Original Article

One carbon metabolism in human lung cancer

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Background: Lung cancer remains the major cause of cancer related death worldwide. The discovery of targeted therapies against activating mutations in genes like EGFR considerably improved the prognosis for a subgroup of patients but still leaves a large part without a targeted therapy. One carbon metabolism (1CM) has been investigated in several cancer entities and its increased activity has been linked to higher tumor aggressiveness and reduced prognosis. In spite of 1CM enzymes role and correlation to cancer cells progression, comprehensive analysis for the diagnostic and functional role of the complete 1CM enzymes in lung cancer has not been conducted so far.

Methods: We investigated the prognostic and functional relevance of five major 1CM factors (MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS) in the three major subclasses of lung cancer [pulmonary adenocarcinoma (AC), squamous cell lung cancer (SQCLC) and small cell lung cancer (SCLC)]. We analyzed 1CM enzymes expression and clinicopathological correlation in patient derived tissue samples of 103 AC, 183 SQCLC and 37 SCLC patients by immunohistochemistry. Furthermore, the effect of 1CM enzymes expression on lung cancer cell proliferation and the response to chemotherapy was investigated in 15 representative AC, SQCLC and SCLC cell lines.

Results: Expression of MTHFD2 and PGDH3 was significantly correlated to a worse overall survival only in AC patients. Cell proliferation assays resolved that all 1CM enzymes have a significant impact on cell growth in AC cell lines and are partially involved in cell proliferation in SQCLC and SCLC cell lines. In addition, expression of MTHFD2 correlated significantly with an increased pemetrexed chemoresistance.

Conclusions: Expression of MTHFD2 significantly reduces the prognosis of AC patients. Furthermore, MTHFD2 expression is crucial for survival of AC cell lines and its expression correlates with resistance against Pemetrexed. As MTHFD2 is almost not expressed in healthy adult tissue, we therefore suggest that the inhibition of MTHFD2 might be a potential therapeutic strategy to surround pemetrexed resistance in AC.

Keywords: One carbon metabolism (1CM); MTHFD2; pemetrexed; lung cancer; chemoresistance

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Introduction

With more than 1.4 million deaths annually, lung cancer is the leading cause of cancer related deaths worldwide (1). In addition to the histological differentiation into small cell lung cancer (SCLC) and non-small cell lung carcinomas (NSCLC) with further differentiation into adenocarcinoma (AC) and squamous cell carcinoma (SQCLC), several genetic subgroups of lung cancer have been discovered. AC is the most common subtype accounting for about 30% of cases and 420,000 deaths per year worldwide. They are characterized by a high number of genomic alterations including 32% activating mutations in the *KRAS* gene as the largest genetic subgroup, followed by activating mutations in the *EGFR* gene (11%). Genomic fusions of the ALK, Ros and Ret genes occur in 1–3% of cases. In about 25% of cases, no genetic driver mutations have yet been identified (2). Discovery of activating genetic mutations in the EGFR and ALK kinases has led to a marked improvement in the prognosis of these specific subgroups of patients through the use of targeted kinase inhibitors (3,4). Unfortunately, no similar therapeutic successes have yet been achieved for the other subgroups and most patients still receive conventional chemotherapy (5). The chemotherapeutic drug pemetrexed in combination with platinum based chemotherapies (e.g., cisplatin) is one of the current first-line therapeutic strategies for non-operable pulmonary adenocarcinoma. Pemetrexed, like methotrexate and 5-FU, affects the folate metabolism of tumor cells and leads to clinical response in ~30% of patients with AC (6).

One carbon metabolism (1CM) includes the methionine and folate cycles and plays an important role in nucleotide metabolism and genomic maintenance (7-9). The 1CM metabolic network includes mitochondria and cytoplasm. Five major steps are processed by the key enzymes: D-3-phosphoglycerate dehydrogenase 3 (PGDH3), Methylenetetrahydrofolate Dehydrogenase 1 and 2 (MTHFD2/1), Serine methyltransferase 2 (SHMT2), and Thymidylate Synthase (TYMS) (*Figure 1*).

Single 1CM enzymes have been shown to play an important role in tumor initiation and progression. MTHFD2 catalyzes the transformation of methylene tetrahydrofolate and is strongly expressed in embryonic development, but is absent in most healthy adult tissues (10,11). Increased expression level of MTHFD2 is associated with poor prognosis of hepatocellular carcinoma, breast cancer and colorectal cancer (12-15). In addition to its role as a mitochondrial enzyme of the folic acid metabolism, nuclear localization close to newly synthesized DNA and a cell proliferation-enhancing effect of MTHFD2 were also found (16). Especially, the importance of MTHFD2 in lung cancer was manifested by modulation redox homeostasis and cell cycle progression (17,18). Furthermore, Takezawa *et al.* described that the increased expression of TYMS in highly proliferative tumor cells has been implicated in pemetrexed resistance in NSCLC (19).

Even though single factors have been evaluated in several cancer types, a comprehensive correlation between expression, proliferation and response towards chemotherapy in the three main lung cancer subgroups has not been conducted so far.

In this study we characterized the expression levels and clinical impact of the 1CM enzymes MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS in pulmonary AC, SQCLC and SCLC patient tissue samples. Furthermore, we functionally analyzed the 1CM enzymes according to cell proliferation and their role in the response towards pemetrexed and cisplatin in 15 lung cancer cell lines including all subgroups. We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/tlcr-20-1039).

Methods

Human tissue samples

Tissue samples were obtained from surgical resections at the Department of Thoracic and Cardiovascular Surgery of the University Medical Center, Göttingen. All procedures performed in this study were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the University Medical Center Göttingen (#1-2-08, 24-4-20) and informed consent was taken from all the patients.

Immunohistochemical staining

Tissue samples were assembled in tissue microarrays and IHC staining was performed as described before (20-22). Briefly, 2-µm tissue sections were incubated in EnVision Flex Target Retrieval Solution with either pH low or high (Dako). Tissue slices were incubated with primary antibody against MTHFD2 (dilution: 1:100, pH low, Abnova, #H00010797-M01), PGDH3 (1:500, pH low, Abcam, #AB57030), SHMT2 (1:200, pH low, Cell signaling, #37004), MTHFD1 (1:500, pH low, Atlas Antibodies, #HPA001290), TYMS (1:50, pH high, Abcam, #AB232021)

