Published in final edited form as:
J Thorac Oncol. 2012 May ; 7(5): 833–840. doi:10.1097/JTO.0b013e31824de09c.

High Expression of Folate Receptor Alpha in Lung Cancer Correlates with Adenocarcinoma Histology and *EGFR* Mutation

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Abstract

Introduction—Folate receptor alpha (FRα) and reduced folate carrier-1 (RFC1) regulate uptake of folate molecules inside the cell. FRα is a potential biomarker of tumors response to antifolate chemotherapy and a target for therapy using humanized monocloncal antibody. Information on the protein expression of these receptors in non–small cell lung carcinoma (NSCLC) is limited.

Material and Methods—Expressions of FRα and RFC1 were examined by IHC in 320 surgically resected NSCLC (202 adenocarcinomas and 118 squamous cell carcinomas) tissue specimens and correlated with patients' clinicopathologic characteristics. *FOLR1* mRNA expression was examined using publicly available microarray datasets. FRα expression was correlated with thymidylate synthase (TS) and p53 expression in NSCLCs, and with *EGFR* and *KRAS* mutations in adenocarcinomas.

Results—NSCLC overexpressed FRα and RFC1. In a multivariate analysis, lung adenocarcinomas were more likely to express FRα in the cytoplasm (odds ratio [OR] = 4.39; $P<0.0001$) and membrane (OR = 5.34; $P<0.0001$) of malignant cells than squamous cell carcinomas. Tumors from never-smokers were more likely to express cytoplasmic (OR = 3.35; $P<0.03$) and membrane (OR = 3.60; $P=0.0005$) FRα than those from smokers. In adenocarcinoma, *EGFR* mutations correlated with higher expression of membrane FRα and *FOLR1* gene expressions. High levels of FRα expression was detected in 42 NSCLC advanced metastatic tumor tissues.

Conclusions—FRα and RFC1 proteins are overexpressed in NSCLC tumor tissues. The high levels of FRα in lung adenocarcinomas may be associated to these tumors' better responses to antifolate chemotherapy and represents a potential novel target for this tumor type.

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Financial disclosure: there are no financial disclosures from any authors

Keywords

non–small cell lung carcinoma; EGFR; membrane transporter; FRα; FRC1

INTRODUCTION

Lung cancer represents the first cause of death for cancer worldwide [1]. Most patients with lung cancer are diagnosed at advanced metastatic stage (IV), requiring systemic treatment.[1] Two types of non-small cell carcinoma (NSCLC), adenocarcinoma and squamous cell carcinoma (SCC), are the most frequent (~80%) histological types of lung cancer.[2] Despite intensive research on molecular targeted therapy, chemotherapy still represents the main treatment option for patients with advanced NSCLC.[3] In addition, over recent years chemotherapy after surgical resection has become the standard of care for treatment of selected patients with early stage (i.e., stage IB, II, or IIIA) NSCLC.[4] However, a subset of tumors does not respond to chemotherapy, and most tumors develop drug resistance, leading to chemotherapy failure.[2] The factors associated with chemotherapy resistance are not well understood, but some phenomena have been associated with this resistance, including, among others, decreases or alterations in the membrane transporters involved in drug uptake systems or increase in drug efflux pumps.[5]

Folate receptor alpha (FRα) and reduced folate carrier-1 (RFC1) regulate cellular uptake of folate molecules inside the cell.[5–7] Folates are required in the synthesis of nucleotide bases, amino acids, and other methylated compounds, and consequently, they are required in larger quantities by proliferating cells.[5] FRα is a glycoprotein that is anchored to the apical cell membrane of normal epithelial cells,[8] and binds folate at a high affinity to mediate transport into the cytoplasm of cells.[5] RFC1 is more ubiquitously expressed in normal cells, binds folate at low affinity, and represents the sole folate uptake pathway for most normal cells [7].

