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Different histological types of non-small cell lung cancer have distinct folate and DNA methylation levels

MingJi Jin,[1] Kazuyuki Kawakami,[1,5] Yousuke Fukui,[2] Sayaka Tsukioka,[2] Makoto Oda,[3] Go Watanabe,[3] Teiji Takechi,[4] Toshinori Oka[2] and Toshinari Minamoto[1]

[1]Division of Translational and Clinical Oncology, Molecular and Cellular Targeting Translational Oncology Center, Cancer Research Institute, Kanazawa University, Kanazawa; [2]Tokushima Research Center, Taiho Pharmaceutical Co., Hiraishi, Tokushima; [3]Department of General and Cardiothoracic Surgery, Kanazawa University Graduate School of Medical Science, Kanazawa; [4]Product Planning and Lifecycle Management Department, Taiho Pharmaceutical Co., Tokyo, Japan

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Aberrant DNA methylation is a commonly observed epigenetic change in lung cancer. Folate has been suggested to play a role in the homeostasis of DNA methylation and has also been implicated in cancer chemotherapy. We investigated a possible role for folate in DNA methylation by measuring folate concentrations in tumors and adjacent normal tissues from 72 non-small cell lung cancer (NSCLC) patients. These were compared to DNA methylation levels and to clinicopathological features. Folate concentrations were determined as the sum of 5,10-methylenetetrahydrofolate and tetrahydrofolate. The MethyLight assay was used to quantitate methylation in promoter regions of *P16(CDKN2A)*, *APC*, *CDH13*, *RARB*, *RASSF1*, *RUNX3*, and *MYOD1*. Methylation of *LINE-1* repeats was used as a surrogate for global methylation. Folate levels in tumors correlated positively with *LINE-1*, *CDH13*, and *RUNX3* methylation. Folate concentrations and methylation of *LINE-1*, *RASSF1*, and *RUNX3* were significantly higher in adenocarcinoma compared to squamous cell carcinoma (SCC). Two sets of array-based data retrieved from the Gene Expression Omnibus consistently showed that expression of *FOLR1*, a folate transport enzyme, and *GGH*, an enzyme that prevents folate retention, were higher and lower, respectively, in adenocarcinomas compared to SCC. This was independently validated by quantitative RT-PCR in 26 adenocarcinomas and 13 SCC. Our results suggest that folate metabolism plays a role in aberrant DNA methylation in NSCLC. The histological subtype differences in folate concentration and DNA methylation observed here were associated with distinct expression patterns for folate metabolizing enzymes. These findings may have clinical applications for histology-directed chemotherapy with fluoropyrimidine and anti-folates in NSCLC. (*Cancer Sci* 2009; 100: 2325–2330)

Lung cancer is a leading cause of cancer death worldwide. Genetic and epigenetic aberrations accumulate throughout lung carcinogenesis. Global hypomethylation of genomic DNA and hypermethylation of gene promoter regions occur simultaneously in a wide variety of malignancies including lung cancer.[1,2] Little is known however about the mechanism leading to these epigenetic alterations in human primary lung cancer.

Epidemiological studies have demonstrated that dietary folate supplementation can prevent the development of lung cancer.[3] Folate is an important precursor of one-carbon units required for DNA methylation. Therefore, folate metabolism has been suggested to influence epigenetic alterations in lung cancer and this could provide a mechanism to explain the prevention of lung cancer by folate supplementation. High folate might contribute to the maintenance of global methylation through an adequate supply of one-carbon units for the methylation machinery, thereby stabilizing the genome. Although the status of dietary folate intake has been analyzed in relation to DNA methylation,[4] an association between folate concentration and global methylation in human lung tissue has so far not been reported.

In addition to influencing global methylation, folate metabolism could also affect promoter hypermethylation, as demonstrated previously in primary colorectal cancer. The subset of colorectal cancers showing concurrent hypermethylation of a large number of CpG islands has been termed the CpG island methylator phenotype, or CIMP.[5] We previously reported high folate concentrations in the tumor tissue of CIMP+ colorectal cancers,[6] suggesting that folate metabolism is involved not only in global methylation but also in promoter hypermethylation. Previous reports suggest that concurrent promoter methylation can also occur in lung cancer.[7,8] This type of lung cancer could therefore be associated with high tissue folate concentrations.

