Differential Thymidylate Synthase Expression in Different Variants of Large-Cell Carcinoma of the Lung

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Abstract

Purpose: In non–small cell lung cancer, higher thymidylate synthase (TS) levels have been reported in squamous cell carcinoma (SCC) compared with adenocarcinoma (ADC). Data on TS expression in large-cell carcinoma (LCC) are scanty.

Experimental Design: TS mRNA and protein levels were analyzed in 42 surgical cases of pulmonary LCC, including 8 large-cell neuroendocrine carcinomas, and were compared with controls represented by ADC (n = 41), SCC (n = 30), and small-cell lung carcinoma (SCLC; n = 33). TS levels were also correlated with the expression of Ki67 and E2F1. Moreover, the reliability of TS expression analysis was assessed in 22 matched cytologic and surgical specimens of non–small cell lung cancer.

Results: TS mRNA levels of LCC were comparable with those of control SCC, but significantly higher than those of ADC (P < 0.001) and lower than SCLC (P < 0.001). A correlation between TS mRNA and protein levels was observed in control ADC and SCC, but not in LCC. Large-cell neuroendocrine carcinomas had the highest TS expression, whereas in non-neuroendocrine LCCs, TS protein levels were significantly higher (P = 0.02) in LCC immunoreactive for p63 and desmocollin3 (markers of squamous differentiation) than those expressing TTF-1 (a marker of ADC). Both E2F1 and Ki67 levels were not correlated with TS in LCCs. Finally, a linear correlation in TS protein levels was observed between matched cytologic and surgical specimens.

Conclusion: The pulmonary LCC immunoprofile may resemble that of SCCs or ADCs. This immunoprofile is associated with differential TS expression levels, which may support a more appropriate therapeutic strategy decision. (Clin Cancer Res 2009;15(24):7547–52)

Under the commonly used term of non–small cell lung cancer (NSCLC), there are three major histologic types according to the current WHO classification for lung tumors: (a) adenocarcinoma (ADC), characterized by glandular differentiation and mucin production; (b) squamous cell carcinoma (SCC), characterized by keratinization and/or intracellular bridges; and (c) large-cell carcinoma (LCC), which accounts for <10% of lung cancers and is represented by poorly differentiated tumors lacking the cytologic and architectural features of the two above histotypes, including variants of neuroendocrine and nonneuroendocrine types (1).

Although histology has not consistently been associated with clinical outcomes in advanced NSCLC (2), it has recently emerged as a potential predictive factor of response to or survival with pemetrexed. In phase III studies on NSCLC, pemetrexed, alone or in combination with cisplatin, showed a statistically superior activity in nonsquamous histotypes (3–5), which was also supported by additional retrospective analyses from phase II data (6, 7). Preliminary molecular evidence indicated that this differential activity may be related to a lower baseline expression of thymidylate synthase (TS), the main molecular target of pemetrexed, in ADC compared with squamous cell (8) and small-cell (9) lung carcinoma. In different tumors (9–12), preclinical data support the hypothesis that overexpression of TS correlates with reduced sensitivity to pemetrexed (13), and high TS expression levels were associated with poor outcome of patients treated with antifolate drugs.

In one of the above-mentioned studies, the combination of cisplatin and pemetrexed showed a clinical efficacy in LCC comparable with that observed in ADC (3), but data on TS gene or protein expression in LCC of the lung are extremely limited to tiny case series.
Translational Relevance

Baseline thymidylate synthase (TS), one of the main targets of pemetrexed, is higher in squamous cell carcinoma than in adenocarcinoma of the lung, and this differential expression has been related to the higher clinical efficacy of this agent in nonsquamous non–small cell lung cancer (NSCLC).

In 42 consecutive surgical cases of large-cell carcinoma (LCC), a differential TS expression according to the different immunophenotypes was observed, with the highest median mRNA TS value in neuroendocrine LCC and in nonneuroendocrine LCC cases expressing the squamous cell carcinoma lineage markers p63/DSC3, whereas a lower median mRNA TS value was detected in nonneuroendocrine LCC positive for the adenocarcinoma lineage marker TTF-1 ($P < 0.0008$).

A statistically significant association between TS immunoreactivity in cytologic preoperative samples, diagnosed as generic NSCLC not otherwise specified, and the corresponding histologic surgical specimens were detected. The present study confirms the relevance of TS assessment as a supportive tool to determine an appropriate therapeutic strategy in NSCLC.

