Immunohistochemical characterization of pyrimidine synthetic enzymes, thymidine kinase-1 and thymidylate synthase, in various types of cancer

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Received October 30, 2009; Accepted January 15, 2010

DOI: 10.3892/or_00000770

Abstract. Thymidine kinase-1 (TK-1) and thymidylate synthase (TS) are key enzymes for salvage and *de novo* pyrimidine synthesis, respectively. Numerous studies have suggested that increased TS levels are associated closely with resistance to fluoropyrimidine-based chemotherapy. TAS-102 is a novel drug containing trifluorothymidine, which is phosphorylated by TK-1 to its active monophosphated form, that in turn can inhibit TS. TAS-102 has been shown to exhibit antitumor activity in fluoropyrimidine-resistant human cancer cells. TAS-102 is currently undergoing clinical trials for use in gastrointestinal cancers. In the present study, we used immunohistochemistry to investigate the expression of TK-1 and TS in various types of cancer. TK-1 and TS expression was markedly different between cancer types. High TK-1 expression was detected prominently in gastrointestinal adenocarcinomas and esophageal and uterine squamous cell carcinomas. Gastrointestinal adenocarcinomas and squamous cell uterine carcinomas were often accompanied by high TS expression, indicating activation of pyrimidine synthesis through both the salvage and *de novo* pathways. These results led us to consider that TAS-102 may also be effective for esophageal and uterine squamous cell carcinomas, as well as for gastrointestinal adenocarcinomas, even in fluoropyrimidine-resistant cases with high TS expression. In contrast, thyroid papillary carcinomas, lung adenocarcinomas, hepatocellular carcinomas, pancreatic ductal carcinomas, and renal cell carcinomas, which exhibit low TK-1 expression, may be resistant to TAS-102. In non-small cell lung cancers, high TK-1 expression was demonstrated in squamous cell carcinomas, but not in adenocarcinomas. This result suggests that TAS-102 efficacy and the pyrimidine synthetic pathway may differ depending on histological type. Our results indicate that administration of TAS-102 could be selected on the basis of the immunohistochemical evaluation of TK-1 and TS.

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Key words: thymidine kinase-1, thymidylate synthase, TAS-102, fluoropyrimidine, immunohistochemistry

Introduction

Thymidine kinase (TK) and thymidylate synthase (TS) are key enzymes for pyrimidine synthesis, which is necessary for DNA synthesis (Fig. 1). TK catalyses the phosphorylation of thymidine for the salvage synthesis of deoxythymidine monophosphate (dTMP) (1). Human cells contain two different TK isozymes that can be distinguished by their biochemical properties and cellular distribution. The cytosolic isozyme, TK-1, is associated with the cell cycle, with its activity increasing at the border of the G1/S-phase, reaching a peak in the late S-phase and becoming undetectable in the M-phase (2,3). In contrast, the levels of the mitochondrial isozyme, TK-2, remain stable throughout the cell cycle. There is a good correlation between TK activity and the amount of TK-1 protein or mRNA during the cell cycle (2,4).

Clinical studies using biochemical techniques have demonstrated that increased serum TK activity in a variety of cancers is related to the clinical stage and outcome of the disease (5-7). However, the origin and function of serum TK-1 is unclear, and therefore there is a need for direct assays of TK-1 that could be applied to cell or tissue samples. Wang *et al* developed a polyclonal anti-TK-1 antibody and demonstrated that TK-1 positive cells were distributed in areas of proliferation activity of various normal and malignant tissues (3). Moreover, some immunohistochemical investigations have shown that the TK-1 labeling index is a marker of proliferation, especially for evaluating high-risk grades of tumor and advanced stages of colorectal and breast cancers (8,9).

TS catalyses the methylation of deoxyuridine monophosphate (dUMP) required for the *de novo* synthesis of dTMP. TS is a crucial target for fluoropyrimidines [5-fluorouracil (5-FU) and its prodrugs] that have been used widely for the treatment of solid cancers. 5-FU is metabolized ultimately to