FRα expression is upregulated in a range of human tumors, including ovarian, mesothelioma, lung and colorectal cancer.[9–13] However, the level of expression of RFC1 in tumors is less known. FRα has emerged as a potential marker for response to treatment of human carcinomas with the drug pemetrexed,[14] a potent inhibitor of thymidylate synthase (TS) and other folate-dependent enzymes.[15–17] Interestingly, FRα has been also investigated as a potential novel molecular target for human tumors.[18–20] Recently, a humanized monoclonal antibody against FRα has been tested in a Phase I clinical trial in patients with advanced chemorefractory ovarian carcinomas.[19]

In this study, we aimed to characterize the expression of FRα and RFC1 proteins in a large series (n=320) of surgically resected NSCLC tissue specimens with annotated clinicopathologic features. In addition, we investigated the expression of FRα in a small series (n=42) of advanced metastatic NSCLC tumor tissues. In surgically resected tumors we correlated the expression of FRα with the expression of TS. Our findings of higher expression of FRα expression in lung tumors with adenocarcinoma histology and tumors obtained from never-smokers prompted us to correlate the expression of FRα with tumors' epidermal growth factor receptor (*EGFR*) and *KRAS* mutation status in adenocarcinomas, and with tumors' p53 protein expression in all NSCLCs.

METHODS

Case selection and tissue microarray (TMA) construction

We obtained archived formalin-fixed and paraffin-embedded (FFPE) NSCLC tissues from the Lung Cancer Tissue Bank at The University of Texas M. D. Anderson Cancer Center

(Houston, TX). We selected lung cancer tissue specimens from surgically resected NSCLCs with curative intent between 1997 and 2001, and constructed TMAs using three 1-mm diameter cores. Detailed clinico-pathologic information was available for most cases (Table 1). In addition, we selected FFPE NSCLC tumor tissues from diagnostic tissue specimens from 42 advanced metastatic NSCLCs. The tissue specimens were histologically classified according to the 2004 World Health Organization classification.[2] The institutional review board at M. D. Anderson Cancer Center approved our study.

Immunohistochemical staining and evaluation

To test the expression of the membrane transporters we used a monoclonal homemade antibody against FRα (clone Mb343, IgG), dilution 1:500, [13] and a polyclonal antibody against RFC1 (Abcam, Cambridge, MA), dilution 1:100. To assess the expression of TS, we used a monoclonal antibody (Zymed Carlsbad, CA, USA), dilution 1:100. For p53 analysis, we used mouse monoclonal antihuman p53, clone DO7 (Dako, Carpinteria, CA), dilution 1:400.

For FRα we used a previously published immunohistochemistry protocol.[13] For RFC1 and TS, immunohistochemical staining was performed as follows: 5-μM FFPE tissue sections were deparaffinized and hydrated, and underwent heat-induced epitope retrieval in a DAKO antigen retrieval bath at 121°C for 30 seconds and 90°C for 10 seconds in a decloaking chamber (Biocare, Concord, CA), followed by a 30-min cool down. Prior to antibody immunostaining, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min. To block nonspecific antibody binding, tissue sections were incubated with 10% fetal bovine serum in Tris-buffered saline solution with Tween 20 for 30 min. The slides were incubated with primary antibody at ambient temperature for 60 min for all antibodies. This was followed by incubation with biotin-labeled secondary antibody (Envision Dual Link +, DAKO) for 30 min. Staining was developed with 0.05% 3′,3-diaminobenzidine tetrahydrochloride, which had been freshly prepared in 0.05 mol/L Tris buffer at pH 7.6 containing 0.024% H_2O_2, and then the slides were counterstained with hematoxylin, dehydrated, and mounted.

Two observers (M.N. and I.W.) jointly quantified the immunohistochemical expression of the membrane transporters (magnification 20×) in normal bronchial epithelium and lung tumor malignant epithelial cells. For each membrane transporter and TS, we defined 3 categories of intensity of immunostaining (0 to 3+). Next, an expression score (range, 0–300) was obtained by multiplying the intensity of staining by the percent of cells (0–100%) staining. p53 expression was categorized by percentage of tumor cells expressing nuclear p53 as positive (≥5%) or negative (0–5%).