In the present study we investigated whether folate concentration correlates with DNA methylation in a series of primary NSCLC and adjacent normal tissues. Tissue folate concentrations were found to correlate with global methylation level and with the methylation level of some promoters. However, the most interesting observation was that lung cancers with different histological types showed differences in folate concentrations and global DNA methylation levels. We also observed differential expression of folate metabolizing enzymes between adenocarcinomas and SCC, thus providing an explanation for the differences in folate concentration and global DNA methylation between these histological subtypes.

Materials and Methods

Patients and samples. Matched pairs of tumor and adjacent normal tissues were obtained from the surgical specimen of 72 patients with NSCLC. The patients comprised 51 men and 21 women and ranged in age from 36 to 86 years (mean 66.7 years). Smoking history was obtained from the health interview questionnaire. Current and former smokers were classified as 'smoker' ($n = 51$) and never smokers as 'non-smoker' ($n = 21$). No patients received neo-adjuvant chemotherapy and folate supplementation before undergoing operation. Tissues were frozen in liquid nitrogen immediately after resection and stored at −80°C until processed for analysis. Remaining portions of sample were fixed with formalin and diagnosed histologically following staining with H&E. This study was approved by the Kanazawa University Medical Ethics Committee and written informed consent was obtained from all patients.

[5]To whom correspondence should be addressed.
E-mail: kawakami@med.kanazawa-u.ac.jp

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Folate measurement. The assay for determining the reduced folate concentration is based on entrapment of CH_2FH_4 into a stable ternary complex with excess recombinant human TS protein and excess [6-^{3}H]FdUMP.[9] Because CH_2FH_4 can potentially be dissociated to form tetrahydrofolate under these conditions, the sum of the two folates was measured. Tumor tissues were homogenized with three volumes of ice-cold 10 mM phosphate buffer (pH 7.0) containing 2 mg/mL ascorbic acid and 40 mM 2-mercaptoethanol. After centrifugation at 105 000*g*, supernatants were collected and an aliquot was used for the measurement of soluble protein by the method of Bradford. The remaining supernatant was diluted with homogenization buffer to a final concentration of 4 mg protein/mL, placed immediately in a boiling water bath for 1 min and then centrifuged to remove precipitated protein. The resultant supernatants were used for folate measurement. The reaction mixture contained 5 μg of recombinant human TS protein, 125 nM [6-^{3}H]FdUMP (555 GBq/mmol) and 6.5 mM formaldehyde in 200 μL of 50 mM Tris-HCl buffer (pH 7.4). Fifty microliters of the supernatant was added to 200 μL of reaction mixture and incubated at 30°C for 50 min. The radioactivity in the acid-insoluble fraction was measured with a liquid scintillation counter (TRI-CARB 2000CA; Packard Instruments, Meriden, CT, USA).

Methylation analysis. DNA was extracted using a QIAamp DNA mini kit according to the manufacturer's protocol (Qiagen, Hilden, Germany) and was then treated with bisulfite as described previously.[10] Methylation of *LINE-1* was analyzed as a surrogate marker of global methylation using a quantitative real-time PCR method described previously.[11] Methylation in the promoter regions of *P16(CDKN2A)*, *APC*, *CDH13*, *RARB*, *RASSF1*, *RUNX3*, and *MYOD1* was analyzed quantitatively using the fluorescence-based real-time PCR MethyLight assay.[12] The amount of methylated DNA sequence was normalized against the amount of *ALU* element used as a DNA loading control. The PMR value was calculated using DNA that was fully methylated with *Sss*I methylase as the positive control reference.[13] The primer and probe sequences used for the MethyLight assay are listed in Supplementary Table S1.