Most of the non-neuroendocrine LCCs (non-NE LCC) may well represent extremely dedifferentiated cases of ADC or SCC, and immunohistochemical markers of ADC (e.g., TTF-1) or squamous (e.g., p63, desmocollin-3) lineages trace residual SCC, and immunohistochemical markers of ADC (e.g., TTF-1) in non-NE LCC or squamous lineages in non-NE LCC cases.

The sequences of primers and probes for TS and the β-actin (ACTB) housekeeping gene were as follows: TS forward 5′-GTCGCGCAATCATCTGAGCT-3′, TS reverse 5′-GATGTCGCGAATCATCTGACGT-3′, TS forward 5′-ACCAGTGCGGCTACAGCTT-3′, TS reverse 5′-GTCGGCGGGGCCTGGCTT-3′, TS forward 5′-CCCTTTATGAGCGCCGCTGCTTTG-3′, TS reverse 5′-CTCCATATGAGCGCCGCTGCTTTG-3′, TS forward 5′-ACATCGATCCGGGCTGTG-3′, TS reverse 5′-CCGGAATTCATTCGATCTCCTCCTTAAAGAGCA-3′ (TAMRA); β-actin forward 5′-TGACCGCTGGTGGGTCTT-3′, β-actin reverse 5′-TCCATATGAGCGCCGCTGCTTTG-3′, β-actin forward 5′-TGACCGCTGGTGGGTCTT-3′, β-actin reverse 5′-TCCATATGAGCGCCGCTGCTTTG-3′, β-actin forward 5′-ACCAGCAGGCGGCGCTGCTTTG-3′ (TAMRA).

Materials and Methods

Patients and samples

LCC cases. Forty-two consecutive surgical cases of LCC (~5% of the total surgical cases), all having annotate clinical information and sufficient amount of tissue available for histologic review, immunohistochemistry, and molecular analyses, were retrospectively collected from the pathology files of the University of Turin at San Luigi Hospital, from May 2000 to February 2008. These included 8 large-cell neuroendocrine carcinomas (LCNEC) and 34 non-NE LCCs, classified as clear cell variant ($n = 9$), basaloïd variant ($n = 3$), and NOS type (ICD-O: 8012/3; $n = 22$). Twenty-six of the non-NE LCC cases were also included in a previous study on LCC immunophenotyping (15).

Control groups. Control cases of ADC ($n = 41$) and SCC ($n = 33$), consecutively selected from the same surgical database in a period of 6 mo, were assessed for TS expression. Conversely, all SCLC control cases were represented by consecutive cases of bronchial biopsies. In addition, 22 preoperative cytologic specimens diagnosed as NSCLC-NOS (16 bronchial washings and 6 trans-thoracic fine-needle aspiration biopsies) and their corresponding surgical specimens were collected.

All histologic materials were deidentified and cases were anonymized by a pathology staff member not involved in the study. Clinical data were compared and analyzed through coded data only.

Microdissection, RNA isolation, and retrotranscription

One 10-μm-thick section was used for RNA extraction. The section was serial to three previous sections, 4-μm thick, from the same formalin-fixed paraffin-embedded tumor block that were used for H&E staining to select appropriate neoplastic areas, and for TS and Ki67 immunohistochemistry, respectively. The 10-μm-thick section was dried at 56°C overnight, deparaffinized, and stained with Nuclear Fast Red solution (Sigma-Aldrich), then rehydrated through graded alcohols and dissected using a scalpel.

RNA isolation and retrotranscription were done as previously reported (8).

Quantitative real-time PCR

Quantitative PCR was done on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a 384-well plate. All quantitative PCR mixtures contained 1 μL of cDNA template (~20 ng of retrotranscribed total RNA) diluted in 9 μL of distilled-stereile water, 1,200 nmol/L of each primer, 200 nmol/L of internal probe, and TaqMan Gene Expression Master Mix (Applied Biosystems) to a final volume of 20 μL. The sequences of primers and probes for TS and the β-actin reference gene were as follows: TS forward 5′-GCGCCCTCGTGCTGCCCTTT-3′, TS reverse 5′-GATTGCGCAATCATCTGACGT-3′, TS probe (FAM)-5′-AACATCGG-CAGCTACGGCCTGC-3′ (TAMRA); E2F1 forward 5′-CTCATCCAGGCTGTGACCTCTT-3′, E2F1 reverse 5′-ACATCGATCCGGGCTGTG-3′, E2F1 probe (FAM)-5′-CCGGAATTCATTCGATCTCCTCCTTAAAGAGCA-3′ (TAMRA); β-actin forward 5′-TGACCGCTGGTGGGTCTT-3′, β-actin reverse 5′-TCCATATGAGCGCCGCTGCTTTG-3′, β-actin probe (FAM)-5′-ACCAGCAGGCGGCGCTGCTTTG-3′ (TAMRA).

Cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 46 cycles at 95°C for 15 s, and 60°C for 1 min. Baseline and threshold for cycle threshold (Ct) calculation were set manually with ABI Prism SDS 2.1 software. A mixture containing Human Total RNA (Stratagene) was used as control calibrator on each plate. The fold change in gene expression levels was evaluated using the 2$^{-\DeltaΔCt}$ method (16).

Immunohistochemistry

Serial 5-μm-thick sections were collected on charged slides; after paraffin removal, rehydration through graded alcohols, and endogenous peroxidase activity quenching, the sections were treated in a pressure cooker for 5 min at 125°C, followed by a quick 10-s step at 90°C using EDTA buffer (pH 8.0). The slides were then incubated for 40 min at room temperature with primary mouse anti-TS antibody (clone 106, dilution 1:100; Zymed) or anti-Ki67 (clone MIB-1, dilution 1:150; Dako). The immune reaction was revealed by the En Vision Mouse system (Dako), using 3,3′-diaminobenzidine (Dako) as chromogen.

To overcome heterogeneity of TS expression within individual tumors, the entire tissue section was evaluated to assess TS immunoreactivity, the percentage of tumor cells positive was scored semiquantitatively as: “low” if TS-positive cells were <10%; “moderate” if TS-positive cells were between 11% and 39%; and “high” if TS-positive cells were >40%. The reactivity pattern was mainly nuclear, but occasional cytoplasmic staining was also considered positive (8, 17). The Ki67 index was determined by counting positive nuclei in 1,000 cells, randomly selected in areas of highest labeling density and expressed as percentage of proliferating cells.
In non-NE LCC, TTF-1 (dilution 1:75; NeoMarkers, LabVision) desmocollin-3 (DSC-3; dilution 1:30; PROGEN Biotechnik), and p63 (dilution 1:100; Neomarkers) immunostainings were done in all newly collected cases, using the same antibodies and procedures described for the 26 previously reported LCC cases (15).

### Statistical analysis

To test the significant association between gene expression (continuous variable) and clinicopathologic features (dichotomous variables), Kruskal-Wallis and Mann-Whitney U tests were used. The correlation between two continuous variables was estimated using Spearman's test. Correlation between TS protein expression subgroups (low, moderate, and high scores) and clinicopathologic variables were analyzed by the $\chi^2$ test. Comparisons between TS expression levels and the phenotypic profiles of individual LCC cases were done both using the marker expression data on TTF-1, p63, and DSC-3 already reported in a previous study (15) and carrying out the same immunoprofiling in the additional LCC cases enrolled in this study. Statistical analysis was done with Statistica 7.0 software with a level of significance of $P = 0.05$; graph design was done excluding outlier values.

### Results

**Patients' characteristics.** The LCC group included 33 males and 9 females, with a median age of 65 years (range, 45-80 years). Most tumors were stage I ($n = 33$), whereas the remaining cases were stage II and III (5 and 4, respectively). The control group of ADC and SCC included 54 males and 17 females with a median age of 70 years (range, 43-84 years). The tumors were grade 1 ($n = 11$), grade 2 ($n = 30$), or grade 3 ($n = 30$), and the tumor stage was I ($n = 24$), II ($n = 24$), or III ($n = 16$). In the SCLC control group, the median age of the 24 male and 9 female patients was 65 years (range, 48-79 years). Finally, the separate group of 22 patients (20 males and 2 females), on whom a cytology-histology TS correlation study was done, had a median age of 64 years (range, 48-83 years) and a final histologic diagnosis of ADC ($n = 13$), SCC ($n = 4$), and LCC ($n = 5$).

**TS expression levels in LCCs and control groups.** The 42 LCCs showed a median TS mRNA level of 0.40 (range, 0.04-2.56). These figures were comparable with those of the control group of SCC (median level, 0.33; range, 0.09-3.12), significantly higher ($P < 0.001$) than those of control ADCs (0.15; 0.03-2.95), and significantly lower than those of SCLC (2.91; 0.44-5.37; $P < 0.001$; Fig. 1A). An analysis of TS expression within the LCC subtypes showed the median mRNA level of LCNEC to be 1.24 (0.42-2.04), whereas that of non-NE LCC was 0.32 (0.04-2.56), a statistically significant difference ($P < 0.001$; Fig. 1B). The group of LCNEC was therefore excluded in the subsequent statistical analysis.