EGFR and *KRAS* mutation analysis

Exons 18 through 21 of *EGFR* and exon 1 of *KRAS* were amplified by polymerase chain reaction (PCR) using intron-based primers as previously described.[21, 22]

Assessment of membrane transporter expression in microarray datasets

The cancer microarray database and integrated data-mining platform Oncomine[23] was utilized to analyze the expression of *FOLR1* (FRα) and *SLC19A1* (RFC1), and in microarray databases of NSCLC available online.[24–27] The statistical significances of differences in expression of the genes were provided by Oncomine and confirmed by a two-tailed t-test with random variance. Gene expression data of lung adenocarcinomas with annotated mutation data of *EGFR* and *KRAS* were obtained from the Ladanyi and Gerald laboratories at the Memorial Sloan-Kettering Cancer Center (MSKCC) (http://cbio.mskcc.org/Public/lung_array_data/).[28] Available Affymetrix® raw data files of

the transcriptomes of 190 adenocarcinomas (set I, n=88; set II, n=102) were analyzed using the BRB-ArrayTools version 3.7.0 software developed by using the BRB-ArrayTools v. 3.7.0 developed by Dr. Richard Simon and BRB-ArrayTools Development Team.[29] Robust multi-array analysis (RMA) was used for normalization of gene expression data using the R language environment.[30] *FOLR1* mRNA expression levels in both MSKCC datasets were median-centered by the Cluster v.2.11 software. Differences in normalized median-centered *FOLR1* expression levels were assessed for statistical significance by the two-tailed test and $P < 0.05$ were considered statistically significant.

Statistical methods

Associations between biomarker expression scores and patient clinico-pathologic data were assessed using the Wilcoxon's rank sum test or Kruskal-Wallis test, as appropriate, for continuous variables and the chi square test for categorical variables. The immunohistochemical expression of the markers was dichotomized in negative (score=0) and positive (score>0) expressions based on the graphical distribution of the scores. For recurrence free survival (RFS) and overall survival (OS) analyses, we tested binary cutoff points of biomarkers using the median expression score for each marker. Univariate and multivariate Cox proportional hazards regression models were used to assess the effects of covariates on survival. All statistical tests were two-sided, and *P* values <0.05 were considered statistically significant.

RESULTS

Expression of FRα and RFC1 in surgically resected tumors

Both adenocarcinoma and SCC expressed relatively high levels of FRα and RFC1 in the malignant cells (Fig. 1 and Table 2). For FRα, the average expression scores and frequency of any expression (score >0) were significantly higher in adenocarcinomas than in SCCs at membrane ($P<0.001$) and cytoplasmic ($P<0.001$) localizations (Fig. 2). Both NSCLC histologies demonstrated similar levels of cytoplasmic and membrane RFC1 expression. For both markers the tumor cells exhibited stronger immunohistochemical expression than the 11 samples of normal bronchial epithelia adjacent to tumors (data not shown).

Correlation of FRα and RFC1 expression with clinicopathologic features in surgically resected tumors

The multivariate analysis of the immunohistochemical expression of the two membrane transporters as a dichotomized variable (positive, score >0, vs. negative, score =0), after adjustment for patient's tumor histology, smoking history, sex, and disease stage, revealed that adenocarcinomas were more likely than SCCs to express cytoplasmic (odds ratio [OR] = 4.39; $P<0.0001$) and membrane (OR = 5.34; $P<0.0001$) FRα. In addition, tumors from never-smokers were significantly more likely to express cytoplasmic (OR = 3.35; $P < 0.03$) and membrane (OR = 3.60; $P=0.0005$) FRα than those of smokers. In the multivariate analysis, the patient's sex was not an independent significant factor influencing tumor expression of FRα. No correlation was found between expression of both membrane transporters and RFS or OS in 230 patients with stage I or II NSCLCs (median follow up, 7.2 years).