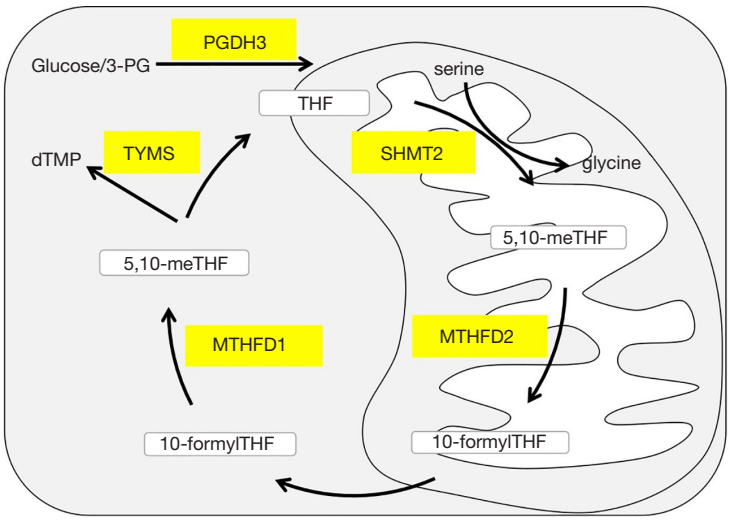

Figure 1 Schematic representation of the one-carbon metabolic cycles including the *de novo* serine synthesis pathway and the folate metabolism.

at room temperature for 20 min. Sections were incubated with secondary antibody (EnVision Flex+, Dako) according to the manufacturer's protocol and visualized through DAB (Dako). Slides were counterstained with Hematoxylin and staining were evaluated under light microscopy according to intensity: zero = negative staining; one = weak staining intensity; two = strong staining intensity.

Cell culture

H1993, H2228, HCC44, HCC78, HCC15, H2170, H520, EBC-1, HCC33, H1339, H82 human lung cancer cell lines were purchased from American Type Culture Collection (ATCC), DMS114, H3122 and H69 cell lines were obtained from the National Cancer Institute (NCI) of the National Institute of Health (NIH). EBC-1 KRAS cells with a heterozygous knock-in of a KRAS gene with an activating mutation was obtained from Horizon Discovery. Cells were maintained in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin and 1% L-Glutamine. Cells were incubated in humidified atmosphere of 5% CO_2 at 37 ℃. Medium was refreshed every 2–3 days and culture was passaged approximately at 80% confluency.

Transfection

All siRNAs were obtained from Qiagen, Hilden, Germany. Cells were transfected with 20 nM siRNA against either MTHFD2 (Nr. #1 SI02664928, #2 SI02664921), PGDH3 (Nr. #1 SI00090405, #2 SI05040994), SHMT2 (Nr. #1 SI04171237, #2 SI04176501), MTHFD1 (Nr. #1 SI02653084, #2 SI00083027) or TYMS (Nr. #1 SI02780757, #2 SI00021609). HiPerFect Transfection Reagent (Qiagen) was used according to the manufacturer's protocol. Allstars negative siRNA was used as a scrambled control. Either a pcDNA3-MTHFD2 vector (CloneID: OHu18706, GenScript) or an empty pCDNA3 vector backbone (138209, Addgene) was transfected into AC cell lines for overexpression of MTHFD2. Cells expressing de-novo MTHFD2 were selected with G418 (at concentration of 700 µg/mL) for at least one week and MTHFD2 protein levels were confirmed by Western Blotting.

Western blotting

Cells were lysed with NP-40 buffer (1% NP40, pH 7.6, 150 mM NACL, 50 mM Tris, 1 mM Sodium orthovanadate, 1x Complete-EDTA, 0.2% Lauryl Maltoside) on ice for 30 min followed by centrifuging at 14,000 RPM at 4 ℃ for 20 min and protein content was determined using DC™ Protein Assay kit according to the manufacturer's protocol. Proteins were immunoblotted as described before (23). Briefly, 20 µg protein were denatured at 95 ℃ for 5 min in Laemmli buffer, separated on a gradient polyacrylamide gel (4–15%) (BioRad) and transferred onto a nitrocellulose membrane

with the Trans-Blot Turbo system (BioRad) according to manufacturer's guideline. Membranes were blocked in 5% milk for 1 h following primary antibody exposure over night at 4 ℃. The following antibodies and related secondary antibodies (DAKO) were use with a dilution of 1:1,000 in TBST for Anti-PGDH3 (#HPA021241, Sigma), Anti-SHMT2 (#HPA020549, Atlas Antibodies), Anti-MTHFD1 (#HPA001290, Atlas Antibodies), TYMS (#AB232021, Abcam) and Anti-MTHFD2 (#H00010797-M01, Abnova) were used. Anti-PARK7 (1:1,000, #AB76008, Abcam) was used as loading control (24).

Cell viability assay

For siRNA viability assay, 25 µL transfection medium containing 0.1 µL siRNAs/negative siRNA, 0.4 µL HiPerFect and 24.5 µL medium serum-free was incubated for 10 min at RT and added onto $[1–5]\times10^4$ cells/well with 175 µL culture medium after seeding. For treatment viability assay, 2,500 cells/well were seeded in 96-well plate with 100 µL medium overnight and treated with the absence or presence of increasing concentrations of cisplatin (0.1, 1, 5, 10, 50, 100 µM) (Hexal AG, Holzkirchen, Germany) or pemetrexed (0.00632, 0.0125, 0.025, 1.5625, 3.125, 6.25, 12.5, 25, 50 µM) (Sigma-Aldrich, Taufkirchen, Germany) for indicated time as specified in the results. Cell proliferation was determined by MTS assay (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were incubated with 20 µL of CellTiter 96 Aqueous One Solution Reagent at 37 ℃, 5% CO_2 for 2 hours and 96-well plates were measured on a Tecan Reader Infinite 2000 using the i-control™ Microplate Reader Software at 490 and 650 nm.

Statistical analysis

Statistical analyses were performed using GraphPad Prim 8. Correlation between clinical parameters and 1CM enzymes expression was analyzed using chi-square test. Survival curves were drawn using Kaplan-Meier analyses and the significance was calculated by log-rank test. Students *t*-test was used for two group comparisons. More than two matched groups were analyzed using one-way ANOVA and Tukey's multiple comparisons tests. Correlation between 1CM enzymes and half maximal inhibitory concentration (IC50) was performed by the Pearson's correlation test. Statistical differences were considered significant at $P<0.05$.

Results

Correlation between expression of one-carbon metabolism enzymes and clinicopathologic characteristics

We performed immunohistochemical staining of the 1CM enzymes MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS in a patient cohort of in total 323 lung cancer patients (*Figure 2* and Figure S1A). All patients were treated with surgical tumor resection without prior chemotherapy. The clinical characteristics of the patients are summarized in *Table 1*. Male patients (AC 57%, SQCLC 84%, SCLC 73%) were more frequent than female patients. The median age at time of diagnosis among the three groups was 66 years (range, 34–85 years). The majority of patients with AC (74%) and SQCLC (77%) showed a moderately differentiated disease. Frequency of T1-2 stage was 80%, 72% and 90% in AC, SQCLC and SCLC patients, respectively. Frequency of patients without lymph node metastasis (AC: 63%, SQCLC: 56%, SCLC: 75%) was higher than frequencies of N1, N2 and N3 together. Median follow-up time was 23, 26 and 34 months and death cases were reported in 48, 131 and 22 patients of AC, SQCLC and SCLC, respectively.