Quantitative real-time RT-PCR. Total RNA was isolated by the single-step guanidinium isothiocyanate method using Isogen (Nipon Gene, Toyama, Japan). Random hexamer-primed complementary DNA was generated from total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantification of mRNA for the genes of interest and for the internal reference gene (*GAPDH*) was conducted using a real-time detection method with SYBR Premix Ex Taq (Takara Bio, Otsu, Japan). The quantities of *FOLR1* and *GGH* mRNA were expressed as ratios relative to that of *GAPDH* mRNA. The primer sequences used for *FOLR1*, *GGH*, and *GAPDH* are listed in Supplementary Table S1.

Immunohistochemistry. The representative paraffin sections of tumor tissues were examined for expression of FOLR1 and GGH proteins by the avidin–biotin–peroxidase complex method with a mouse monoclonal antibody to FOLR1 (diluted 1:100; R&D Systems, Minneapolis, MN, USA) and a chicken polyclonal antibody to GGH (diluted 1:100; GenWay Biotech, San Diego, CA, USA) as described previously.[14] Biotinylated horse anti-mouse IgG (diluted 1:200; Vector Laboratories, Burlingame, CA, USA) and biotinylated rabbit anti-chicken IgY (diluted 1:200; Open Biosystems, Huntsville, AL, USA) were used as secondary antibody to detect FOLR1 and GGH, respectively.

Statistics. The results were expressed as median values (25th percentile–75th percentile). The Mann–Whitney *U* test or Kruskal–Wallis test was used to compare levels between two or three categorical variables, respectively. Correlations between two numerical variables were analyzed by Spearman's rank test. All *P*-values shown are two tailed with $P < 0.05$ taken as significant. Statistical analyses were carried out using the R software package (version 2.7.2; http://www.r-project.org/).

Results

Folate concentration and DNA methylation level in NSCLC and adjacent normal tissue. The concentration of folate was significantly higher in NSCLC compared to adjacent normal lung tissue, whereas the global methylation level as indicated by *LINE-1* was lower (Table 1). Methylation levels for *p16*, *CDH13*, *RARB*, *RASSF1*, and *MYOD1* were significantly higher in tumor tissue, but showed no difference for *APC* or *RUNX3* (Table 1). All of the analyzed promoter regions in the genes other than *APC* were more frequently methylated in tumors than in normal tissue when the promoter methylation levels were dichotomized using a cut-off value of 10 PMR (Supplementary Table S2).

No significant associations were observed between folate concentration and DNA methylation levels (global or gene specific) in normal lung tissue (Table 2). In tumor tissue, the folate concentration correlated positively with *LINE-1*, *CDH13*, and *RUNX3* methylation (Table 2), but not with the other methylation sites examined. We next analyzed whether the folate concentration in tumor tissue was associated with concurrent hypermethylation of promoter regions. Hypermethylation was defined as a PMR value of >10 and tumors were classified as showing low (0 or 1 gene methylated), medium (2–4 genes methylated), or high (>4 genes methylated) frequency of methylation. This analysis failed to reveal any significant association between folate concentration and

Table 1. Folate concentrations and DNA methylation in non-small cell lung cancer and adjacent normal tissue

	Normal	Tumor	*P*-value
Folate	1.87 (1.12–2.84)	3.23 (1.21–6.13)	<0.01
LINE-1	88.5 (85.1–90.3)	77.8 (63.0–90.1)	<0.001
p16	0.08 (0.01–0.20)	0.29 (0.03–1.45)	<0.001
APC	7.42 (4.89–8.12)	3.84 (0.38–54.8)	0.252
CDH13	3.26 (1.41–4.34)	7.55 (2.53–15.7)	<0.001
RARB	1.60 (0.41–4.02)	12.5 (4.96–33.0)	<0.001
RASSF1	0.86 (0.24–1.94)	7.28 (0.10–40.6)	<0.01
RUNX3	0.15 (0.00–0.80)	0.00 (0.00–13.2)	0.725
MYOD1	1.89 (0.89–3.20)	3.92 (1.51–12.6)	<0.001

Values for the median and 25th–75th percentile range (in parentheses) are shown for folate concentration (pmol/g protein), *LINE-1* methylation (%), and gene promoter methylation.