Correlation between FRα expression and tumors' p53 expression and *EGFR* and *KRAS* mutation status

Our findings of higher expression of FRα expression in lung tumors with adenocarcinoma histology and tumors obtained from never-smokers prompted us to correlate the expression

of FRα with tumors' *EGFR* and *KRAS* mutation status in adenocarcinomas, and with tumors' p53 protein expression in all NSCLCs.

In lung adenocarcinomas, *EGFR* mutant tumors demonstrated significantly higher expression scores for membrane FRα (mean scores: mutant 134.8 vs. wild-type 67.1; P=0.002) than wild-type tumors (Figure 3, A). No correlation between FRα expression and adenocarcinoma tumors' *KRAS* mutation status was detected.

Of all NSCLCs tested, 38% (75/195) of adenocarcinomas and 69% (80/116) of SCCs had a positive p53 level (≥5%). Interestingly, we found that the scores for FRα expression in both membrane (P=0.001) and cytoplasm (P<0.001) were significantly lower in malignant cells from NSCLC tumors with positive p53 expression (mean score: membrane 33.4, SD 59.9, and cytoplasm 58.3, SD 60.0) than in tumors with negative p53 expression (mean score: membrane 65.3, SD 90.6, and cytoplasm 83.55.3, SD 65.3).

Expression of FRα in advanced metastatic tumors

To determine the levels of FRα expression in the entire spectrum of NSCLC, we examined FRα expression in 42 tumor tissues obtained from advanced NSCLCs (27 from lung/pleural tumors and 15 from metastatic sites). The tumor histologies corresponded to 23 adenocarcinomas, 5 SCCs, and 14 tumors classified as NSCLCs without features of specific histology (NSCLC-no otherwise specified [NOS]). We found that advanced tumors demonstrated similar levels of FRα expression than surgically resected tumors by examining the average expression scores and frequency of any expression (score >0). Although small numbers, in the advanced tumors the FRα average expression scores were higher in adenocarcinomas than SCCs at membrane (mean score: adenocarcinoma 62.2. SD 81.2; SCC 20.0, SD 44.7; P=0.042) and cytoplasmic (mean score: adenocarcinoma 104.1, SD 88.5; SCC 22.0, SD 39.0; P=0.319) locations. NSCLC-NOS showed intermediate levels of FRα expression (mean score: membrane 20.7, SD 56.9, and cytoplasm 64.6, SD 96.5). In advanced tumors, any membrane expression (score > 0) of FRα was detected in 48% (11/23) of adenocarcinomas and 20% (1/5) of SCCs, whereas cytoplasmic expression was observed in 78% (18/23) and 40% (2/5) SCCs. No significant difference in the expression of FRα was detected when comparing lung/pleural tumors with metastatic sites (data not shown).

FOLR1 mRNA expression in tumor tissues

Our findings that protein levels of FRα was greater in adenocarcinomas than in SCCs incited us to analyze expression levels of the mRNA of the *FOLR1* in published microarray datasets of surgically resected (satges I–III) NSCLC tumor specimens and compare them by histologic type.[24–27] In accordance with our immunohistochemistry data, *FOLR1* mRNA expression levels were significantly higher in adenocarcinomas (n=197) than in SCCs (n=210) in all four datasets available: 1.8 vs. 1.0 (P<0.0001),[25] 0.81 vs. 0.73 (P=0.03),[27] 2.61 vs. 0.98 (P<0.0001),[26] and 0.93 vs. 0.31 (P<0.0001). [24]

To confirm our findings on the increased FRα immunoreactivity in tumors obtained from *EGFR* mutant lung adenocarcinomas compared to wild type tumors, we probed this association using the mRNA expression levels of *FOLR1* in publicly available microarray datasets with information on *EGFR* and *KRAS* mutation status.[28] Notably, the analysis of the microarray data further revealed the statistically significant up-regulation of *FOLR1* mRNA levels in *EGFR* mutant lung adenocarcinomas compared to wild-type tumors in both available datasets (P=0.00016 and P =0.003) (Fig. 3, B and C). In addition, no statistically significant differences were found in *FOLR1* expression levels between *KRAS* mutant lung adenocarcinomas and wild-type tumors (data not shown). These findings confirm the close

positive association between *FOLR1* expression and *EGFR* mutation status which we had found at the protein level by assessment of FRα immunoreactivity.