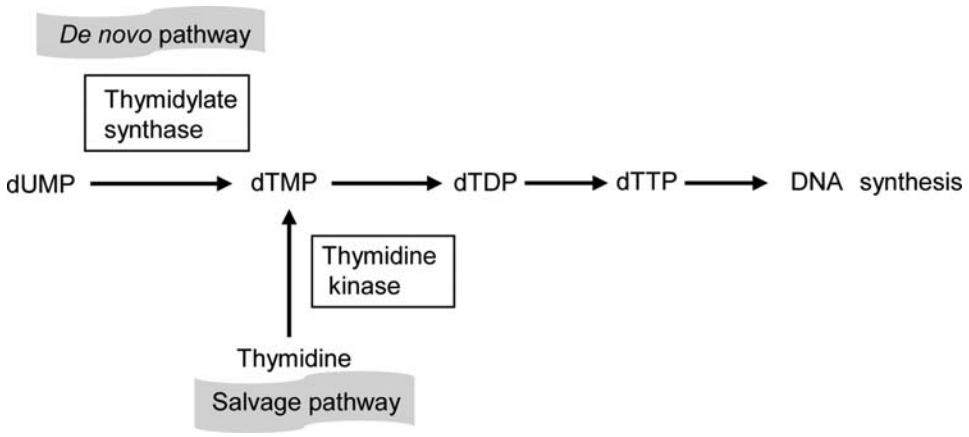

Figure 1. The *de novo* and salvage pathways of DNA synthesis. dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate; dTDP, deoxythymidine diphosphate; dTTP, deoxythymidine triphosphate.

fluorodeoxyuridine monophosphate, which then binds to both TS and methylene tetrahydrofolate to form a ternary complex that inhibits TS activity (10). Resistance to fluoropyrimidines has been associated with TS gene amplification and increased TS protein levels (11-13). Increased TS expression has also been found to be a significant independent prognostic factor for disease-free survival and overall survival in patients treated with adjuvant fluoropyrimidine-based chemotherapy (14,15). In addition, a positive correlation has been observed between the expression levels of TS mRNA or protein, disease stage, and lymph node metastasis in certain types of cancer (14,16).

Trifluorothymidine (TFT) is a fluorinated thymidine analog, which is phosphorylated by TK to its active monophosphate form TF-TMP, that has an inhibitory effect on TS (17). TF-TMP differs from fluoropyrimidines in that it does not form a ternary complex and binds covalently to the active site of TS thereby inhibiting its activity. TF-TMP is further phosphorylated to triphosphate TFT (TF-TTP) that can be incorporated into DNA. TAS-102, a novel drug composed of TFT and a thymidine phosphorylase inhibitor, has been shown to exhibit antitumor activity in fluoropyrimidine-resistant human cancer cells (17,18). TAS-102 is currently undergoing clinical phase II trials in the US and Japan to evaluate its efficacy in patients with gastrointestinal cancers.

Simultaneous analysis of the levels of TK-1 and TS may be useful for predicting sensitivity to TAS-102. However, to the best of our knowledge there are only a small number of clinical studies that have evaluated the activities of the two enzymes in gastric and mammary cancers (19,20). The purpose of this study was to investigate the expression of TK-1 and TS in various types of cancer using immunohistochemistry and to discuss the potential usefulness of these two pyrimidine synthetic enzymes in TAS-102 chemotherapy.

Materials and methods

Specimens. Surgically resected tumor tissues were obtained from a total of 175 cases of advanced cancer (12 tongue, 12 thyroid, 23 lung, 14 breast, 12 esophagus, 18 stomach, 14 colorectum, 12 liver, 15 gallbladder, 10 pancreas, 10 kidney, 13 urinary bladder, and 10 uterus). The tissues were fixed in 10% formalin and embedded in paraffin. Next, 3-μm-thick sections were cut, mounted on aminopropyltriethoxysilane-coated slides, and stored at -20˚C until use. Informed consent was obtained from all the patients for the use of tissues for analyzing antigen expression.

Immunostaining for TK-1 and TS. The sections were deparaffinized in four changes of xylene, followed by rehydration in a series of graduated alcohol. Endogenous peroxidase activity was blocked by incubating the sections in 0.03% hydrogen peroxide in methanol for 30 min. To retrieve the antigenicities of TK-1 and TS, the sections were subjected to hydrated heating for 10 min in a pressure cooker in 1 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 8.0) (T-Fal, Clichy, France). After antigen retrieval, the sections were left at room temperature to cool in the EDTA solution for 30 min. The sections were washed in running tap water, followed by 10 mM phosphate-buffered saline (PBS; pH 7.2) and then incubated overnight at room temperature in mouse monoclonal antibody against TK-1 (clone F12, diluted 1:200; Abnova, Taipei, Taiwan) or rabbit polyclonal antibody against TS (diluted 1:200; Taiho Pharmaceutical, Tokushima, Japan). After washing in PBS, the sections were incubated with immunoenzyme polymer reagent (Nichirei, Tokyo, Japan) at room temperature for 60 min. The reaction products were visualized in 0.05% diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide. The nuclei were lightly counterstained with Mayer's hematoxylin. Negative control studies were performed with PBS instead of the primary antibody.