In SQCLC and SCLC, all 1CM enzymes meanwhile in AC only MTHFD1 and TYMS were expressed (either strong or weak) in more than 80% of cases. MTHFD2, SHMT2 and PGDH3 expression were significantly less abundant in AC with 50%, 63.4% and 65.7% respectively (*Table 2*). Only the expression of MTHFD2 and PGDH3 significantly correlated with a shorter overall survival only in AC (P=0.044 and P=0.036, respectively, *Figure 2*, *Table 3*). Furthermore, MTHFD2 expression was significantly associated with male gender, poorly differentiation grade and the occurrence of lymph node metastasis in AC patients (P=0.024, 0.0003 and 0.040, respectively, *Table 4*) in AC. Even though some factors showed a tendency to significance no other relevant difference or association to clinical parameters was detected (Figure S1B,C,D, and *Tables 3*,*4*).

Proliferation of pulmonary adenocarcinoma cells strongly depend on 1CM activity

For a functional analysis of the 1CM proteins *in vitro* we used five AC cell line (H1993, H2228, H3122, HCC44 and HCC78), five SQCLC cell lines (HCC15, H2170, H520, EBC-1 and EBC-1 KRAS) and five SCLC cell lines (DMS114, H1339, H69, H82 and HCC33). Protein expression was evaluated by western blot and quantified

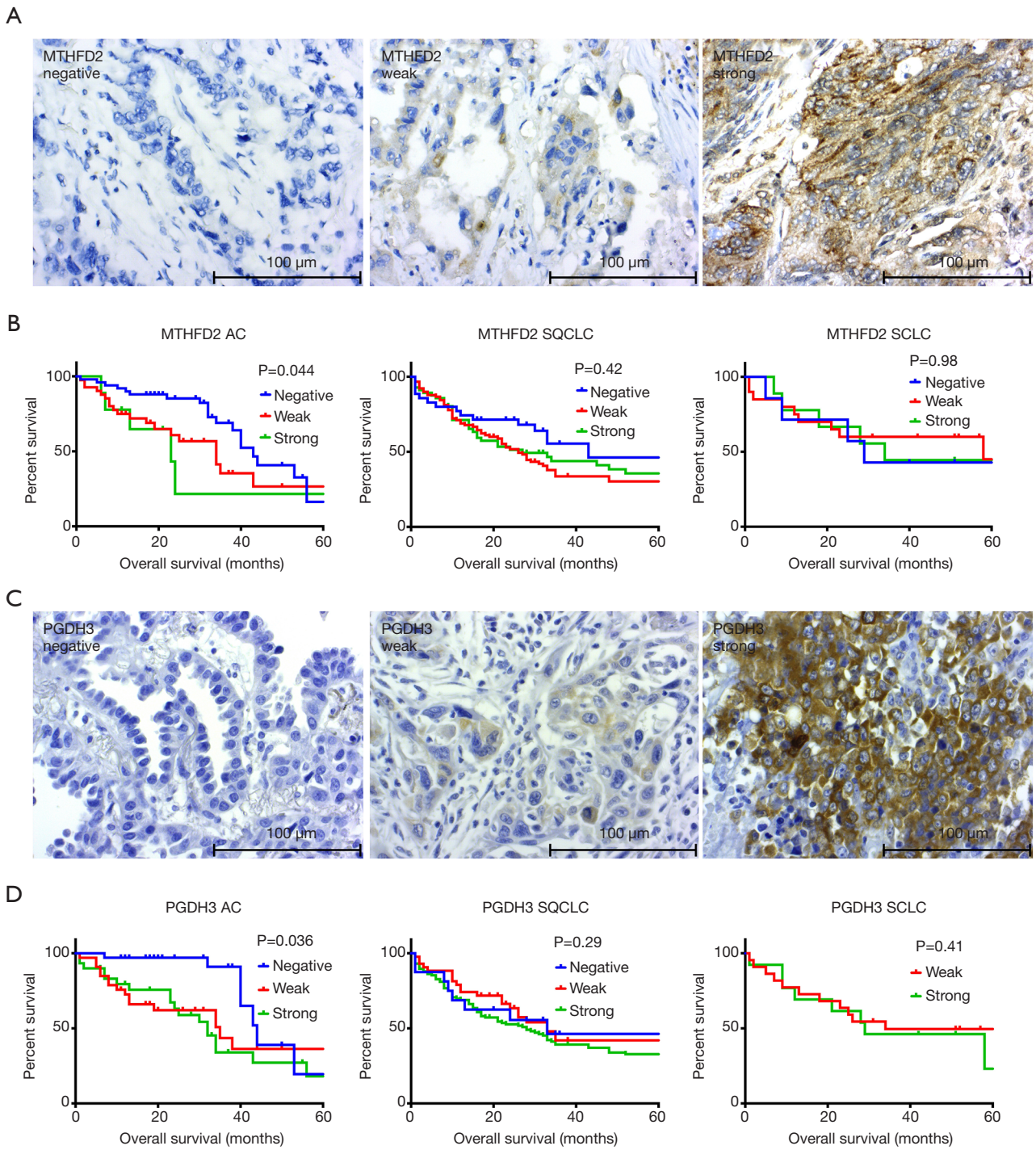

Figure 2 Prognostic expression of MTHFD2 and PGDH3 in human lung cancer samples. (A,C) Human lung cancer tissues were immunohistochemically stained to show expression of MTHFD2 (A) or PGDH3 (C) in AC, SQCLC and SCLC. All images were captured at 40× magnification. (B,D) Survival analysis using Kaplan-Meier estimate grouped by IHC score. Blue, red and green lines depict survival curves of patients with negative, weak and strong expression of MTHFD2 and PGDH3 protein, respectively. P values were calculated with a log-rank test.

Table 1 Clinical data summary

Characteristic	AC (n=103)	SQCLC (n=183)	SCLC (n=37)
Gender, n (%)			
Male	59 (57.3)	154 (84.2)	27 (73.0)
Female	44 (42.7)	29 (15.8)	10 (27.0)
Median age [range] (years)	67 [34–85]	66 [42–83]	65 [50–77]
Tumor grade, n (%)			
G1–2	74 (73.8)	140 (76.5)	0 (0.0)
G3	27 (26.2)	43 (23.5)	37 (100.0)
Tumor stage, n (%)			
T stage			
T1–2	82 (80.0)	131 (72.4)	35 (89.7)
T3–4	20 (20.0)	50 (27.6)	2 (10.3)
N stage			
N0	62 (63.3)	99 (55.6)	21 (75.0)
N1–3	36 (36.7)	79 (44.4)	7 (25.0)
Median survival time (months)	40	30	58
Median follow-up time [range] (months)	23 [1–128]	26 [1–196]	34 [1–125]
Reported deaths (%)	48 (46.7)	131 (71.6)	22 (59.5)

AC, pulmonary adenocarcinoma; SQCLC, squamous cell lung cancer; SCLC, small cell lung cancer.

over three replicates (*Figure 3* and Figure S2). Meanwhile the expression of MTHFD1, MTHFD2 and SHMT2 was comparable in most of the cell lines, the expression of TYMS and PGDH3 demonstrated a higher variability. Interestingly, the expression of MTHFD2 and TYMS was significantly higher in the cell line HCC44 that harbors an activating KRAS mutation than in the other AC cell lines whereas SHMT2 was only moderately increased (*Figure 3A*,*B*,*C*).