Correlation of immunohistochemical expression of TS and FRα

TS was expressed frequently in the nucleus and cytoplasm of malignant NSCLC cells. However, the frequency of any TS expression (score >0) was higher in the cytoplasm (212/267, 79%) than in the nucleus (117/267, 44%) of these cells. Although cytoplasmic expression of TS was similar in both NSCLC histologic types (Table 2), nuclear expression was significantly higher (P=0.003) in SCCs (mean score: 13.8, SD 27.7) than in adenocarcinomas (mean score: 9.3, SD 27.1). The level of TS expression did not correlate with clinicopathologic characteristics, including RFS and OS. In all NSCLC, significantly (P=0.02) higher expression of nuclear TS immunostaining was detected in tumors with positive p53 expression (67/114, 58%) than in those with negative p53 staining (65/147, 44%). In adenocarcinomas, there was no correlation between TS expression and *EGFR* or *KRAS* mutation status.

We correlated the expression of TS and FRα in NSCLC tissue specimens. The score for nuclear TS expression correlated negatively with the score for cytoplasmic FRα expression in SCCs (r = −0.20; P=0.04), and showed marginally significant negative correlation with membrane FRα expression in adenocarcinomas (r = −0.16; P=0.05). When we examined the correlation of any expression (score >0) of both markers in tumors, we found that in SCCs expression of nuclear TS was significantly inversely correlated (P=0.03) with membrane expression of FRα, and that most tumors positive for TS (62/79, 79%) lacked membrane FRα. This correlation was not detected in adenocarcinomas.

DISCUSSION

Membrane transporters FRα and RFC1 are considered potential biomarkers of tumor response to antifolate chemotherapy.[14] Additionally, FRα represents a novel targets for therapy in human carcinomas utilizing monoclonal antibodies.[19, 20] Information on the protein expression of these receptors in NSCLC is limited. Here, we report for the first time that NSCLC frequently overexpress both FRα and RFC1 proteins by studying a large series of cases with annotated clinico-pathologic information. Importantly, we report that tumor cells from lung adenocarcinoma histology expressed significantly higher levels of cytoplasmic and membrane FRα than SCC, and tumors from never-smokers were significantly more likely to express cytoplasmic FRα than those from smokers. In lung adenocarcinomas, the presence of *EGFR* mutations correlated with higher expression of membrane FRα and *FOLR1* gene expression. NSCLC tissue specimens from advanced metastatic tumors showed similar levels of FRα expression than surgically resected tumors. We postulate that this information may be useful in selecting which patients with NSCLC may benefit from receiving treatment with antifolate inhibiting agents and monoclonal antibodies against FRα.

Our study showed that RFC1 is expressed frequently in the membrane and cytoplasm of malignant cells of NSCLC tumor tissues. While RFC1 performs its important biological functions at the cell membrane, the cytoplasmic expression can be explained as part of synthesis of the protein.[31] The only report available on the expression of RFC1 in human tumors showed relatively high levels of mRNA gene expression in NSCLC, with similar expression in adenocarcinomas and SCCs.[11] These data are consistent with our protein expression data showing that levels of expression of RFC1 were similar in the two histologic types.