Table 2. Associations between tissue folate concentration and DNA methylation levels in non-small cell lung cancer and adjacent normal tissue

Genes	Normal		Tumor	
	Spearman's rho	*P*-value	Spearman's rho	*P*-value
LINE-1	−0.049	0.705	0.243	0.041
p16	−0.034	0.792	0.007	0.953
APC	0.110	0.390	0.083	0.490
CDH13	0.027	0.832	0.251	0.034
RARB	−0.035	0.783	0.024	0.841
RASSF1	−0.076	0.550	0.081	0.499
RUNX3	−0.053	0.680	0.247	0.038
MYOD1	0.028	0.826	0.055	0.652

Spearman's rank test was used to correlate tissue folate concentrations with DNA methylation levels.

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the frequency of gene methylation (Fig. 1). The use of different PMR cut-off values also failed to reveal any significant associations (data not shown). These results indicate that folate concentrations in NSCLC tissue correlate with the global DNA methylation level and with the methylation level of some CpG islands, but not with the concurrent promoter hypermethylation of multiple genes.

Positive correlations were observed between *LINE-1* methylation and promoter methylation of *CDH13* and *RUNX3* in tumor tissue (*LINE-1* and *CDH13*, Spearman's rho 0.278, $P = 0.018$; *LINE-1* and *RUNX3*, Spearman's rho 0.299, $P = 0.011$). No associations were observed between *LINE-1* methylation and promoter methylation in normal tissue.

Folate concentration and DNA methylation in normal lung tissue: Associations with clinical and histological features. Methylation of *CDH13* in normal tissue was positively correlated with patient age (Spearman's rho 0.269, $P = 0.033$). The methylation of several other genes in normal tissue also showed trends for positive correlation with patient age (*RASSF1*, Spearman's rho 0.234, $P = 0.065$; *RARB*, Spearman's rho 0.225, $P = 0.077$; *RUNX3*, Spearman's rho 0.216, $P = 0.089$). These results are consistent with previous studies that have reported increased promoter methylation with older age in several types of normal tissues.[15–17]

Previous studies have demonstrated an association between smoking and promoter methylation in NSCLC.[18,19] In the current study of normal tissue from NSCLC patients, significantly higher methylation levels of *CDH13*, *RARB*, and *MYOD1* (Mann–Whitney *U* test; *CDH13*, $P = 0.021$; *RARB*, $P = 0.009$; *MYOD1*, $P = 0.033$) and marginally higher methylation levels of *p16* (Mann–Whitney *U* test, $P = 0.064$) were observed in smokers compared to non-smokers. The methylation level for these genes was also higher in men compared to women (Mann–Whitney *U* test; *CDH13*, $P = 0.0027$; *RARB*, $P = 0.0005$; *MYOD1*, $P = 0.0098$; *p16*, $P = 0.049$), partly due to the close association between smoking history and sex (Chi-square test; $P < 0.0001$).

Folate concentrations and the level of *LINE-1* methylation in normal lung tissue from NSCLC patients showed no association with any clinical feature. No difference was observed in folate concentration and methylation levels between the normal lung tissues from patients with adenocarcinoma and those from SCC.

Folate concentration and DNA methylation in NSCLC tissue: Associations with clinical and histological features. Patients' age was not correlated with folate concentration and DNA methylation in tumor tissue. Table 3 and Supplementary Table S3 show the associations of folate concentration and of DNA methylation with smoking history, sex, and tumor histology. Tumors from non-smokers showed significantly higher folate concentrations, higher global methylation, and lower *p16* methylation. In accordance with this, NSCLC from women showed the same associations, although the higher *LINE-1* methylation level did not reach significance. *CDH13* was more frequently methylated in smokers and in men when the promoter methylation levels were dichotomized using a cut-off value of 10 PMR (Supplementary Table S4).

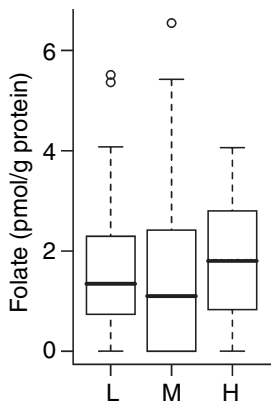

Fig. 1. Folate concentration and the frequency of promoter hypermethylation in non-small cell lung cancer. The methylation frequency was categorized as low (0 or 1 gene methylated; L), medium (2–4 genes methylated; M) or high (>4 genes methylated; H). No relationship was observed between folate concentration and the frequency of promoter hypermethylation (Kruskal–Wallis test).

