Quantitative Analysis of Thymidine Kinase 1 and 5'(3')-Deoxyribonucleotidase mRNA Expression: The Role of Fluorothymidine Uptake

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Abstract. *Background: Thymidine kinase 1 (TK1) and 5'(3')-deoxyribonucleotidase (dNT1) regulate the metabolism of thymidine and its analogs. However, the expression patterns and roles of these enzymes in fluorothymidine (FLT) uptake have not been systemically studied. Materials and Methods: TK1 and dNT1 mRNAs were determined by quantitative PCR in 20 asynchronously growing lung and colon cancer cell lines. [3H]FLT uptake and doubling time were measured. Results: The TK1/GAPDH values varied from $3.09\times10^{-3}\pm6.62\times10^{-4}$ to $2.44\times10^{-2}\pm8.49\times10^{-4}$ and dNT1/GAPDH values from $5.84\times10^{-4}\pm4.88\times10^{-5}$ to $1.59\times10^{-2}\pm1.20\times10^{-3}$. The correlation coefficient of TK1/GAPDH versus dNT1/GAPDH was 0.669 ($p<0.001$). [^{3}H]FLT uptake showed negative moderate correlation with dNT1/GAPDH levels ($r=-0.563$; $p<0.01$), but no significance with TK1/GAPDH levels. Doubling time had no relationship with the TK1 or dNT1 expression. Conclusion: This study profiled correlative expression of TK1 and dNT1 in cancer cell lines, and showed that dNT1 expression determines the lower uptake of FLT, providing a basis for understanding deoxyribonucleotide metabolism.*

The homeostatic regulation of deoxyribonucleotides is important for the balance of DNA replication and repair. *De novo* synthesis of all four deoxyribonucleotides is allosterically regulated *via* ribonucleotide reductase, whereas salvage synthesis of deoxyribonucleotides by nucleoside kinase is catabolically antagonized by nucleotidase (1). The deoxythymidine/ deoxythymidine monophosphate (dTMP) pool in the cytosol is mainly regulated by thymidine kinase 1 (TK1) and 5'(3')-deoxynucleotidase 1 (dNT1, P5N-II) (1). TK1 is the most extensively analyzed salvage enzyme among the nucleoside kinases, including deoxycytidine kinase, thymidine kinase 2 and deoxyguanosine kinase, as it shows S phase-correlated expression and prognostic characteristics in patients with malignancy (2). Conversely, the nature of dNT1 is poorly understood at present. dNT1 is a cytosolic enzyme and has unique substrate specificity to thymidine and deoxyuridine monophosphates differing from ectonucleotidase, high Km-nucleotidase (cN-II), and 5'-nucleotidase Ia/b (cN-Ia/b) (1, 3-5) Other pyrimidine nucleotide-catabolizing 5'-nucleotidases are localized in erythrocytes (P5N1) (6) and mitochondria (dNT2) (7). dNT1 was initially identified in 1971 (8), but its cloning and catalytic properties were described after 2000 (9). Despite accumulating data on TK1, systematic elucidation of the mechanisms underlying salvage regulation of the deoxythymidine/dTMP pool has been difficult owing to the limited information available on dNT1.

TK1 and dNT1 are critical for the regulation of nucleoside analogs as well as endogenous substrates (2, 10). Nucleoside analogs include anti-HIV drugs, such as 3'-azido-3'-deoxythymidine (AZT) and [^{18}F]fluorothymidine ([18F]FLT) used as a radiotracer for molecular imaging with positron emission tomography. [^{18}F]FLT-positron emission tomography can be applied for the early assessment of response to anti-cancer therapy, as [^{18}F]FLT is accumulated in tumors after phosphorylation by TK1 (11-13). [^{18}F]FLT monophosphate is thought to be dephosphorylated by dNT1, since AZT monophosphate, a structurally close analog, displays a high V_{max}/K_m value for dNT1 (10, 14). Therefore, further studies on TK1 and dNT1 are required to elucidate the mechanism of [^{18}F]FLT metabolism in cancer cells. However, the activity profiles of TK1 and dNT1 have been determined only in lymphocytes, adipocytes and myocytes, as their expression patterns are associated with anti-HIV drug-induced adverse effects (*i.e.*, peripheral neuropathy, myopathy, and lipodystrophy syndrome) (15, 16). In this study, we evaluated the mRNA levels of TK1 and dNT1 in 20 lung and colon cancer cell lines. Moreover, [^{3}H]FLT uptake and doubling

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time were estimated to determine the roles of TK1 and dNT1 expression in the evaluation of proliferation with FLT.