Next we aimed to investigate the effect of 1CM enzymes on cell proliferation using siRNAs that lead to a specific knockdown of the five described 1CM enzymes (Figure S3). The knockdown with two different siRNA against every single factor resulted in a significant decrease in cell proliferation in four of the five AC cell lines (*Figure 4* and Figure S4). Especially in the KRAS mutated HCC44 with high MTHFD2 expression the inhibitory effect was stronger than 90%. In contrast, the reduction of the low expressed PGDH3 and TYMS in the AC cell line H1993 only moderately inhibited cell growth (less than 50%). In the SQCLC cell lines only the knockdown of MTHFD1 and MTHFD2 showed a constant reduction of cell proliferation and in SCLC there were only three cell lines (DMS114, H1339, H69) that showed a reduced cell growth after siRNA treatment against MTHFD1 and MTHFD2 (*Figures 5*,*6*).

MTHFD2 expression marks resistance to pemetrexed in AC cell lines

In order to evaluate the sensitivity of lung cancer cell lines to clinically used chemotherapeutics the described cell lines were treated with increasing concentrations of pemetrexed and cisplatin. Pemetrexed treatment resulted in a broad range of responses in the AC cell lines with IC50 levels between 0.027 to 113 µM (*Figure 7A*). Especially HCC44 showed a strong resistance to pemetrexed with an IC50 of 113 µM. Compared to the AC cell lines the SQCLC and the SCLC cell lines responded in a narrower range with an in average much higher IC50 (*Figure 7B* and Figure S5A). All cell lines showed a comparable response to cisplatin with IC50 ranging from

Table 2 1CM expression in lung cancer

Tumor type	PGDH3				SHMT2				MTHFD1				MTHFD2				TYMS			
	Cases	–	+	P	Cases	–	+	P	Cases	–	+	P	Cases	–	+	P	Cases	–	+	P
AC	108	37 (34.3%)	71 (65.7%)	0.00001	112	41 (36.6%)	71 (63.4%)	0.00001	116	7 (6.0%)	109 (94.0%)	0.037	114	57 (50.0%)	57 (50.0%)	0.00001	116	16 (13.8%)	100 (86.2%)	0.22
SQCLC	191	17 (8.9%)	174 (91.1%)		191	27 (14.1%)	164 (85.9%)		191	29 (15.2%)	162 (84.8%)		197	37 (18.8%)	160 (81.2%)		190	38 (20.0%)	152 (80.0%)	
SCLC	38	0 (0%)	38 (100%)		38	3 (7.9%)	35 (92.1%)		34	6 (17.6%)	28 (82.4%)		38	7 (18.4%)	31 (81.6%)		37	4 (10.8%)	33 (89.2%)	

P values are calculated according to Chi-Square test. PGDH3, 3-phosphoglycerate dehydrogenase; SHMT2, serine hydroxymethyltransferase 2; MTHFD1/2, methylenetetrahydrofolate dehydrogenase 1/2; TYMS, thymidylate synthase. AC, adenocarcinoma; SQCLC, squamous cell lung cancer; SCLC, small cell lung cancer.

1.88 to 27.11 μM (Figure S5B,C,D). In comparison to the expression of one-carbon enzymes, MTHFD2 was the only enzyme that correlated to the response to pemetrexed in AC cell lines (r=0.87, P=0.03, *Figure 7C*). Although, there was a tendency for better response to pemetrexed treatment in MTHFD2 low expression SCLC cell lines (r=0.85, P=0.07, *Figure 7D*) there was not a significant correlation neither with pemetrexed nor with cisplatin (Figures S6,S7). However, we did not observe any further significant correlation between 1CM proteins expression and drug sensitivity, there was a tendency for better response to pemetrexed treatment in TYMS low expression AC and SCLC cells (r=0.27, P=0.06, and r=0.84, P=0.06, respectively, Figure S6A,B).

To explore the Pemetrexed resistance function of MTHFD2 in AC cells, we transfected a pcDNA-MTHFD2 or an empty vector or a specific siRNA against MTHFD2 or a non-target control siRNA in AC cells and treated the resulting cells with increasing concentrations of Pemetrexed. Overexpression of MTHFD2 in H1993 cells markedly increased viability of cells when treated with Pemetrexed compared to empty vector treated cells (IC50 for Pemetrexed: 0.30 and 0.081 μM, respectively, *Figure 7E*, Figure S8A,B). In contrast, MTHFD2 silencing significantly reduced cell viability after Pemetrexed treatment when compared to control treatment in HCC44 cells (IC50 Pemetrexed: 30.2 and 110.7 μM, respectively, *Figure 7F*). This indicated that resistance to Pemetrexed was induced by MTHFD2 overexpression and restored by its knockdown in AC cells.

Discussion

Increased 1CM and specifically MTHFD2, PGDH3 and TYMS activity has been associated with poor prognosis of cancer indicating the essential role of 1CM in saturating the need of newly synthesized nucleotides in proliferating cancer cells (25-29). Targeting 1CM metabolism with pemetrexed has been shown to be beneficial for patients with pulmonary adenocarcinoma (AC) (30). However, a comprehensive study of the main 1CM factors expression, function and correlation to chemoresistance in lung cancer was still missing.

In our cohort of lung cancer samples, expression of MTHFD2 and PGDH3 in AC group was significantly less common than in squamous cell lung cancer (SQCLC) or SCLC groups indicating a subtype specific diversity of 1CM activity in lung cancer. Previous studies have shown

Table 3 Kaplan-Meier survival analysis according to SHMT2, MTHFD1 and TYMS protein expression levels in AC, SQCLC and SCLC patients

Tumor type	SHMT2				MTHFD1				TYMS			
	Negative	Weak	Strong	P	Negative	Weak	Strong	P	Negative	Weak	Strong	P
AC (months)	43	32	40	0.06	–	43	35	0.49	40	35	44	0.28
SQCLC (months)	33	23	29	0.42	22	29	31	0.98	33	33	22	0.34
SCLC (months)	9	34	58	0.92	58	50	27	0.96	–	58	24	0.47

SHMT2, serine hydroxymethyltransferase 2; MTHFD1, methylene tetrahydrofolate 1; TYMS, thymidylate synthase; AC, pulmonary adenocarcinoma; SQCLC, squamous cell lung cancer; SCLC, small cell lung cancer.