Interestingly, in our study the expression of membrane and cytoplasmic FRα was significantly higher in surgically resected lung adenocarcinomas compared with SCCs. FRα is bound to the cell membrane where binds to folate and internalize it in the cytoplasm *via* endocytosis.[6] Similar trend was detected in a small set of advanced metastatic NSCLC tumor tissues. FRα has been shown by immunohistochemical studies to be overexpressedpreviously in small sets of NSCLC tissue specimens.[11, 12] However, to the best of our knowledge, there is not published report of FRα protein expression in NSCLC tumors and correlation with clinical and pathological features. Our protein expression findings agree with the significantly higher levels of expression of *FOLR1* (FRα gene) mRNA in adenocarcinomas than in SCCs in all four public microarray datasets available.[24–27] Similar findings have been reported in a quantitative (q)PCR study of mRNA expression of 119 NSCLC tissue specimens.[11]

The findings of higher levels of FRα protein and *FOLR1* mRNA expression in adenocarcinomas than in SCCs of the lung may have important clinical implications. The higher level of FRα protein expression in adenocarcinoma cells may explain the better response of advanced NSCLC of non-squamous histology when treated with the combination of cisplatin and the multitargeted antifolate agent pemetrexed.[32] However, this needs to be further tested in NSCLC tumor tissue specimens obtained from patients treated with pemetrexed. In addition, FRα is currently considered an attractive target for biologic therapy in tumors in which it is overexpressed compared to corresponding normal epithelium such as ovarian cancer by using the humanized monoclonal antibody against FRα Farletuzumab.[19, 20] Our findings of high expression of FRα in NSCLC compared with normal bronchial epithelium suggest that this protein could be considered a novel potential target for NSCLC, particularly in lung adenocarcinomas.

Our finding that NSCLCs of never-smokers have a higher expression of FRα than those of smokers is of interest. Our data showing significantly higher cytoplasmic and membrane FRα expression in NSCLCs obtained from never-smokers are in agreement with the previous report of higher levels of mRNA *FOLR1* by qPCR in adenocarcinomas from non-smokers and light smokers than in those from heavy smokers.[11] These differences in the expression of FRα by smoking status are consistent with our findings of higher FRα expression in NSCLCs lacking p53 expression and in adenocarcinomas harbouring *EGFR* mutation, two features associated with the pathogenesis of non–smoking-related lung cancer.[33] Of interest, the analysis of the publicly available microarray data confirmed at mRNA gene expression level our observation that *EGFR* mutant adenocarcinoma tumors expressed higher levels of FRα protein. There are not data available on the response to antifolate chemotherapy agents in lung adenocarcinomas based on *EGFR* mutation status. However, it has been shown that advanced stage adenocarcinoma harbouring this mutation showed improved response to other type of (carboplatin and paclitaxel) chemotherapy.[34]

Because of their roles in metabolism of the chemotherapy agent pemetrexed,[14, 35] we correlated the expressions of TS and FRα in NSCLC tissue specimens by histologic type. As previously reported,[36, 37] TS protein was expressed frequently in the nucleus (44%) and cytoplasm (79%) of malignant NSCLC cells. In our analysis we determined that nuclear expression was significantly higher in SCCs than in adenocarcinomas. Ceppi et al[36] previously reported that immunohistochemical expression of TS mRNA and protein was significantly higher in SCCs of the lung than in adenocarcinomas. In this previously reported immunohistochemical analysis, however, expression of TS in the malignant cells was not distinguished as nuclear or cytoplasmic. It has been shown that low levels of TS mRNA expression significantly correlated with *in vitro* chemosensitivity of freshly explanted human tumor specimens to pemetrexed.[38] It has been hypothesized that the higher mRNA and protein expressions of TS observed in SCCs explains the lower rate of response

to pemetrexed in this NSCLC type.[32] Recently, Sun et al[39] reported that low immunohistochemical TS protein expression in tumors correlated with worse progression-free survival in stage IIIB and IV patients with non-squamous cell lung carcinomas treated with pemetrexed,

When we correlated FRα and TS protein expression in NSCLC tumors, we found that in SCCs the expression of nuclear TS had a significant inverse correlation with expression of membrane FRα, and most TS-positive SCCs (79%) lacked membrane FRα immunostaining. Furthermore, we speculate that the more frequent occurrence of the FRα membrane-negative/TS nuclear-positive expression pattern in lung SCCs than in adenocarcinomas could be associated with the lower response rate to pemetrexed in this tumor type. While FRα is most biologically active at the cell membrane,[6] there is strong evidence of the important role of TS as translational regulation in the nucleus of cells.[40]