Materials and Methods

Cell cultures. The 13 lung cancer cell lines (H522, H358, A549, EKVX, H292, H460, H1975, Hop92, Calu6, H226, Hop62, H23, and H322M) and 7 colon cancer cell lines (SW620, HCT116, Colo205, KM12, HT29, HCT15, and HCT8) were obtained from the American Type Culture Collection (Manassas, VA, USA). All cell lines were maintained in RPMI-1640 containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 g/ml streptomycin at 37˚C in a humidified atmosphere with 5% CO_2 as described previously (12). Cells were seeded at a density of 1~5×10^5 per 6-well plate, and incubated for 24 h to reach 60% confluency for mRNA analysis and [3H]FLT uptake assay. Cells were seeded at the density of 1×10^5 per 6-well plate and incubated for 72 h for doubling time measurement.

Reverse transcription-quantitative polymerase chain reaction (qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Aliquots (2 μg) were reverse-transcribed in mixtures containing AMV-reverse transcriptase, OligodT, dNTP, and RNasin (Promega, Madison, WI, USA). The resulting cDNA was used for real-time PCR analysis with Solaris qPCR gene expression master mix (Dharmacon Inc., Lafayette, CO, USA) and specific primers for human dNT1 (NM_014595), TK1 (NM_003258) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (NM_002046) (Dharmacon Inc.), according to the manufacturer's instructions, using a LightCycler 480 System (Roche, Indianapolis, IN, USA). All experiments were independently repeated 3 to 4 times.

[^{3}H]FLT uptake assay. Asynchronously growing cells were incubated for 2 h in 1 ml fresh medium containing [3H]FLT or [^{3}H]Thd (0.001 μCi/ml media) (Moravek Biochemicals, Brea, CA, USA) (12). Cells were centrifuged, and radioactivity in cells and supernatant fractions measured using a liquid scintillation counter (Perkin-Elmer, Waltham, MA, USA). The aliquots of cells were used for counting of cell number with the trypan blue exclusion assay. [^{3}H]FLT or [^{3}H]Thd uptake was calculated as $100\times CPM_{cell}/(CPM_{cell}+CPM_{sup})/1\times10^5$ viable cells. All experiments were independently repeated 3 to 4 times.

Doubling time measurements. Cell number was counted at 24 h (t1), 48 h, and 72 h (t2) after seeding. The cell number showed a constant growth rate during the assay period. Doubling time was calculated as $(t_2-t_1) \times \log(2)/\log(q_2/q_1)$, where q_1 represents cell quantity at time $t1$ and q_2 at time t_2.

Statistical analysis. Statistical analysis with SPSS 12.0 KO for windows (SPSS Inc, Somers, USA). Data were expressed as means±standard deviation. The Pearson's correlation coefficient was applied to determine the strength of linear association between TK1 and dNT1 mRNA expression (n=66) and between mRNA expression and [3H]FLT uptake test (n=25). Spearman's correlation coefficient was used for analysis of mRNA expression and doubling time (n=8) ($r\geq0.70$, strong correlation, $0.5\leq r<0.7$, moderately strong correlation, $0.3\leq r<0.5$, weak to moderate correlation, and $0.1\leq r<0.3$, weak correlation) (17). The criteria for statistical significance were set at $p<0.05$, $p<0.01$ or $p<0.001$.

Results

TK1 and dNT1 mRNA expression in 20 cancer cell lines. We investigated the relationship of mRNA expression levels between TK1 and dNT1 by quantitative analysis in asynchronously growing cancer cells. Expression of TK1 and dNT1 mRNA was detected in all cancer cell lines, and was not tissue-specific. The TK1 transcript level normalized to GAPDH was the highest in H522 cells and lowest in Calu6 cells, with a 7.90-fold difference among the lung cancer cell lines (Figure 1A). Decreasing expression of TK1 was observed in the following order: H522, H358, H226, Hop62, H292, H1975, A549, Hop92, EKVX, H460, H23, H322M, and Calu6 cell lines. Among the colon cancer cell lines, the difference in TK1/GAPDH values was 2.41-fold between HCT116 and Colo205 which is comparable to H358 and H1975 cells, respectively. Analysis of dNT1 mRNA in lung cancer cells revealed greatest abundance in H522 cells (dNT1/GAPDH ratio of $1.59\times10^{-2}\pm1.20\times10^{-3}$). Decreasing expression of dNT1 was observed in the following order: H358, A549, EKVX, H292, H460, H1975, Hop92, Calu6, H226, Hop62, H23, and H322M cell lines which showed 27.2-fold lower expression than that in H522 cells (Figure 1B). Among the colon cancer cell lines, dNT1 mRNA levels showed a 1.83-fold difference as a mean dNT1/GAPDH ratio of $5.66\times10^{-3}\pm2.83\times10^{-3}$. A plot of TK1/GAPDH *vs*. dNT1/GAPDH produced a Pearson's correlation coefficient of 0.669 ($p<0.001$; N=66), suggesting that TK1 and dNT1 mRNA expression are strongly correlated with each other in these cell lines (Figure 1C).