Table 4 PGDH3 and MTHFD2 expression in adenocarcinoma sorted by clinical feature

Feature	PGDH3				MTHFD2			
	Cases	–	+	P	Cases	–	+	P
Gender, n (%)								
Female	47 (43.5)	15 (31.9)	32 (68.1)	0.65	52 (45.6)	32 (61.5)	20 (38.5)	0.024
Male	61 (56.5)	22 (36.1%	39 (63.9)		62 (54.4)	25 (40.3)	37 (59.7)	
Age (years), n (%)								
<60	26 (24.1)	13 (50.0)	13 (50.0)	0.052	27 (23.7)	14 (51.9)	13 (48.1)	0.83
≥60	82 (75.9)	24 (29.3)	58 (70.7)		87 (76.3)	43 (49.4)	44 (50.6)	
Grade, n (%)								
1–2	78 (71.2)	29 (37.2)	49 (62.8)	0.30	156 (8.8)	91 (58.0)	65 (42.0)	0.0003
3	30 (27.8)	8 (26.7)	22 (73.3)		31 (27.2)	7 (22.6)	24 (77.4)	
Lymph node metastasis, n (%)								
No	64 (59.3)	25 (39.1)	39 (60.9)	0.12	64 (56.1)	36 (56.3)	28 (43.8)	0.040
Yes	39 (36.1)	9 (23.1)	30 (76.9)		42 (36.8)	15 (35.7)	27 (64.3)	
Tumor stage (T stage), n (%)								
T1 + T2	87 (82.1)	28 (32.2)	59 (67.8)	0.70	94 (83.2)	47 (50.0)	47 (50.0)	0.83
T3 + T4	19 (17.9%	7 (36.8)	12 (63.3)		19 (16.8)	9 (47.4)	10 (52.6)	

P values are calculated according to Chi-square test. PGDH3, 3-phosphoglycerate dehydrogenase; MTHFD2, methylenetetrahydrofolate dehydrogenase 2.

that MTHFD2 expression is correlated to worse prognosis of lung cancer, hepatocellular carcinoma, human pancreatic cancer, colorectal cancer and renal cell carcinoma patients (12,15,31-33). Similarly, PGDH3 has been proposed to be a prognostic factor in human breast cancer (14) and has been linked to tumorigenesis and poor prognosis in NSCLC (25). Through inclusion of the three major lung cancer subgroups, we could pinpoint that expression of MTHFD2 and PGDH3 was correlated to worse prognosis particularly in AC but not in SQCLC or SCLC patients. In addition, MTHFD2 expression correlated significantly with male gender, poor differentiation grade and lymph node metastasis in AC patients, suggesting an association between MTHFD2 expression and AC disease progression.

1CM enzymes have been described to fulfill the cancer-specific nutrient demand in tumor cell proliferation (28,29,34). Proliferation of AC cell lines was more affected upon siRNA knockdown of MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS than SQCLC or SCLC cell lines. In accordance to these results the down-

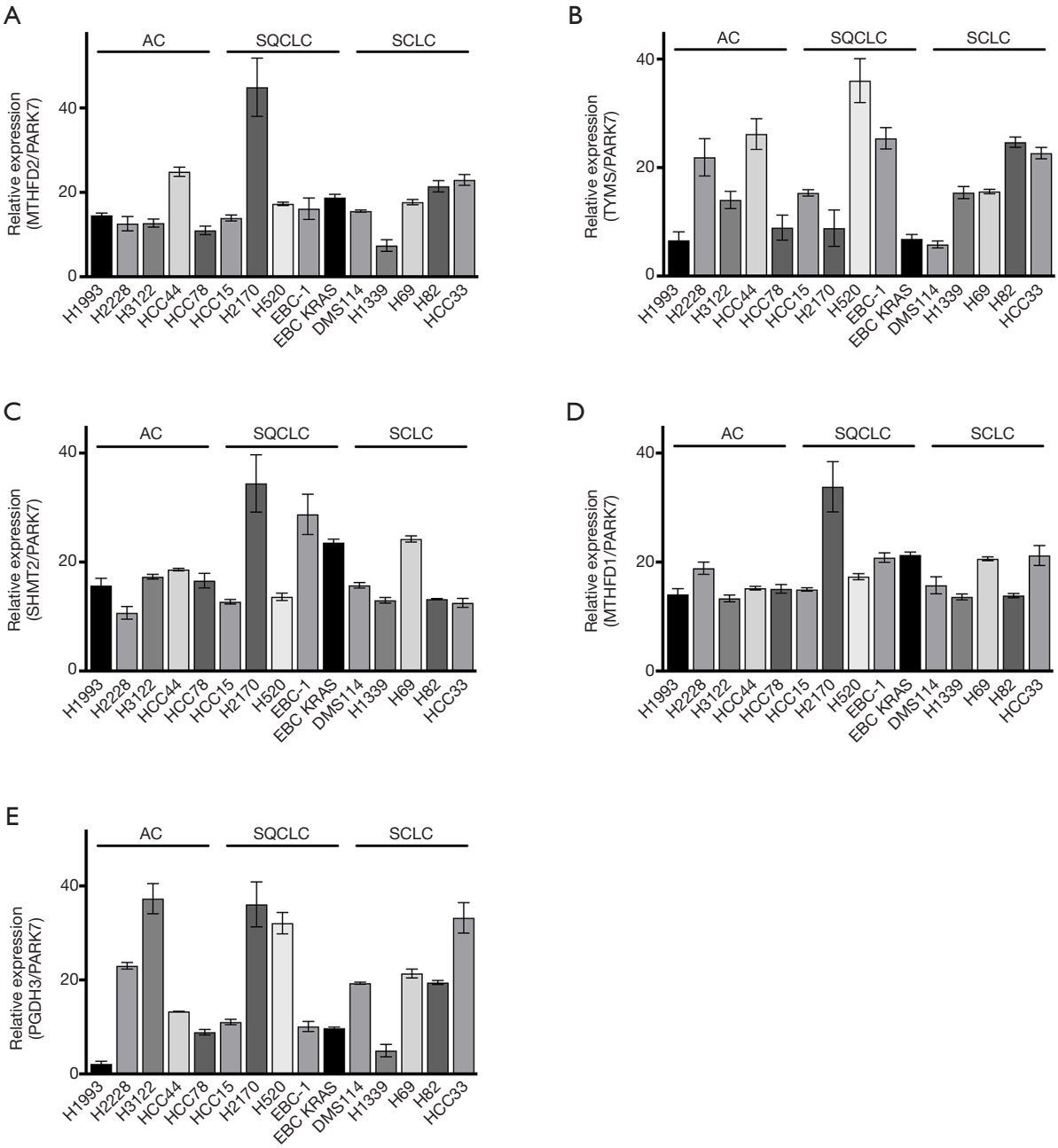

Figure 3 Expression of the five 1CM enzymes in human lung cancer cell lines. Quantification of MTHFD2 (A), TYMS (B), SHMT2 (C), MTHFD1 (D) and PGDH3 (E) protein expression in human lung cancer cell lines. PARK7 was used as loading control. Data is represented as mean ± SEM of at three independent experiments.

regulation of MTHFD2 in NSCLC was shown to inhibit cell proliferation via regulating cell cycle arrest and redox homeostasis (17,18). Dependency of cancer cells proliferation on 1CM was also observed in breast cancer, colorectal cancer (16,35), Ewing sarcoma (36), human hepatocellular carcinoma (13,37) and glioma (38).

