Thymidylate synthase (TS) gene expression in primary lung cancer patients: a large-scale study in Japanese population

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Introduction

Primary lung cancer is the leading cause of cancer-related deaths worldwide and is usually classified into small-cell lung cancer (SCLC), accounting for 15%–20% of all lung cancers, or non-small-cell lung cancer (NSCLC), including squamous cell carcinoma (Sq), adenocarcinoma (Ad) and large-cell carcinoma (La) [1, 2]. Almost all SCLC patients and most NSCLC patients present with advanced disease such as distant metastasis, and systemic chemotherapy is usually prescribed for such patients [3]. For NSCLC patients, platinum agent plus new-generation cytotoxic agent is recommended as first-line chemotherapy, but the therapeutic effect had reached a plateau [2, 4–6]. However, in recent clinical trials, targeting agents including bevacizumab, an antibody against vascular endothelial growth factor [7, 8], as well as gefitinib and erlotinib, epidermal growth factor receptor tyrosine kinase inhibitors [9–11], have been proved to be effective for advanced NSCLC.

In addition to targeting agents, new cytotoxic agents inhibiting thymidylate synthase (TS), pemetrexed [12–14] and S-1 [15, 16] have been introduced in the clinical practice. Pemetrexed, an analogue of folic acid (folate), shows antitumor activity by inhibiting multiple enzymes involved in the metabolic pathway of folic acid as follows: TS, dihydrofolate reductase and glyciamide ribonucleotide formyl transferase [17]. In vitro studies have demonstrated that pemetrexed inhibits most effectively TS among these enzymes and that increased TS activity is correlated with low sensitivity to pemetrexed [17–20], and some clinical studies have revealed that higher TS expression is associated with lower chemotherapeutic effect of pemetrexed in patients with a variety of solid tumors including breast cancer [21], colorectal cancer (CRC) [22] and malignant pleural mesothelioma [23]. In clinical trials for primary lung cancer, pemetrexed showed minimal antitumor activity for SCLC patients [24–26] and diminished antitumor activity for Sq patients as compared with non-Sq patients [27, 28]. Such difference in antitumor activity of pemetrexed according to histologic cell type may be partly explained by very high TS expression in SCLC and high TS expression in Sq, which was documented in a few small-sized studies [29, 30].

S-1 is a novel oral drug in which a prodrug of 5-fluorouracil (5-FU), tegafur (FT) is combined with an inhibitor of dihydropyrimidine dehydrogenase (DPD) that is responsible for the activation of DPD.
for degradation of 5-FU [31]. 5-FU is a chemotherapeutic agent that is widely used for a variety of solid tumors but shows low antitumor activity against primary lung cancer due to rapid degradation of 5-FU caused by high DPD [32]. To enhance antitumor activity by inhibiting DPD activity, FT and a DPD inhibitor, uracil, were combined in tegafur–uracil (UFT), which proved to be effective in adjacent setting for resected early-stage NSCLC [32, 33]. In S-1, a more potent DPD inhibitor, 5-chloro-2,4-dihydroxyppridine (gimeracil) was combined, which resulted in more active antitumor activity against NSCLC [15, 16, 32]. 5-FU shows antitumor activity mainly through inhibition of TS after activation into phosphorylated metabolites such as 5-fluoro-2'-deoxyuridine-5'-monophosphate. Previous experimental and clinical studies showed that TS expression status was correlated with antitumor activity of 5-FU [22] or UFT [22, 32, 34]. These results indicate that TS-inhibiting agents play important roles in the treatment of advanced NSCLC and that TS expression status may be an important factor to predict chemotherapeutic effect of these TS-inhibiting agents, but, only a few small-scale studies on quantitative measurement of TS expression in primary lung cancer have been reported [29, 30, 35]. In a previous study, we analyzed gene expression levels of multiple enzymes including TS in a total of 17,613 surgical specimens of a variety of solid tumors obtained from Japanese patients, which included 816 specimens of primary lung cancer. The average TS gene expression level of primary lung cancer, in which all histologic cell types were included, was comparable to other solid tumors such as CRC [36], but no detailed analysis was conducted in the study. Thus, in the present study, we focused on TS status in primary lung cancer and compared TS gene expression levels according to patient characteristics such as histologic cell types.

