiocarcinoma cell lines and a murine cholangiocarcinoma model cell line were employed for this purpose. The results confirmed the presence of PRMT5 expression in all cell lines along with the concomitant expression of its cofactor MEP50.

Following confirmation of PRMT5 and MEP50 expression, we investigated whether PRMT5 inhibition could significantly reduce cellular proliferation in vitro. Two PRMT5 inhibitors, GSK3326595 and JNJ64619178, were selected for this study. Both compounds exhibited efficacy in all cell lines, allowing the determination of IC50 values.

The effectiveness of PRMT5 inhibition was assessed not only by measuring cell viability but also by examining its impact on PRMT5-mediated methyltransferase activity. Specifically, we analyzed the levels of symmetric dimethylarginine (SDMA) in cholangiocarcinoma cells treated in vitro. Western blot analysis revealed a marked reduction of SDMA levels in cells treated with either GSK3326595 or JNJ64619178, which was sustained for up to 5–7 days of treatment.

To further validate the correlation between pharmacological PRMT5 inhibition and SDMA reduction, SDMA levels were assessed in a cell line subjected to PRMT5 knockdown (both stable shPRMT5 and transient siPRMT5). In cells with stable PRMT5 knockdown, Western blotting demonstrated significant reductions in PRMT5, SDMA and MEP50 compared to control cells. In transient knockdown cells, PRMT5 and MEP50 expression were markedly reduced, while SDMA levels showed only a modest decrease relative to controls.

Beyond demonstrating the efficacy of PRMT5 inhibition alone, we evaluated whether GSK3326595 could exert synergistic effects in combination with standard chemotherapeutics for cholangiocarcinoma, namely cisplatin and gemcitabine. The combination of GSK3326595 with either gemcitabine or cisplatin produced synergistic effects in the majority of tested combinations, supporting the potential utility of PRMT5 inhibitors in clinical trials.

To further explore the effects of PRMT5 inhibition on tumor growth mechanisms, we examined its impact on cellular aggregation and colony formation. The colony formation assay was employed to assess the capacity of cells to organize into colonies. PRMT5-silenced cells (KMCH shPRMT5) exhibited reduced colony formation compared to controls, an effect that was further enhanced in cells treated with GSK3326595. Overall, PRMT5 inhibition not only decreases cellular proliferation but also impairs the ability of cholangiocarcinoma cells to aggregate in vitro, with a more pronounced effect observed following treatment with JNJ64619178.

In conclusion PRMT5 and MEP50 are frequently upregulated in human CCA, and PRMT5-targeting drugs have significant antitumoural efficacy in clinically relevant CCA models. Our findings support the evaluation of PRMT5 inhibitors in clinical trials, including their combination with cytotoxic therapies.

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