Table 3. Folate concentration and DNA methylation in non-small cell lung cancer: associations with smoking history, sex, and histological subtype

	Smoker		Sex		Histology	
	Yes	No	Male	Female	Adeno	SCC
n	51	21	51	21	43	23
Folate	2.49	4.66*	2.47	4.53**	4.57	1.84**
LINE-1	70.5	85.1*	75.6	80.9	86.8	63.4***
p16	0.58	0.10**	0.68	0.15*	0.23	0.72
APC	3.51	4.16	4.30	3.15	4.91	2.78
CDH13	7.88	6.32	8.25	5.31	8.02	5.31
RARB	10.4	14.2	12.4	12.7	12.4	10.4
RASSF1	4.11	16.9	8.81	3.69	16.9	0.89*
RUNX3	0.00	0.00	0.00	0.00	0.67	0.00**
MYOD1	3.63	5.23	3.78	3.95	2.75	5.12

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Median values are shown for folate concentration (pmol/g protein), *LINE-1* methylation (%), and promoter methylation. The Mann–Whitney *U* test was used for statistical analysis. Data showing the median, 25th–75th percentile range, and *P*-value are available in Supplementary Table S3. Adeno, adenocarcinoma; *n*, number of patients; SCC, squamous cell carcinoma.

Adenocarcinomas showed significantly higher folate concentrations and methylation of *LINE-1*, *RASSF1*, and *RUNX3* compared to SCC. These associations were not due to links with smoking history or sex, as neither of these factors was associated with histological type (Chi-square test $P = 0.31$ for each). In addition, the association of histological type with folate and *LINE-1* methylation level was also observed with sub-group analyses stratified by smoking history and sex (Table 4). No significant association between the methylation status of promoter regions and histological types was observed when the promoter methylation levels were dichotomized using a cut-off value of 10 PMR (Supplementary Table S4). Tumors with rare histological types (adenosquamous carcinoma $n = 3$; large cell carcinoma $n = 2$; mucoepidermoid carcinoma $n = 1$) were excluded from the analysis.

mRNA and protein expression of *FOLR1* and *GGH* in adenocarcinomas and SCC. The significant association observed between folate concentration and histological type suggests that cellular folate metabolism may differ between adenocarcinomas and SCC. To test this hypothesis, we analyzed gene expression data for NSCLC contained within public databases. Two array-based datasets (Gene Expression Omnibus accession numbers GSE 3141 and GSE 8894) were selected because of their large sample sizes ($n = 111$ and $n = 138$, respectively). Gene expression data for the folate metabolism genes *RFC1*, *FOLR1*, *FPGS*, and *GGH* was retrieved for analysis. In both data sets *FOLR1* mRNA expression was significantly higher in adenocarcinomas compared to SCC (Mann–Whitney *U* test; $P < 0.0001$ for each). In contrast, *GGH* mRNA expression was significantly lower in adenocarcinomas compared to SCC (Mann–Whitney *U* test; $P < 0.0001$ in both data sets). There were no differences in the expression of *RFC1* or *FPGS* between the two major NSCLC histological types.

Table 4. Association of histological subtypes with folate and *LINE-1* methylation levels by sub-group analyses according to smoking history and sex

	Smoker group		Non-smoker group	
	Adeno	SCC	Adeno	SCC
n	27	18	16	5
Folate	3.88 (0.42–6.78)	1.76 (0.00–2.61)*	4.94 (3.93–6.84)	3.23 (1.84–4.04)
LINE-1	85.7 (71.9–90.2)	60.3 (45.9–69.8)***	90.2 (83.1–91.4)	75.4 (51.3–76.5)**
	Male group		Female group	
	Adeno	SCC	Adeno	SCC
n	27	18	16	5
Folate	3.66 (0.00–6.35)	1.76 (0.00–2.61)	4.94 (3.93–7.38)	3.10 (1.84–3.23)*
LINE-1	86.8 (76.8–90.2)	60.3 (45.9–69.8)***	87.7 (77.4–91.4)	75.4 (51.3–77.2)*

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Values for the median and 25th–75th percentile range (in parentheses) are shown for folate concentration (pmol/g protein) and *LINE-1* methylation (%). The Mann–Whitney *U* test was used for statistical analysis. Adeno, adenocarcinoma; *n*, number of patients; SCC, squamous cell carcinoma.