The specificity of immunostaining with the TK-1 monoclonal antibody was checked by a preabsorption experiment. Prior to immunostaining, the diluted anti-TK-1 antibody was admixed with recombinant TK-1 (Abnova) at final concentrations of 0.01, 0.10, 1.00, or 10.00 μg/ml for 60 min at 37˚C. TK-1 immunoreactivity was abolished completely at a concentration of 10.00 μg/ml, proving the specificity of the immunostaining. The specificity of TS immunostaining has been confirmed previously (21).

Immunostaining evaluation and statistical analysis. The immunostained sections were evaluated by two investigators (M.S. and S.K.). The degree of immunostaining was

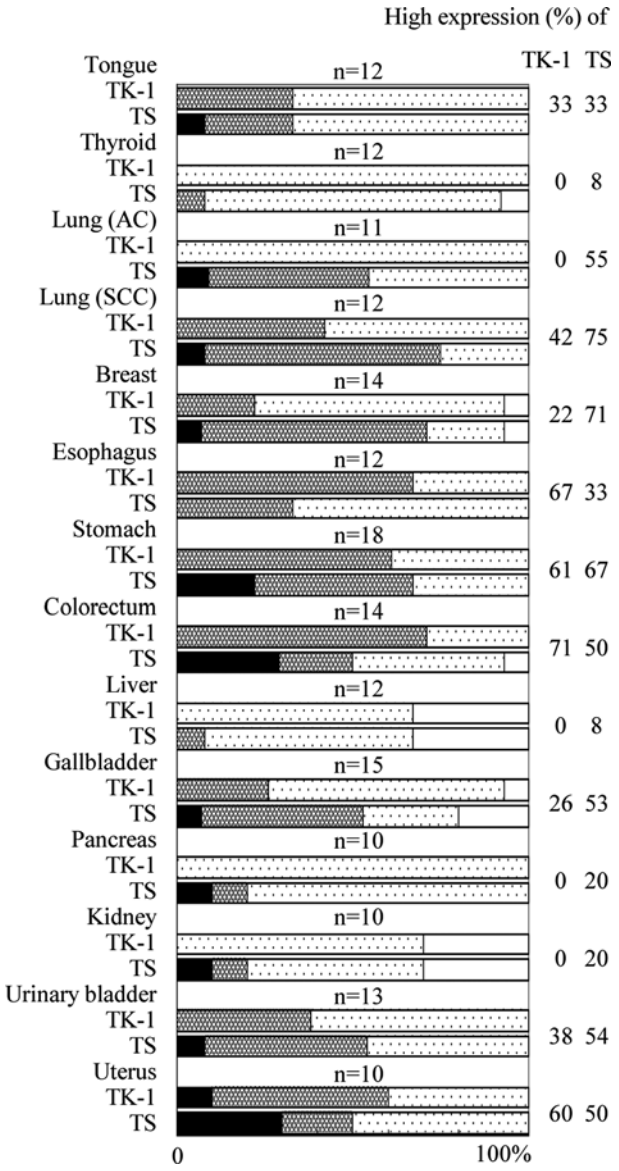

Figure 2. Expression of TK-1 and TS in various types of cancer. AC, adenocarcinoma; SCC, squamous cell carcinoma; open bar, 0; dotted bar, 1+, hatched bar, 2+; black bar, 3+.