MTHFD2 and PGDH3 protein levels in the SQCLC cell line H2170 and the SCLC cell line HCC33 were significantly higher than all other cell lines, however, there was no significant impact on cells viability upon siRNA

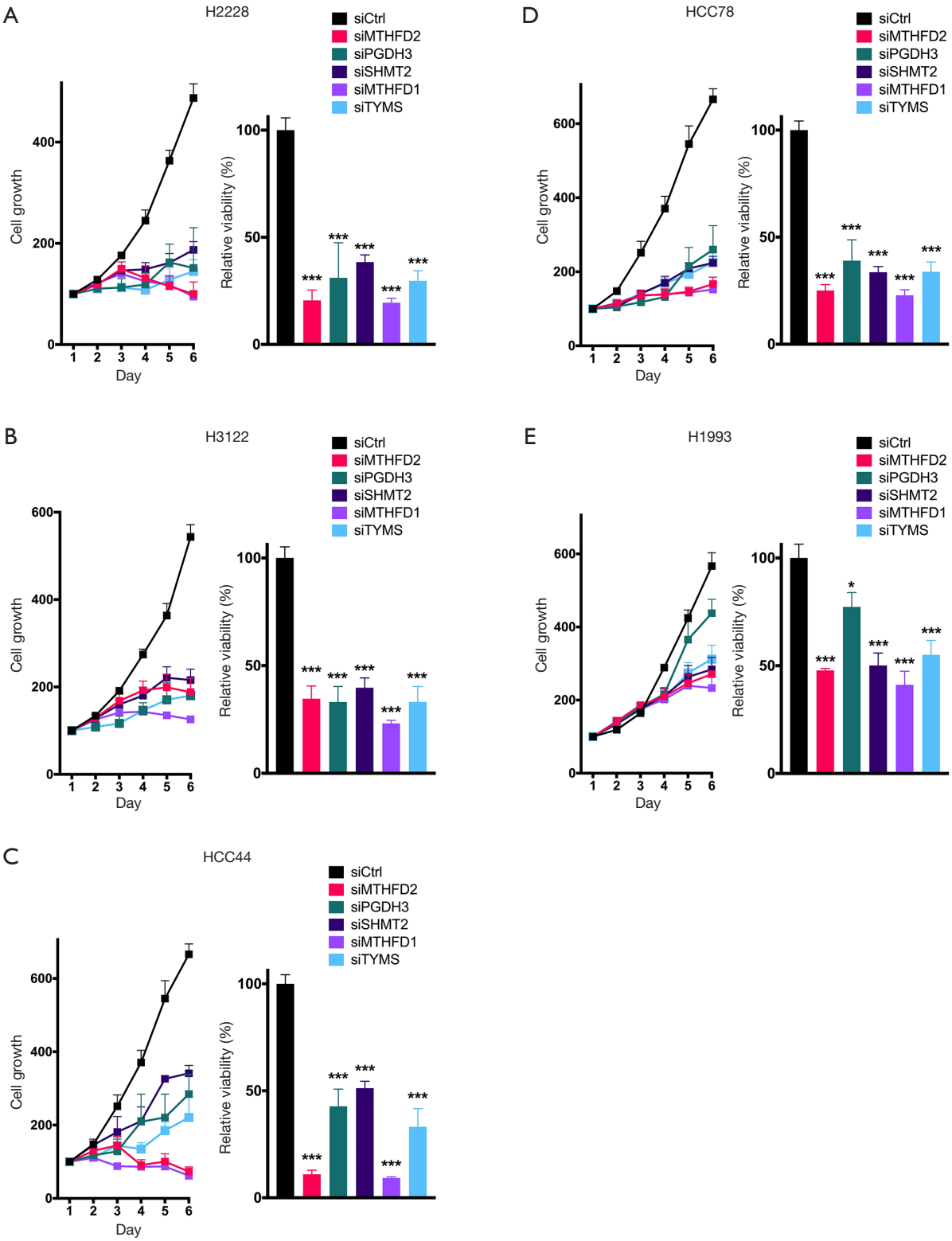

Figure 4 Knockdown of 1CM enzymes reduced cell proliferation in pulmonary adenocarcinoma. (A,B,C,D,E) Growth curve (left side) of AC cell lines transfected with siRNA against MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS and the fraction of cell viability at day 6 (right side). The data are represented as mean ± SEM of three independent experiments. *, P<0.05; ***, P<0.001.