To validate the differences in *FOLR1* and *GGH* expression between histological types, the mRNA level of these genes was measured by quantitative RT-PCR in 26 adenocarcinomas and 13 SCC from the current study. Consistent with results from the two array-based datasets, significant differences in the expression of *FOLR1* and *GGH* were observed in our NSCLC samples (Fig. 2a). FOLR1 and GGH proteins were immunohistochemically detected in these tumor tissues. Both proteins were mainly expressed in tumor cells. The levels of FOLR1 and GGH protein expression examined by immunohistochemistry (Fig. 2b) were concordant with the levels of mRNA expression measured by the RT-PCR (Fig. 2a).

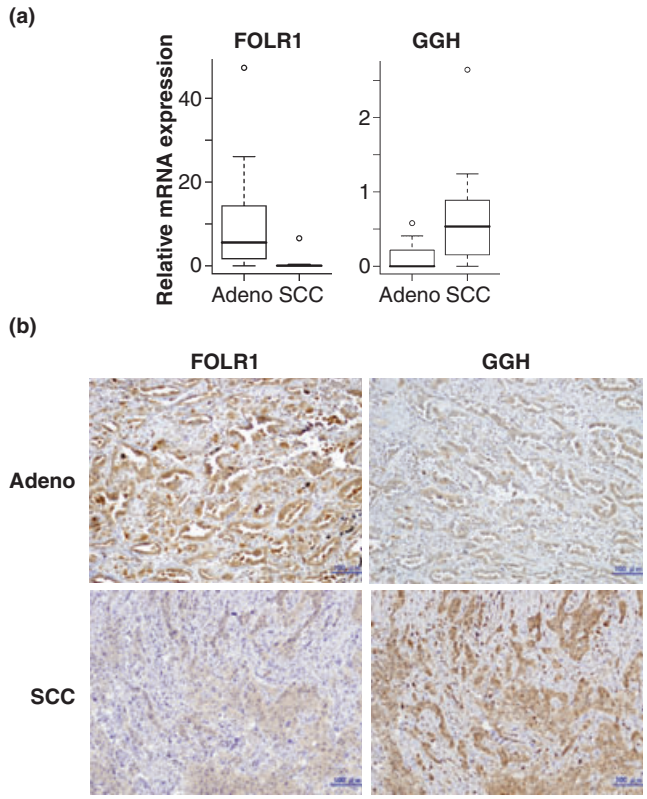

Fig. 2. mRNA and protein expression of *FOLR1* and *GGH* according to the histological type of non-small cell lung cancer. (a) RNA isolated from adenocarcinoma (Adeno; $n = 26$) and squamous cell carcinoma (SCC; $n = 13$) was quantitatively analyzed by real-time RT-PCR. RNA isolated from the HeLa cervical cancer cell line was used as a standard. The mRNA expression level of *FOLR1* and *GGH* in HeLa was set as 1. The expression of *FOLR1* was higher in adenocarcinoma than SCC (Mann–Whitney *U* test; $P < 0.0001$), whereas the expression of *GGH* was lower (Mann–Whitney *U* test; $P < 0.001$). (b) Expression and localization of FOLR1 and GGH proteins in the tumors of Adeno and SCC were examined by immunohistochemical staining. These proteins were mainly expressed in tumor cells. Expression of FOLR1 was higher in Adeno than SCC, whereas the level of GGH was higher in SCC than Adeno. Scale bars = 100 μm.