materials and methods

patients

After enrollment of 816 patients with primary lung cancer in the previous study [36], we continued to recruit patients. As a result, a total of 2,621 Japanese patients from 21 hospitals across Japan (listed in the ‘Acknowledgements’ section) were finally enrolled in the present study. All patients received surgery for primary lung cancer without prior treatment, and all the patients provided written informed consent for the use of the specimens for this study. Primary tumor tissue and surrounding normal lung tissue were obtained from each surgical specimen, and the nodal tissue involved was also obtained if available. Each tissue was fixed in formalin and was used for measurement of TS gene expression.

measurement of TS gene expression

Measurement of TS gene expression was carried out with a real-time reverse transcription–PCR analysis in laser-captured microdissected formalin-fixed and paraffin-embedded sections, as described previously [36]. In brief, serial 10-μm-thick sections were stained with nuclear Fast Red (Sigma-Aldrich, St. Louis, MO), and representative tumor areas were selected by pathologists and only tumor cells were collected with laser capture microdissection for primary tumor samples as well as involved nodal samples; normal lung epithelial cells were collected for normal lung samples. RNA isolation was carried out with a novel proprietary procedure (Response Genetics, Los Angeles, CA; United States Patent Number 6,248,535) [29, 30, 36]. After synthesis of combinational DNA (cDNA), the target cDNA sequences were amplified using quantitative PCR and a fluorescence-based real-time detection method [ABI PRISM 7900 Sequence Detection System (TaqMan), Applied Biosystems, Foster City, CA] [29, 30, 36]. The PCR mixture consisted of primers, dATP, deoxyxycytidine triphosphate, dGTP, dUTP, MgCl2, and TaqMan buffer (all reagents were supplied by Applied Biosystems). The PCR conditions were 50°C for 10 s and 95°C for 10 min, followed by 42 cycles at 95°C for 15 s and 60°C for 1 min. The messenger RNA (mRNA) expression levels were expressed as values relative to those of b-actin used as the internal reference.

statistics

The Mann–Whitney U test and the Kruskal–Wallis H test were used to test significant association between TS gene expression and dichotomous variables and that between TS gene expression and multiple variables, respectively. The Wilcoxon signed rank test was used to test significance of difference in TS gene expression between tumor tissue and surrounding normal lung tissue obtained from patients with primary lung cancer. For a multivariate analysis of significant factors to predict TS gene expression level, logistic regression analysis was employed. All statistical analysis was carried out using the JMP version 5.1 package software.

results

TS gene expression in primary tumor, involved node and surrounding normal lung

Among all 2,621 lung samples, quantitative TS gene expression was successfully carried out in 2,150 samples (Table 1). TS gene expression in primary tumor (the mean and median TS/b-actin values, 3.408 and 1.997, respectively) was significantly higher than that in the surrounding normal lung (the mean and median TS/b-actin values, 0.989 and 0.856, respectively; P < 0.001) (Figure 1).

TS gene expression on tumor cells was also quantitatively evaluated in 66 involved nodal samples, which was significantly higher (the mean and median TS/b-actin values, 7.685 and 4.058, respectively) than that in primary tumor or that in normal lung tissue (Figure 1).

TS gene expression in primary tumor according to patients’ characteristics

In a univariate analysis (Table 1), TS gene expression in primary tumor was extremely high for SCLC (the mean and median TS/b-actin values, 13.839 and 11.362, respectively), which was significantly higher than that for NSCLC (the mean and median TS/b-actin values, 3.145 and 1.924, respectively; P < 0.001). A multivariate analysis confirmed that SCLC showed a higher TS gene expression than NSCLC (P < 0.001).