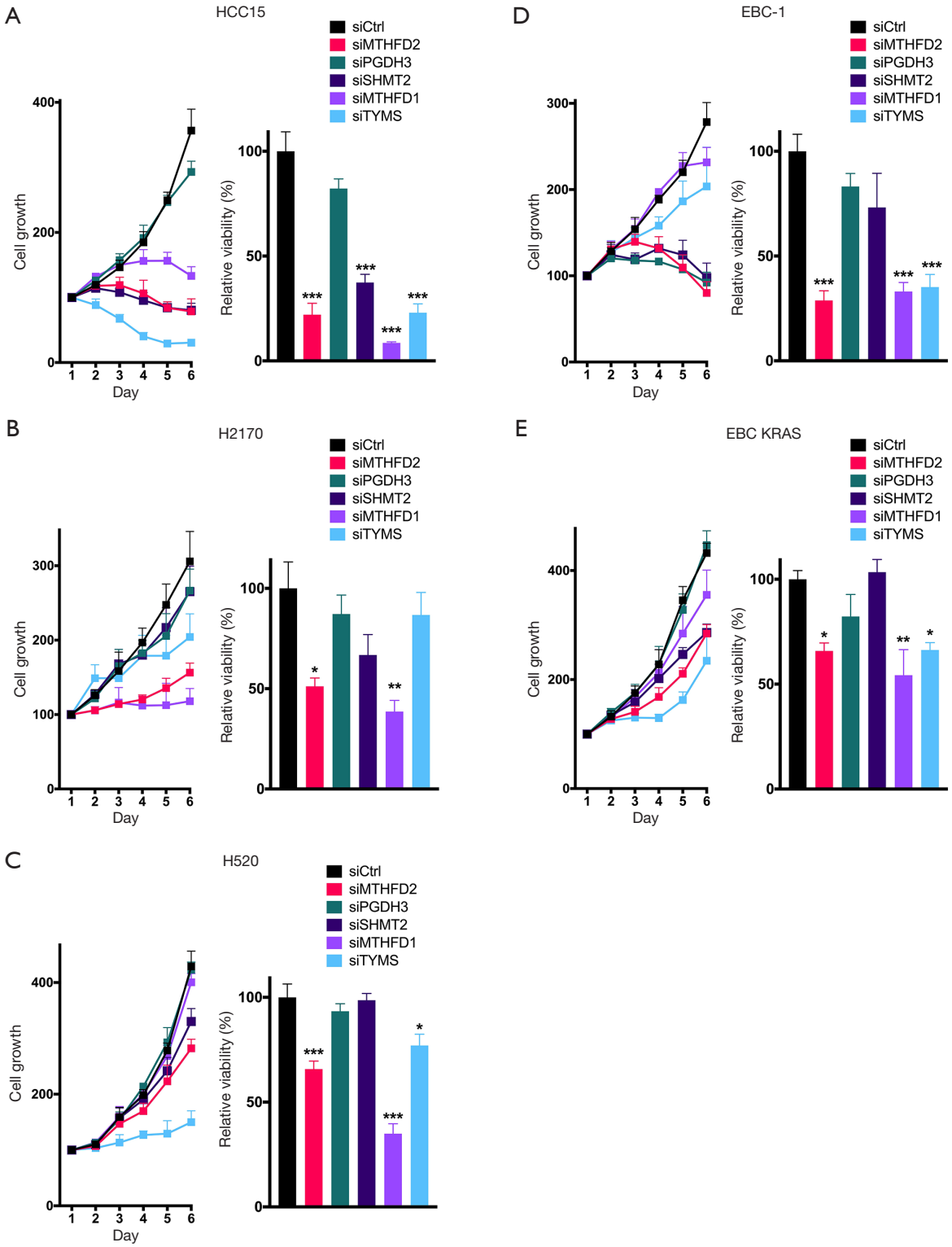

Figure 5 Knockdown of 1CM enzymes reduced cell proliferation in squamous cell lung cancer. (A,B,C,D,E) Growth curve (left side) of SQCLC cell lines transfected with siRNA against MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS and the fraction of cell viability at day 6 (right side) The data are represented as mean ± SEM of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

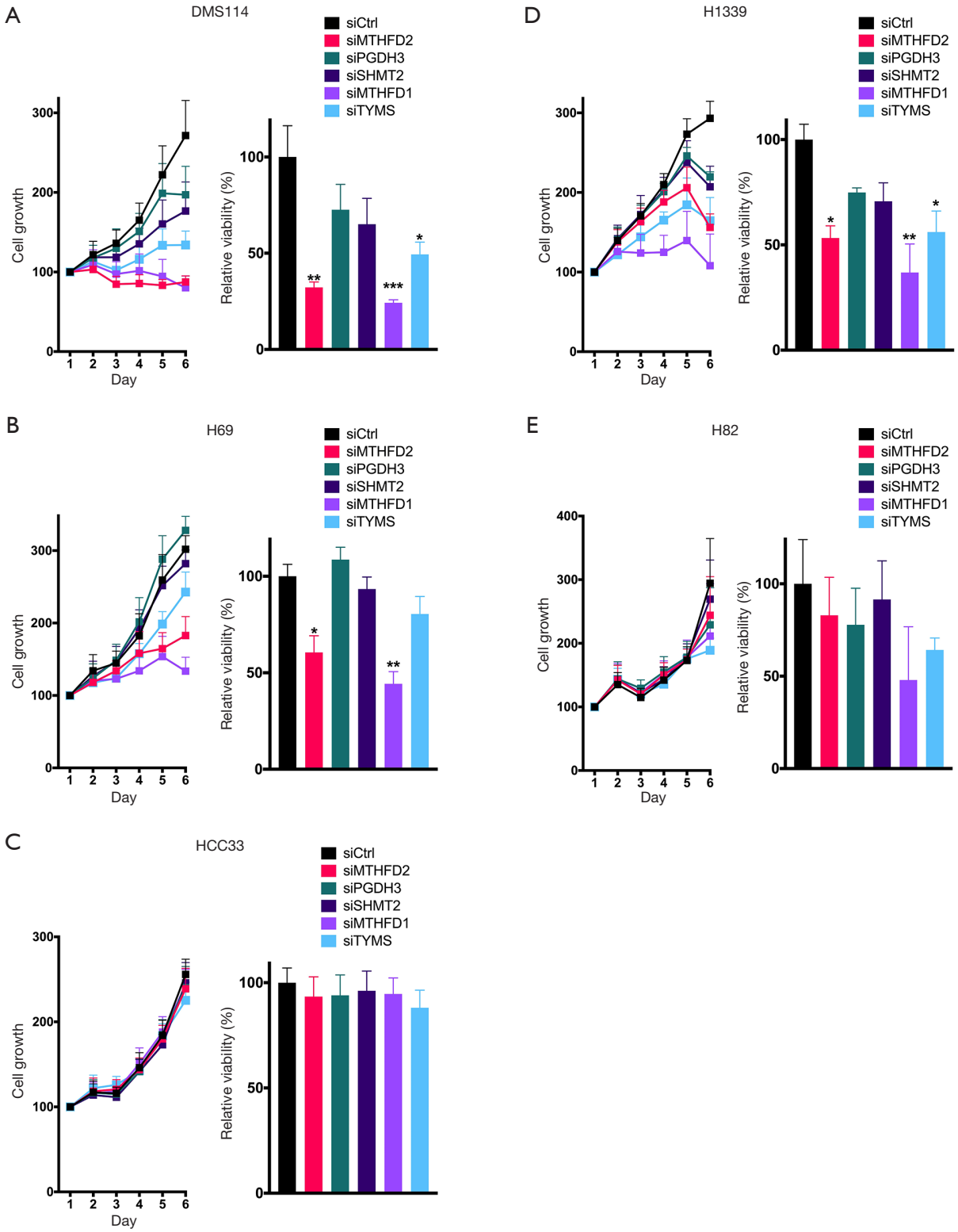

Figure 6 Knockdown of 1CM enzymes reduced cell proliferation in small cell lung cancer. (A,B,C,D,E) Growth curve (left side) of SCLC cell lines transfected with siRNA against MTHFD2, PGDH3, SHMT2, MTHFD1 and TYMS and the fraction of cell viability at day 6 (right side). The data are represented as mean ± SEM of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