Discussion

The significant differences between normal and tumor tissues observed in this study for global (*LINE-1*) and promoter methylation (Table 1) indicate the central role of aberrant DNA methylation in lung carcinogenesis. In addition, we found a positive correlation between folate concentration and DNA methylation of *LINE-1* and of several promoter regions in NSCLC samples. This correlation supports the notion that folate metabolism plays a role in acquiring and/or maintaining aberrant DNA methylation.

Global hypomethylation acts as a risk factor for the development of cancer by giving rise to genomic instability.[20] High concentrations of folate in lung tissue may contribute to the prevention of global hypomethylation because folate is a precursor molecule of the one-carbon units required for DNA methylation. Dietary folate supplementation is likely to result in higher tissue folate concentrations, therefore preventing lung carcinogenesis by maintaining an adequate global methylation level. However, a positive correlation between folate concentration and global methylation level in human lung tissue has not been demonstrated to date. The positive correlation observed in the present study between folate concentration and *LINE-1* methylation in NSCLC tissue (Table 2) is consistent with a role for folate in the maintenance of global methylation. Stronger evidence for a role of folate supplementation in lung cancer prevention may have been provided by an association between folate concentration and DNA methylation level in normal lung tissue; however, this was not observed in the present study (Table 2). The narrow range of *LINE-1* methylation in normal lung tissue (5.2% for the 25th–75th percentiles) could explain the failure to observe an association with folate concentration. Analysis of a larger number of samples and/or a different method of measuring global methylation may reveal a link between folate and global methylation in normal lung tissue.

Unlike colorectal cancer,[6] no relationship was observed here between tissue folate concentration and the frequency of concurrently hypermethylated genes in NSCLC (Fig. 1). The

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methylator phenotype in colorectal cancer, or CIMP, has well-defined genetic and clinicopathological features, including frequent *Braf* mutation, microsatellite instability, and mucinous pathology.[21–23] Previous studies have suggested the existence of a methylator phenotype in lung cancer;[7,8] however, distinctive biological characteristics for this phenotype have yet to be described. No confirmative evidence or defined CpG sites for methylation analysis have been put forward to demonstrate the existence of a methylator phenotype in NSCLC. Currently, the existence of a methylator phenotype defined by concurrent promoter methylation and showing an association with tumor folate concentrations is unlikely in NSCLC. Nevertheless, further large-scale analyses of promoter methylation are required to establish whether a methylator phenotype exists in NSCLC.

The most important finding in this study was that folate concentration and global DNA methylation were significantly higher in adenocarcinoma compared to SCC. The higher folate concentration in adenocarcinomas suggested a difference in folate metabolism and this led us to investigate mRNA expression levels for several folate metabolizing enzymes in relation to NSCLC histology. Two public databases of array-based gene expression analysis showed consistent results with regard to histologically related differences in the expression of folate metabolizing enzymes. Quantitative RT-PCR analysis of tumor samples from the present NSCLC series confirmed that *FOLR1* expression was higher and *GGH* expression was lower in adenocarcinomas compared to SCC (Fig. 2a). Immunohistochemical observation of FOLR1 and GGH proteins in the tumor tissues supports that the histological subtype differences in these mRNA expressions were attributed to their distinct expression in tumor cells (Fig. 2b). FOLR1 transports folate into cells[24] and its elevated expression in adenocarcinomas relative to SCC was consistent with the higher folate concentration observed for this histological type (Table 3). The GGH enzyme removes polyglutamate chains from polyglutamylated folate,[25] thus facilitating the escape of folate from within cells. The low GGH expression level observed in adenocarcinomas is therefore also consistent with the higher folate concentrations observed in this histological type.

Another possible explanation for the associations between folate, global methylation, and histological type (Table 3) is a common link with smoking history. The development of SCC has been linked with smoking.[26,27] Smoking has also been associated in previous reports with low folate[28] and low global methylation.[29] However, in the present study histological type was not associated with smoking history. Furthermore, significant associations between histological type and folate concentration or global methylation were observed in both the smoking and non-smoking groups (Table 4). Although the link between histological type and folate level did not reach statistical significance in the non-smoker group probably due to the small number of SCC cases, the results of sub-group analysis suggest that these links were independent of smoking. Larger-scale analysis for NSCLC cases without smoking history is needed to rule out the influence of smoking.