With regard to tumor grade, TS expression levels were significantly higher in poorly differentiated than in moderately and well-differentiated cases of the control ADC and SCC groups ($P < 0.0001$). Indeed, TS expression in LCC was significantly higher when compared with TS levels of moderately/well-differentiated ($P = 0.005$) but not of poorly differentiated control ADC or SCC ($P = 0.6$). TS mRNA and protein levels were not associated with pathologic stage in LCC, ADC, or SCC patients.

**Correlation between TS protein and mRNA expression.** A significant correlation was observed between TS mRNA and protein levels in control ADC and SCC, but not in LCC. Both non-NE LCC and ADC/SCC case series were divided into three groups (low, moderate, and high) according to the TS protein expression score (see Materials and Methods). In control cases, the median gene expression values were 0.11 (range, 0.03-1.97) for the "low TS score", 0.29 (0.09-2.95) for the "moderate TS score", and 0.52 (0.07-3.12) for the "high TS score" subgroups. These differences were statistically significant between low TS score versus moderate ($P = 0.002$) or high ($P < 0.0001$) cases, respectively, and between moderate and high TS score groups ($P = 0.04$). Conversely, in non-NE LCCs, no significant correlation between TS mRNA and protein expression levels was found: the median gene expression of "low-moderate TS protein" group was 0.38 (0.06-1.42) versus 0.54 (0.13-1.91) for the high TS group ($P = 0.17$; Fig. 1C).

**Differential TS expression in non-NE LCC subtypes.** After excluding LCNEC from further analysis, the remaining non-NE LCC cases were further characterized into two subgroups based on the mutually exclusive expression of immunohistochemical markers commonly associated with the ADC (i.e., TTF-1) or SCC (i.e., p63 and DSC-3) subtypes. The median percentage of TS protein–positive cells was higher (60%) in DSC-3/p63–positive tumors compared with the median value of protein expression observed in TTF-1–positive tumors (30%), a difference that was statistically significant ($P = 0.02$; Fig. 1D). This difference did not reach statistical significance when the TS mRNA, rather than protein, values were compared. No significant correlation was detected between TS expression and gender, age, or pathologic stage ($\chi^2$ test, $P = 0.585$ and $P = 0.686$, respectively).

**Correlation of TS expression levels with E2F1 and Ki67 proliferation index.** In the LCC group, E2F1 mRNA median expression levels were significantly higher in the LCNEC subtype (0.72; range, 0.21-1.36) than in the non-NE LCC variants (0.20; 0.05-1.30; $P = 0.004$). Both non-NE LCC and LCNEC groups showed a significantly lower E2F1 expression compared with SCLC (1.31; 0.49-6.23; $P = 0.02$ and $P < 0.001$, respectively). Conversely, no significant differences were found between E2F1 mRNA expression in non-NE LCC and either the ADC (0.13; range, 0.03-0.66; $P = 0.19$) or the SCC (0.26; range, 0.05-1.25; $P = 0.4$) control group.

In comparing E2F1 and TS mRNA expression levels, a significant linear correlation was found in ADC only ($R = 0.49$ and $P = 0.004$), whereas both SCC and non-NE LCC cases failed to show any linear correlation. Similarly, comparing Ki67 proliferation index and TS absolute percentage value of protein expression, a significant linear correlation was observed in ADC ($R = 0.53$ and $P = 0.007$), but not in the SCC and non-NE LCC groups.

**Correlation between TS protein expression in preoperative and postoperative specimens.** In comparing the immunohistochemical expression of TS in 22 NSclc cytologic samples with those of the corresponding resected specimens, a significant correlation was observed ($R = 0.89; P < 0.001$). The percentage of TS expression in tumor cell nuclei ranged from 0 to 60 in cytologic samples and from 0 to 80 in matched surgical specimens. A representative case is shown in Fig. 2.

### Discussion

This study showed that in a LCC series, considered as a single group of tumors, the median TS gene expression levels were comparable with those observed in SCC ($P = 0.24$), higher than the median value in ADC ($P < 0.001$) and significantly lower.
than those reported in SCLC ($P < 0.001$). A significant correlation was found between TS immunohistochemical and quantitative PCR detection methods, suggesting the reliability of either technique for TS analysis in lung cancer specimens. These results are in agreement with previous reports (8, 18, 19) and may further provide a molecular explanation for the documented superior efficacy of pemetrexed in nonsquamous compared with squamous tumors (3, 6, 20).