A univariate analysis also showed a significant difference in TS gene expression, which was not confirmed by a multivariate analysis, according to histologic cell type as follows: (i) Sq showed a significantly higher TS gene expression (the mean and median TS/b-actin values, 4.324 and 3.093, respectively) than Ad (the mean and median TS/b-actin values, 2.296 and 1.611, respectively; P < 0.001); (ii) La also showed a significantly higher TS gene expression (the mean and median TS/b-actin values, 8.437 and 4.812, respectively) than Ad (Figure 2A).
When patients were divided into younger patients and older patients by using the cut-off age of 67 years (the median age of all patients included in the study), a univariate analysis revealed that older patients showed a significantly higher TS gene expression (Table 1), but a multivariate analysis failed to show such significant difference.

In a univariate analysis, TS gene expression was significantly increased along with a decrease in grade of tumor cell differentiation (Figure 2B). A univariate analysis suggested some difference in TS gene expression according to tumor progression (Figure 3A–C, Table 1), but a multivariate analysis failed to show a significant difference in TS gene expression according to pathologic stage.

**discussion**

In the present study, we showed that TS gene expression was significantly increased in tumor cells as compared with surrounding normal lung epithelial cells (Figure 1), which is consistent with results observed in previous immunohistochemical studies as follows: TS protein expression was observed not only in tumor cells but also in normal cells such as bronchial smooth muscle and endothelial cells, but apparent TS expression was not observed in normal lung epithelial cells [37, 38]. As TS is an essential enzyme for de novo DNA synthesis, TS expression is usually up-regulated in cells with active proliferation [39]. Accordingly, such up-regulated TS gene/protein expression in tumor cells as compared with normal cells represents active proliferative activity of tumor cells and may account for mechanism of action of TS-inhibiting antitumor agents such as pemetrexed and 5-FU. In the present study, we also showed that tumor cells in involved nodes had significantly higher TS gene expression than tumor cells in primary tumor (Figure 1), which had not been reported previously. Very high TS expression in tumor cells in involved nodes should be verified in future studies, and, if it is true, different responses to TS-inhibiting antitumor agents between tumor cells in primary tumor and tumor cells in involved nodes may occur in correlation with different TS expression levels.

**Table 1. Patient characteristics and TS gene expression (univariate analysis)**

<table>
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<tr>
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<th>Number of patients</th>
<th>TS gene expression</th>
<th>P value</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>Total number of patients</td>
<td>2150</td>
<td>3.408</td>
<td>1.997</td>
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<td>Age (mean, 66 years; median, 67 years; range, 20–90 years)</td>
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<tr>
<td>&lt;67</td>
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<tr>
<td>≥67</td>
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<td>Small cell</td>
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<td>Large cell</td>
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</tr>
<tr>
<td>IV</td>
<td>56</td>
<td>3.599</td>
<td>2.313</td>
</tr>
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</table>

TS, thymidylate synthase.

**Figure 1.** Thymidylate synthase gene expression in primary tumor, involved node and surrounding normal lung tissue.

When patients were divided into younger patients and older patients by using the cut-off age of 67 years (the median age of all patients included in the study), a univariate analysis revealed that older patients showed a significantly higher TS gene expression (Table 1), but a multivariate analysis failed to show such significant difference.