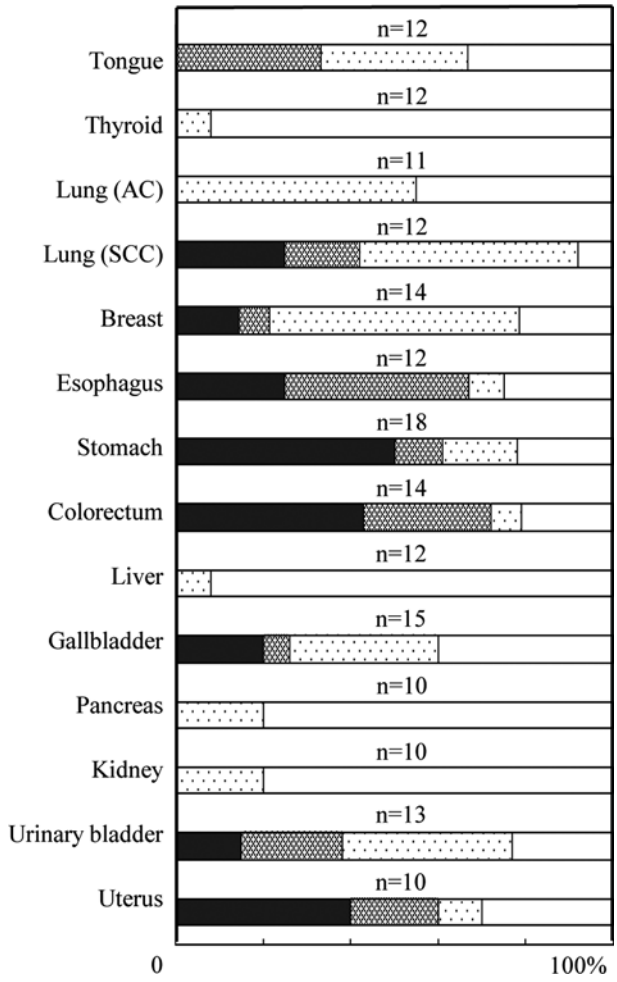

Figure 3. Co-expression of TK-1 and TS in various types of cancer. AC, adenocarcinoma; SCC, squamous cell carcinoma; black bar, TK-1-high and TS-high; hatched bar, TK-1-high and TS-low; dotted bar, TK-1-low and TS-high; open bar, TK-1-low and TS-low.

categorized using a four point scale with 0 representing negative or very weak staining; 1+, <30% positive cancer cells; 2+, 30-60% positive cancer cells; and 3+, >60% positive cancer cells. The 0 and 1+ categories were classified further into a low expression group and the 2+ and 3+ categories into a high expression group (13,14,22). Student's t-test and Welch's t-test were used to determine the statistical difference of antigen expression between adenocarcinomas and squamous cell carcinomas of the lung or between intestinal type and diffuse type adenocarcinomas of the stomach. Differences with $p<0.05$ were considered significant.

Results

TK-1 and TS expression in cancer tissues. The results of immunostaining for TK-1 and TS in various types of cancer are summarized in Fig. 2. There were marked differences in TK-1 and TS expression between different types of cancers.

TK-1 was distributed in both the cytoplasm and nuclei. TK-1 expression categorized as 2+ or 3+ (i.e. high expression) was detected in 67% of esophageal squamous cell carcinomas, 61% of gastric adenocarcinomas (78% of intestinal type tumors and 44% of diffuse type tumors; no significant difference), 71% of colorectal adenocarcinomas, and 60% of squamous cell uterine carcinomas. TK-1 expression categorized as 0 or 1+ (i.e., low expression) was noted in all cases of thyroid papillary carcinoma, lung adenocarcinoma, hepatocellular carcinoma (33% of the cases completely lacked TK-1 expression), pancreatic ductal carcinoma, and renal cell carcinoma (30% of the cases completely lacked TK-1 expression). In lung cancers, 2+ (high) expression of TK-1 was found in 42% of squamous cell carcinomas, whereas adenocarcinomas invariably showed 1+ (low) expression ($p<0.02$).

The staining pattern of TS was fundamentally cytoplasmic, but infrequently nuclear. High expression of TS (i.e. 2+ or 3+) was detected in 75% of squamous cell lung carcinomas, 71% of mammary ductal carcinomas, and 67% of gastric adenocarcinomas. In colorectal adenocarcinomas and squamous cell uterine carcinomas, TS expression categorized as 3+ was detected in about 30% of the cases. In