The observed differences in folate metabolism between the two major histological types of NSCLC may be of clinical importance for individualized chemotherapy. A recent clinical trial has shown that fluoropyrimidine is active for NSCLC.[30] The activity of fluoropyrimidine can be enhanced by simultaneous administration of folate, thereby increasing the intracellular levels of CH_2FH_4 and prolonging the inhibition of TS, the major target enzyme of fluoropyrimidine.[31] An *in vitro* study recently demonstrated that cancer cell lines with higher basal levels of intracellular folate also showed larger increases in CH_2FH_4 following the addition of leucovorin, a folinic acid.[32] Since adenocarcinomas were shown in the present study to have higher folate concentrations than SCC, the administration of folinic acid could therefore be more effective in increasing the CH_2FH_4 concentration and thus providing better sensitization of these tumors to fluoropyrimidine. This hypothesis has not been tested to date and future clinical trials may consider whether the histological type of NSCLC is a determinant for the activity of fluoropyrimidine modulated with folinic acid.

In addition to fluoropyrimidine, the histological type of NSCLC may be important for personalized chemotherapy with anti-folates. Recent clinical studies in NSCLC have demonstrated that pemetrexed is more effective against adenocarcinoma than SCC.[33,34] Pemetrexed is an anti-folate that inhibits multiple enzymes including dihydrofolate reductase and GARFT as well as TS. An earlier study found that the polyglutamylated form of pemetrexed inhibits TS and GARFT more potently than the monoglutamylated form of this agent.[35] The low expression level of GGH observed here in adenocarcinomas (Fig. 2) could therefore contribute to higher concentrations of longer, polyglutamylated forms of pemetrexed. This would imply stronger inhibition of TS and GARFT by pemetrexed in adenocarcinomas compared to SCC. The histological subtype of NSCLC could also influence the efficacy of other anti-folates such as raltitrexed, nolatrexed, and plevitrexed. Future clinical studies should consider histological type when testing the activity of anti-folates against NSCLC.

In conclusion, the current study has demonstrated an association between folate concentration and DNA methylation in NSCLC, thus providing evidence that folate metabolism plays a role in the DNA methylation machinery. However, the concurrent hypermethylation of multiple promoter regions in NSCLC was not associated with elevated folate levels. These results suggest that a methylator phenotype similar to CIMP in colorectal cancer and associated with higher folate concentrations does not occur in NSCLC. Striking histology-related differences in folate concentration and global methylation were apparent in NSCLC and were shown to correlate with a distinct expression pattern for two major folate metabolizing enzymes. Differences in folate metabolism between adenocarcinomas and SCC may be of clinical use in NSCLC patients for histology-directed chemotherapy using fluoropyrimidine and anti-folates.

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Disclosure Statement

There is no conflict of interest to this article.

Abbreviations

ALU	Alu element, a primate-specific SINE element
APC	adenomatous polyposis coli
CDH	cadherin
CH_2FH_4	5,10-methylenetetrahydrofolate
CIMP	CpG island methylator phenotype
FOLR	folate receptor
FPGS	folylpolyglutamate synthase
GARFT	glycinamide ribonucleotide formyltransferase
GGH	gamma-glutamyl hydrolase
LINE	long interspersed nuclear element
MYOD	myogenic differentiation
NSCLC	non-small cell lung cancer
p16(CDKN2A)	cyclin-dependent kinase inhibitor 2A

PMR	percentage of methylated reference
RARB	retinoic acid receptor beta
RASSF	Ras association (RalGDS/AF-6) domain family member
RFC	reduced folate carrier
RUNX	runt-related transcription factor
SCC	squamous cell carcinoma
TS	thymidylate synthase

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers and probes used for real-time detection.

Table S2. Dichotomized promoter methylation statuses in NSCLC and adjacent normal tissue.

Table S3. Folate concentration and DNA methylation in NSCLC: associations with smoking history, gender and histological subtype.

Table S4. Dichotomized promoter methylation statuses in NSCLC: associations with smoking history, gender and histological subtype.

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