In a univariate analysis, TS gene expression was significantly increased along with a decrease in grade of tumor cell differentiation (Figure 2B). A univariate analysis suggested some difference in TS gene expression according to tumor progression (Figure 3A–C, Table 1), but a multivariate analysis failed to show a significant difference in TS gene expression according to pathologic stage.
In addition, we showed that TS gene expression in primary lung cancer was significantly different according to histologic cell type as follows: (i) SCLC showed very high TS expression levels with the mean and median TS/β-actin values of 13.839 and 11.362, respectively (P < 0.001 in both uni- and multivariate analysis), and (ii) among NSCLC cell types, Ad showed the lowest TS expression levels (mean and median TS/β-actin values, 2.296 and 2.611, respectively) and Sd showed higher TS expression levels (mean and median TS/β-actin values, 4.324 and 3.093, respectively) than Ad (Figure 2A) (P < 0.001 in univariate analysis). With regard to clinical relevance, lower TS gene expression in Ad and the highest TS expression in SCLC may account for enhanced antitumor activity of pemetrexed, a TS inhibitor, against Ad and for diminished antitumor activity against Scl when treated with S-1.

Concerning La, the mean and median TS gene expression levels (8.437 and 4.812, respectively) were lower than SCLC, but were higher than Sq and Ad in the present study. According to an Italian study [30], TS gene expression in La was different according to subtypes; large-cell neuroendocrine carcinoma had relatively high TS expression, which was even higher than that in Sq, and non-neuroendocrine large-cell carcinoma had lower TS expression comparable to that in Ad. Unfortunately, no information concerning histologic subtypes was available in the present study, and we did not analyze TS gene expression levels of La according to subtypes.

Although such difference in TS gene expression according to histologic cell type may provide a reason for reduced antitumor effect achieved with pemetrexed for SCLC and Sq, there are many limitations for the use of TS gene expression level as a clinical marker for selection of patients for whom pemetrexed is effective before initiation of chemotherapy. First, TS gene expression may be heterogeneous according to sites of one tumor specimen; TS gene expression may be higher in peripheral region of a tumor where proliferative activity of tumor cells is higher [37]. Thus, TS gene expression examined in a small clinical specimen, such as transbronchial biopsy specimen, may not be representative of TS gene expression level of a tumor of each patient. Secondly, TS gene expression level may be heterogeneous among patients with same histologic cell type. In fact, the majority of the TS gene expression level in each histologic cell type was overlapped; in other words, an Sq patient showed a very low TS expression level, which was lower than an Ad patient. Lastly, methods for evaluation of TS gene expression level in a clinical specimen, including sampling of specimens and measurement of gene expression, are not yet established or validated.

In the present study, we showed that TS gene expression was higher in poorly differentiated tumors and was lower in well-differentiated tumors (Figure 2B), which had not been reported. Such difference in TS expression according to cell differentiation may represent differences in proliferative activity; well-differentiated tumors generally grow slower and show lower TS expression, because TS is an essential enzyme in de novo DNA synthesis for cell proliferation. Finally, we examined TS expression according to tumor progression. However, we failed to show any difference in TS expression, although it might be expected that advanced tumors

Figure 2. Thymidylate synthase gene expression in primary tumor according to histologic cell type (A) and grade of tumor cell differentiation (B).
theoretically show higher TS expression in correlation with higher proliferative activity. One possible explanation for failure to show no significant correlation between TS expression and tumor progression might be a bias in selection of specimens as all specimens served for TS expression were surgically resected. For example, stage IV patients with distant metastasis enrolled in the present study may not usually represent stage IV patients who are not usually candidates for surgery, but may represent highly selected stage IV patients such as patients in whom no metastasis had been detected before surgery. To clarify TS expression according to tumor progression, further studies for resectable disease as well as unresectable disease should be conducted in future. In addition, TS may be a prognostic factor in malignant tumor including primary lung cancer [38]. We could not obtain survival data of patients included in the present study, which will be examined in a future study.

In conclusion, the present large-scale study clearly showed a significant difference in TS gene expression according to histologic cell types in primary lung cancer, which may account for different antitumor activity of pemetrexed, a TS inhibitor, in the therapy of SCLC and NSCLC documented in recent clinical studies, and of UFT in adjuvant therapy of NSCLC. In future clinical trials of chemotherapy with TS-inhibiting agents such as pemetrexed and S-1, evaluation of TS expression status may be encouraged to clarify its clinical significance.

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disclosure

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references