The observed TS expression levels in LCC and SCC apparently challenge the clinical findings reported in a phase III study showing comparable activity of pemetrexed plus cisplatin in ADC and LCC (3). However, when mRNA and protein TS expression levels were separately considered in the different LCC subtypes, LCNEC had significantly higher mRNA and protein amounts than the non-NE LCC subtypes, with average values more closely related to SCLC than to SCC. This finding is not totally unexpected because both LCNEC and SCLC share extremely poorly differentiated features, a neuroendocrine phenotype, as well as a similar clinical behavior. When LCNEC were excluded, a correlation between TS expression and the tumor phenotype was found in the non-NE LCC subtypes. LCCs are poorly differentiated carcinomas lacking morphologic evidence of squamous or glandular differentiation, although the possibility of their further subtyping was previously documented using immunohistochemical differentiation markers, such as TTF-1 and DSC-3, which in the vast majority of cases seemed to be mutually exclusive (15). Interestingly, in the present series, DSC-3–positive non-NE LCC had significantly higher TS protein expression levels that those that were TTF-1 positive.

Overall, in ADC and SCC, a direct correlation was seen between median TS expression and tumor differentiation grade. The median mRNA TS expression level in non-NE LCC was comparable with the value observed in poorly differentiated ADC and SCC.

TS transcription is regulated by several factors, including E2F1, a transcription factor belonging to the E2F gene family (21), which plays a central role during transition from G1 to

![Fig. 1.](#) A, TS mRNA expression levels in LCC (subtyped in B), ADC, SCC, and SCLC. C, correlation between TS mRNA and protein expression in ADC/SCC and non-NE LCCs. In non-NE LCC, low and moderate cases were grouped together for statistical purposes. D, TS protein in non-NE LCC: correlation with glandular (TTF-1) or squamous (DSC3/p63) differentiation markers.
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Fig. 2. A strong TS immunoreactivity was observed in a lung cancer cytologically reported as NSCLC-NOS in a bronchoalveolar washing. The corresponding surgical specimen (inset) showed a poorly differentiated squamous carcinoma having diffuse TS expression (×200).

S phase of cell cycle. In vitro studies have shown that E2F1 overexpression increases both TS mRNA and protein (22). In our study, E2F1 and TS mRNA expression levels correlated only in ADC, whereas no correlation was observed in SCC and LCC, showing in the latter series lower E2F1 levels compared with TS levels.

In LCC, increased expression of genes implicated in cell cycle regulation, such as E2F3, PCNA, and CDK4 (23), may prevent growth arrest contributing to the undifferentiated status and high proliferation of LCC (24). The observed differential expression of TS and E2F1 in the non-NE LCC group only could be related to the activation of an alternative TS transcription pathway, such as the p16INK4A-CDK4 pathway as preliminarily suggested in cell line studies (25).

The need to define a specific histotype for a decision-making process collides with the growing percentage of patients given a generic diagnosis of NSCLC-NOS, especially in extremely scanty biopsies or cytology samples. TS protein expression was tested in 22 cases having a preoperative cytologic diagnosis of NSCLC-NOS and in the corresponding histologic samples obtained at the time of subsequent surgical resection: a highly significant level of correlation of TS protein expression in matched specimens was observed. These data show that when sample cellularity is sufficient, immunohistochemistry may be a reliable tool to assess TS expression levels. These immunohistochemical findings related to TS as well as those for TTF-1 and DSC-3 expression will potentially contribute to a better definition of the role of pemetrexed-based therapies in NSCLC-NOS.

In conclusion, this study confirms that TS is differentially expressed in NSCLC histotypes and, for the first time, shows a different TS mRNA expression in LCC subgroups. Specifically, LCNEC contained the highest TS levels, approaching those observed in the SCLC control group, whereas non-NE LCC cases had a differential TS expression, based on their phenotypes: those expressing the ADC lineage marker TTF-1 had the lowest TS levels within this group, whereas cases expressing the SCC lineage markers p63 or DSC3 had the highest ones. Because only baseline TS expression and preliminary, non-conclusive data on the predictive value for response and survival of TS expression in different NSCLC histotypes are available, these results are hypothesis generating and need a prospective validation, in a large cohort of LCC cases, at the biological and clinical levels through serial assessment of TS levels before and during pemetrexed therapy.

Disclosure of Potential Conflicts of Interest

G.V. Scagliotti declares honoraria from Eli Lilly, AstraZeneca, Sanofi-Aventis, GlaxoSmithKline, Roche, Millennium. The other authors have no conflict of interest to declare.

References

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