In summary, our findings indicate that membrane transporter FRα and RFC1 proteins are frequently overexpressed in NSCLC tissues. The higher level of FRα in adenocarcinomas than in SCCs may help explain differences in efficacy of antifolate chemotherapy between these tumor types. We postulate that this information may be useful in selecting which patients with NSCLC may benefit from and should receive treatment with antifolate inhibiting agents and with monoclonal antibodies against FRα.

Acknowledgments

Acknowledgments of research support: This study was supported in part by grants from the Department of Defense (W81XWH-07-1-0306 to D.J.S., and I.I.W.), the Specialized Program of Research Excellence in Lung Cancer (P50CA70907 to I.I.W.), and the National Cancer Institute (Cancer Center Support Grant CA-16672).

Abbreviations

EGFR	epidermal growth factor receptor
FFPE	formalin-fixed paraffin-embedded
FRα	folate receptor alpha
RFC1	reduced folate carrier 1
TS	thymidylate synthase
NSCLC	non–small cell lung carcinoma
OR	odds ratio

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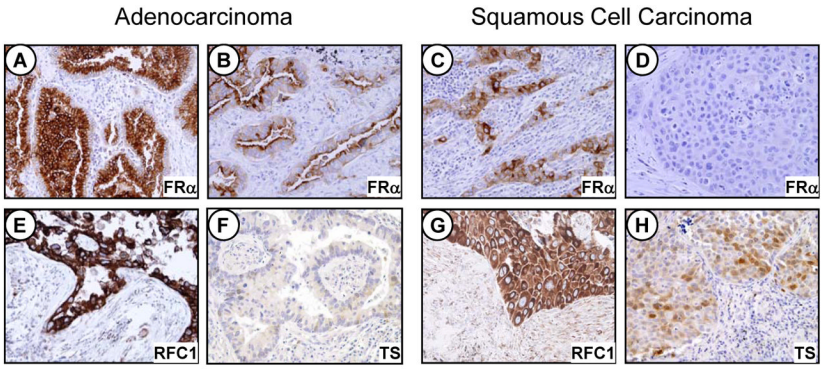

Figure 1.

Photomicrographs showing immunohistochemical expression of FRα, FRC1 and TS in NSCLC tissue specimens by histologic type. FRα: *A*, strong cytoplasmic and membrane expression in tumor cells; *B* and *C*, moderate expression in tumor cells; *D*, lack of expression in malignant cells. RFC1: *E* and *G*, strong cytoplasmic expression in malignant cells. TS: *F* and *H*, negative and moderate cytoplasmic and nuclear expression in tumor cells, respectively. Original magnification, ×200.

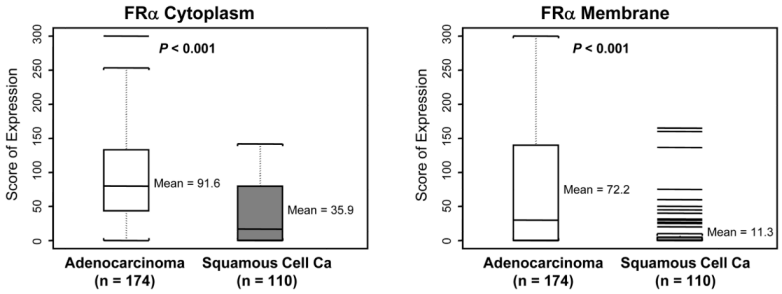

Figure 2.

FRα expression scores by tumor histology. In the box-plots, *black bar* indicates median scores.