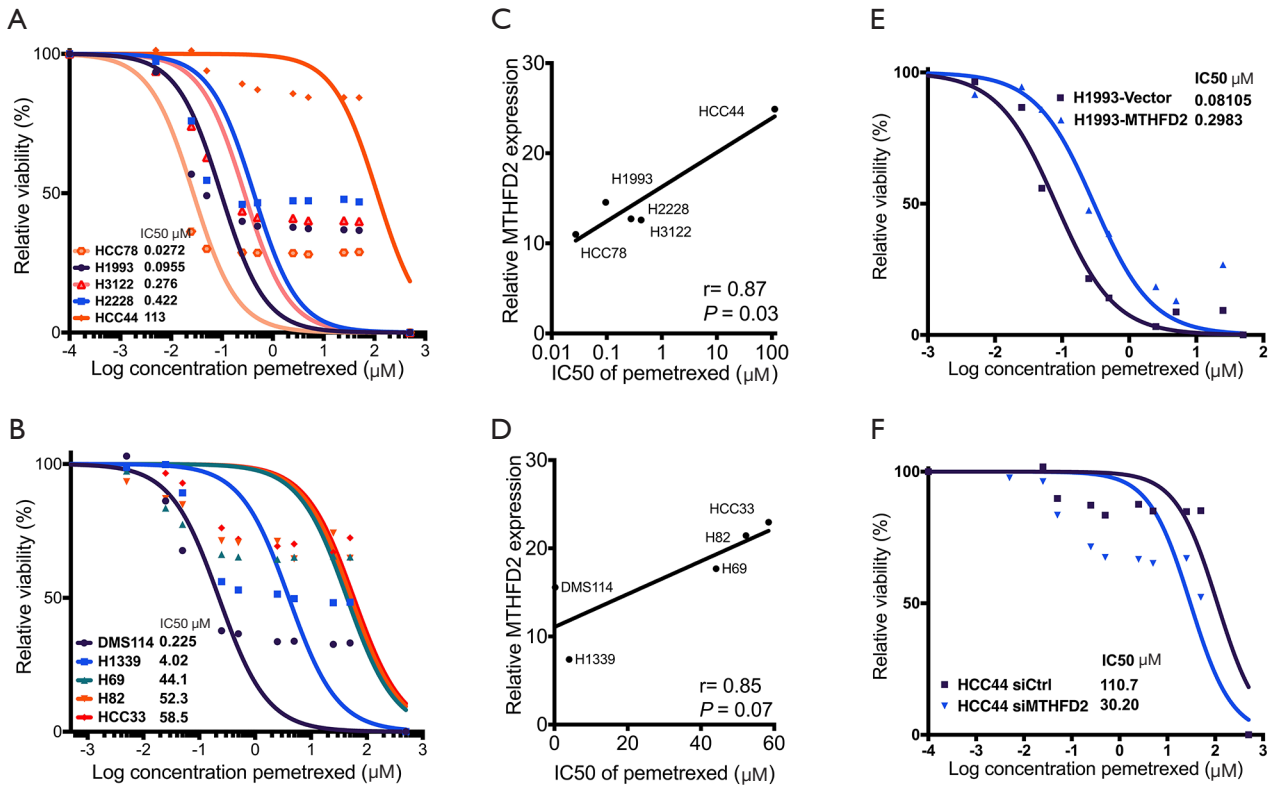

Figure 7 Correlation between pemetrexed sensitivity and the expression levels of 1CM enzymes. IC50 values and inhibitory curve in each cell line after treatment with Pemetrexed tested in lung cancer cell lines of AC (A) and SCLC (B). Scatterplots of relative MTHFD2 expression vs IC50 values of Pemetrexed in 5 AC (C) and 5 SCLC (D) cell lines. Pearson correlation coefficient (r) and P value are displayed. Representative results of cell viability assay after treatment with Pemetrexed in MTHFD2-overexpressing H1993 cells, empty vector transfected H1993 cells (E), MTHFD2-knocked down HCC44 cells and negative control siRNA transfected HCC44 cells (F). Data is represented as mean ± SEM of three independent experiments.

knock down. In summary, according to protein expression levels and functional analyses of 1CM enzymes, we propose a stronger dependency on 1CM activity in AC compared to SQCLC or SCLC.

We observed a stronger expression of MTHFD2, SHMT2 and TYMS in the KRAS mutant cell line HCC44 in comparison to KRAS wild type cell lines. This goes along with recent studies, which have described a higher dependency on folate metabolism in KRAS mutated NSCLC cells (39). Here, we show that four AC cell lines with low levels of MTHFD2 expression exhibited higher response to pemetrexed in comparison to HCC44 with high levels of MTHFD2 expression. We furtherly confirmed the correlation between MTHFD2 expression and resistance to Pemetrexed by showing that overexpression of MTHFD2 leads to increased resistance to Pemetrexed meanwhile knockdown of MTHFD2 reduces resistance to Pemetrexed. Accordingly, Nishimura *et al.* has described that MTHFD2 increased chemotherapy resistance to Gefitinib in pulmonary adenocarcinoma (40). The SQCLC and SCLC cell lines H2170, H520, H82 and HCC33 showed not only the highest expression of MTHFD2 but also the strongest resistance against pemetrexed. These results suggest that high expression of MTHFD2 could shape a primary indicator of treatment resistance against pemetrexed. Moreover, our findings provide additional evidences that high expression of MTHFD2 not only correlates with worse prognosis of AC patients, but also contributes to the sensitivity to Pemetrexed. Therefor MTHFD2 might be a useful biomarker for the decision of a specific treatment strategy in AC.

A persuasive MTHFD2 inhibitor (DS18561882) has already been characterized to have an anti-tumor efficacy *in vivo* in human breast cancer (41). Taking into consideration

that MTHFD2 is only expressed in developmental and neoplastic cells and is almost absent in healthy adult tissues (11) makes MTHFD2 a potential new therapeutic option in AC with further need for validation.

Although the correlative analyses with TYMS expression and Pemetrexed resistance did not produce a statistically significant correlation, there was also a trend for better response to pemetrexed treatment in TYMS low expression AC and SCLC cell lines (r=0.27, P=0.06 and r=0.84, P=0.07, respectively, Figure S6A,B). These findings support previous studies, which suggested that TYMS was a predictive factor for sensitivity to pemetrexed in lung cancer (19,42,43).

Overall, our study revealed a diversity of 1CM enzyme expression and effects in human lung cancer subtypes. In pulmonary adenocarcinoma, MTHFD2 might be both a predictive biomarker that could help for chemotherapy selection and a potential new drug target especially as MTHFD2 is almost not expressed in healthy adult tissue.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee of the University Medical Center Göttingen (#1-2-08, 24-4-20) and informed consent was taken from all the patients.

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