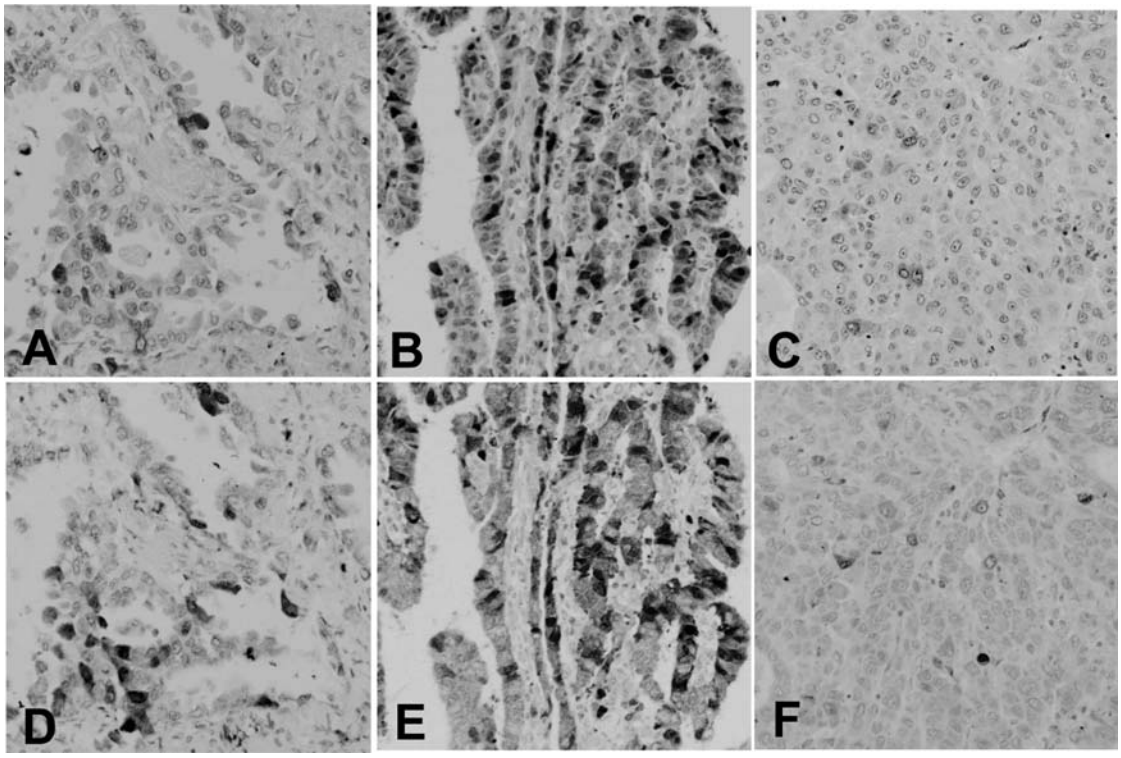

Figure 4. Expression of TK-1 (top panels, A-C) and TS (bottom panels, D-F) in cancer tissues. In the case of lung adenocarcinoma (A and D), a few cancer cells are positive for TK-1, but TS expression is found in a number of the cells. Immunoreactivity for TK-1 and TS is observed in the majority of gastric adenocarcinoma cells (B and E). In the case of hepatocellular carcinoma (C and F), a small number of cancer cells show immunoreactivity for TK-1 and TS.

contrast, low TS expression (i.e., 0 or 1+) was found in 92% of thyroid papillary carcinomas, 92% of hepatocellular carcinomas (33% of the cases completely lacked TS expression), 80% of pancreatic ductal carcinomas, and 80% of renal cell carcinomas (30% of the cases completely lacked TS expression). There were no significant differences in TK-1 and TS expression between adenocarcinomas and squamous cell carcinomas of the lung or between intestinal type and diffuse type adenocarcinomas of the stomach.

Co-expression of TK-1 and TS in cancer tissues. Fig. 3 shows the combined evaluation of TK-1 and TS expression in cancer tissues. Representative expression patterns of TK-1 and TS in cancer tissues are illustrated in Fig. 4. Based on the co-expression of the two enzymes, the tissues were further divided into the following four groups; TK-1-high and TS-high, TK-1-high and TS-low, TK-1-low and TS-high, and TK-1-low and TS-low. The TK-1-high and TS-high patterns were observed in 50% of gastric adenocarcinomas, 42% of colorectal adenocarcinomas and 40% of squamous cell uterine carcinomas. In contrast, 92% of thyroid papillary carcinomas, 92% of hepatocellular carcinomas, 80% of pancreatic ductal carcinomas and 80% of renal cell carcinomas showed the TK-1-low and TS-low patterns.