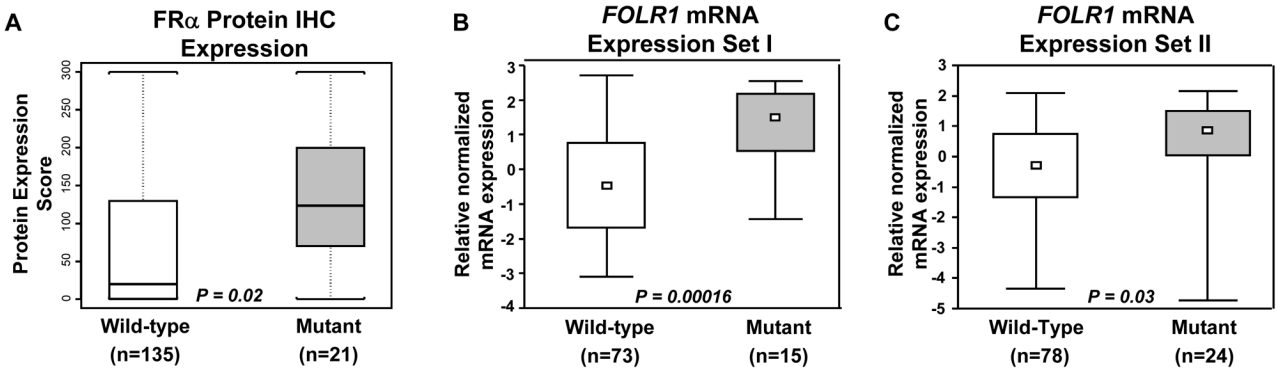

Figure 3.

FRα protein (*A*) and *FOLR1* mRNA (*B* and *C*) expression scores in lung adenocarcinoma by *EGFR* mutation status. *A*, FRα protein expression was determined by immunohistochemistry (IHC). *B* and *C*, *FOLR1* expression is based in 2 publicly microarray datasets available.[28] In the box-plots, *small open box* indicates median mRNA expression scores.

Table 1

Summary of clinicopathologic features of patients with NSCLC examined for membrane transporter and thymidylate synthase expression.

Feature	NSCLC Histologic Type		
	Squamous Cell Carcinoma (*n* = 118)	Adenocarcinoma (*n* = 202)	Total (*n* = 320)
Mean age, years (SD), (range)	68.4 (9.20), (43–90)	64.9 (11.5), (33–88)	66.2 (10.85), (33–90)
Sex			
Male	73	77	150
Female	45	125	170
Smoking status[†]			
Never	4	52	56
Ever	113	150	263
TNM stage			
I	62	134	196
II	36	25	61
III	18	36	54
IV	2	7	9

[†]Smoking status and history were not available for one patient with squamous cell carcinoma.

Table 2

Frequency of membrane transporters and thymidylate synthase (TS) immmunohistochemical expression in NSCLC by tumor histology

Marker	Any Expression (Score > 0)			Average Score		
	Adenocarcinoma Positive/Total (%)	SCC[¥] Positive/Total (%)	*P* Value[*]	Adenocarcinoma Score (SD)	SCC[¥] Score (SD)	*P* Value[†]
FRα						
Cytoplasm	152/174 (87%)	63/110 (57%)	< 0.001	91.6 (66.4)	35.9 (40.3)	< 0.001
Membrane	107/174 (61%)	29/110 (26%)	< 0.001	72.2 (89.0)	11.29 (28.8)	< 0.001
RFC1						
Cytoplasm	181/182 (99%)	110/112 (98%)	0.56	162.7 (83.2)	153.2 (72.0)	0.34
Membrane	164/182 (90%)	103/112 (92%)	0.68	128.1 (95.9)	119.2 (86.1)	0.59
TS						
Cytoplasm	130/165 (79%)	82/102 (80%)	0.75	52.2 (40.1)	55.6 (42.0)	0.565
Nuclear	58/165 (35%)	59/102 (58%)	0.0003	9.3 (21.1)	13.8 (27.7)	0.0043

[¥] SCC = Squamous cell carcinoma

[*] Fisher's exact test

[†] Wilcoxon rank-sum test