TK-1 and TS expression in adjacent normal tissues. Representative staining patterns of TK-1 and TS in normal tissues are presented in Fig. 5. A number of TK-1- and TS-positive cells are demonstrated in the proliferative area of mucosal epithelia of the stomach, large intestine, tongue, esophagus and uterus. However, a small number of cells positive for TK-1 and TS were observed in the epithelia of the lung, liver, kidney, urinary bladder and thyroid. In addition, immunoreactivity for TK-1 and TS was observed in non-epithelial cells. TK-1 and TS were expressed in plasma cells and the germinal center of lymphoid follicles. TS immunoreactivity was also found in fibroblasts, endothelial cells, and smooth muscle cells.

Discussion

TK-1 and TS are key enzymes for pyrimidine synthesis. TK-1 converts thymidine to the monophosphate form dTMP in the pyrimidine salvage pathway (1), whereas TS catalyses the methylation of dUMP for *de novo* synthesis of dTMP (10). TS is a crucial target for fluoropyrimidines that have been used widely as a representative anti-cancer drug in the treatment of solid malignancies. Numerous studies have suggested that increased TS levels are associated closely with inherent or acquired resistance to fluoropyrimidine-based chemotherapy, which is a major clinical problem (11-13). On the other hand, there is disagreement among various investigators concerning the relationship between TK-1 activity or mRNA in human cancer cell lines and resistance to fluoropyrimidines (23-27).

TAS-102 is a novel drug containing TFT, which is phosphorylated by TK to its active monophosphate form, TF-TMP (17). TF-TMP differs from fluoropyrimidines in that it does not form a ternary complex and binds covalently to the active site of TS, thereby inhibiting its activity. TF-TMP is further metabolized to the triphosphate form TF-TTP that can be incorporated into DNA. Theoretically, this implies that intra-tumor levels of TK-1 should be a more important factor than TS for determining TAS-102 efficacy (14). TAS-102 has

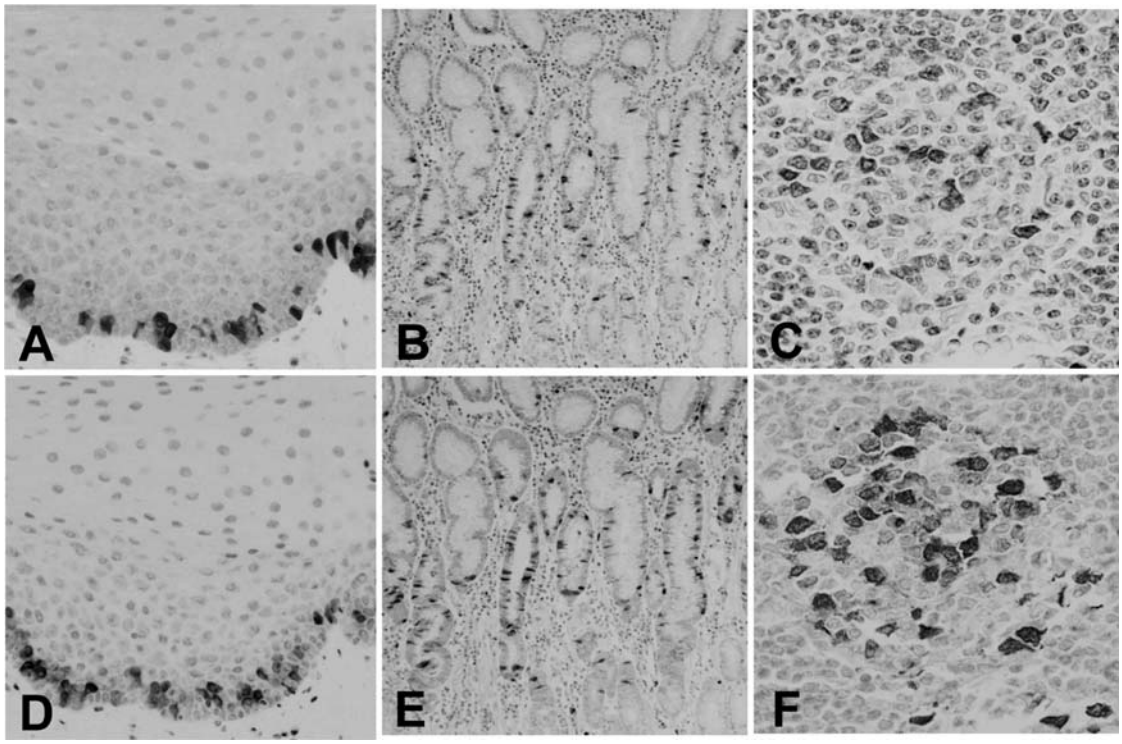

Figure 5. Expression of TK-1 (top panels, A-C) and TS (bottom panels, D-F) in normal tissues. Immunoreactivity for TK-1 and TS is observed in the proliferative area of the tongue epithelium (A and D), gastric epithelium (B and E) and mucosa-associated lymphoid tissue (C and F).

been shown to exhibit antitumor activity in fluoropyrimidine-resistant human cancer cells, and therefore has been suggested as a promising candidate for not only fluoropyrimidine-sensitive patients, but also fluoropyrimidine-resistant cancer patients (14).

If determinants of TAS-102 efficacy in individual cancers could be distinguished prior to commencement of treatment, this would allow responsive and non-responsive patients to be identified, thereby avoiding ineffective therapy with unpleasant side effects. In the present study, we used immunohistochemistry to analyze TK-1 and TS expression in various types of cancer. TK-1 and TS expression was markedly different between cancer types, a finding that suggests strongly that the expression pattern of the pyrimidine synthetic enzymes may influence the therapeutic effects of TAS-102 in different types of cancer. High TK-1 expression was detected prominently in gastrointestinal adenocarcinomas and esophageal and uterine squamous cell carcinomas. The gastrointestinal adenocarcinomas and squamous cell uterine carcinomas were also often accompanied by high TS expression, suggesting activation of pyrimidine synthesis through both the salvage and *de novo* pathways. These results led us to consider that TAS-102 may also be effective for treating esophageal and uterine squamous cell carcinomas, as well as gastrointestinal adenocarcinomas, even in fluoropyrimidine-resistant cases with high TS expression.

Currently, TAS-102 is undergoing clinical phase II trials for use in the latter cases. In contrast, thyroid papillary carcinomas, lung adenocarcinomas, hepatocellular carcinomas, pancreatic ductal carcinomas, and renal cell carcinomas, which exhibited low TK-1 expression, may be resistant to TAS-102. A preclinical study using mouse xenograft models has demonstrated that the rate of tumor growth inhibition caused by TAS-102 is low in tumors derived from pancreatic cancer cells (18).

Konishi *et al* measured the activities of TK-1 and TS in gastric cancers and showed that TK activity was higher in the intestinal type than the diffuse type, whereas TS activity was higher in the diffuse type than the intestinal type (19). However, in our study, we found no significant differences in immunohistochemical expression of TK-1 or TS between these two types of cancers. This discrepancy between studies may be explained by the different methods used for determining the enzyme levels and the number of tumors examined. On the other hand, in non-small cell lung cancers, we demonstrated high TK-1 expression in squamous cell carcinomas, but not in adenocarcinomas ($p<0.02$). These results suggest that TAS-102 efficacy and pyrimidine synthetic pathway may differ according to histological type.

In normal tissues, TK-1 and TS were expressed primarily in the proliferative area of mucosal epithelial and lymphoid tissues. Similar results have been obtained from other immunohistochemical studies (3,22). In addition, a significant positive correlation between TK-1 expression and Ki-67 labeling index (LI) has been observed in breast and non-small cell lung cancers (28,29). Although studies in rodent models indicate that TS is an S-phase-dependent enzyme, studies in human cells have shown that TS levels are high in cycling cells and are largely independent of the cell cycle phase, whilst correlating with Ki-67 LI (16,30,31). Unexpectedly, in our study, both TK-1 and TS expression were low in the majority of hepatocellular carcinomas, pancreatic ductal carcinomas, and renal cell carcinomas, which typically show aggressive behavior and also resistance to cytostatic agents. It is possible

that dysregulation of expression of other proteins involved in control of the cell cycle, proliferation, apoptosis, and invasiveness may contribute to this aggressive behavior (32).

In conclusion, we demonstrated that TK-1 and TS expression was markedly different between cancer types. It is likely that due to high TK-1 expression, TAS-102 is effective for gastrointestinal adenocarcinomas and esophageal and uterine squamous cell carcinomas, even in fluoropyrimidine-resistant cases with high TS expression. To our knowledge, this is the first study to evaluate immunohistochemical expression of TK-1 and TS simultaneously in various types of cancers. Prospective and retrospective clinicopathological trials on a larger number of cases are required to confirm our results.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (No. 20590355) from the Japan Society for the Promotion of Science.

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