[^{3}H]FLT uptake and doubling time in cancer cell lines. Radiolabeled FLT is a suggested substrate of TK1 and dNT1 and is used as a proliferation marker for early monitoring of cancer therapy (11, 14). To investigate whether the basal expression levels of TK1 and dNT1 are related to cellular [^{3}H]FLT uptake, we performed a [^{3}H]FLT uptake test in asynchronously growing 7 types of cancer cell lines (H226, H1975, H460, A549, HCT116, SW620 and HT29 cells). The values of [^{3}H]FLT uptake (%/10^5 cells) varied from 4.18±0.92 to 9.48±1.52 (Figure 2A). Plots of dNT1/GAPDH values against [^{3}H]FLT uptake (%/10^5 cells) from 25 pairs of data (Figure 2B) showed moderate negative correlation ($r=-0.563$, $p<0.001$). [^{3}H]FLT uptake did not show significant correlation with TK1/GAPDH values (Table I). Next, we measured the doubling time in H292, H226, H1975, A549, H460, HT29, HCT116, and SW620 cells (Figure 2C). Cellular doubling time ranged from 15.52±3.19 h to 30.54±1.41 h in our experiments, but also has no relationship with TK1 or dNT1 expression (Table I).

Discussion

To our knowledge, this study is the first to report the dNT1 expression levels in cancer cell lines. Previous investigations on dNT activity have focused on adipocytes, myoblastic cells

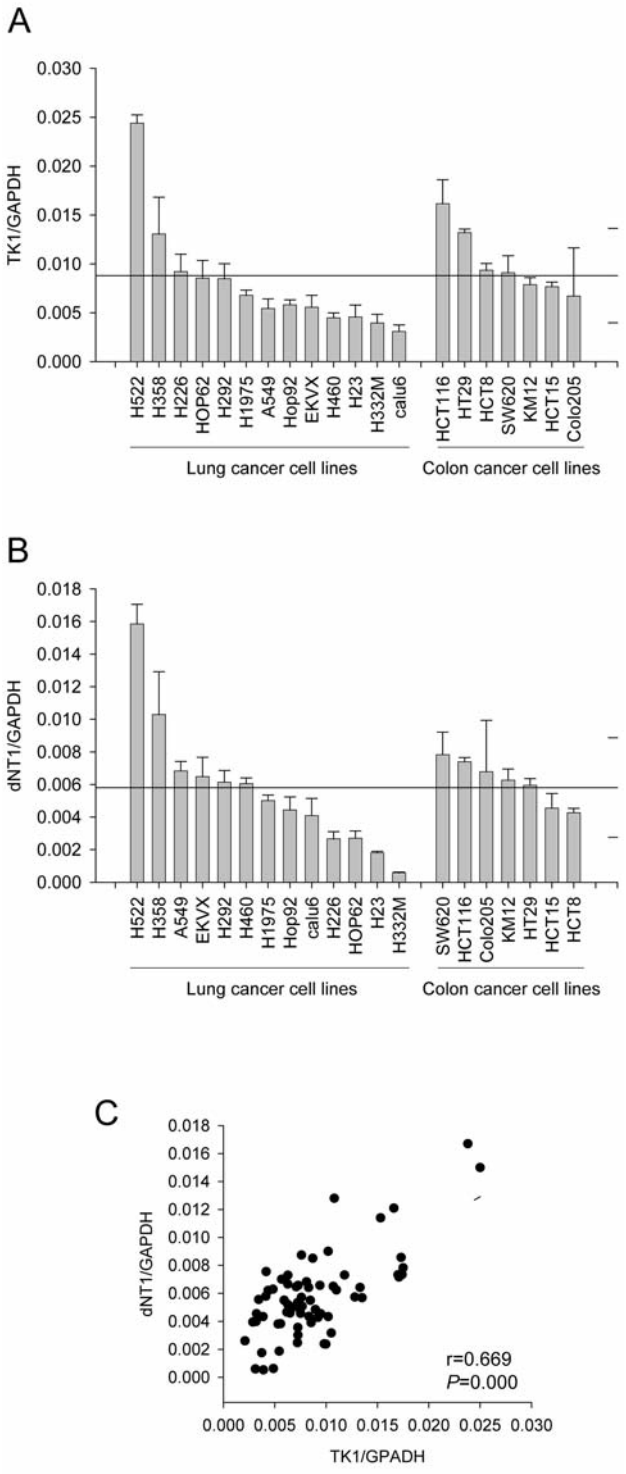

Figure 1. *TK1 and dNT1 mRNA expression in lung and colon cancer cell lines. Asynchronously growing cancer cells with 60% confluency were used for mRNA extraction (n=3~4 per cell line). A: TK1/GAPDH. B: dNT1/GAPDH values were obtained from reverse transcription and qPCR (solid line: mean, dashed line: ±1×SD). C: In total, 66 pairs of data were used for correlation analysis between TK1/GAPDH and dNT1/GAPDH. The Pearson correlation coefficient and P values are shown.*

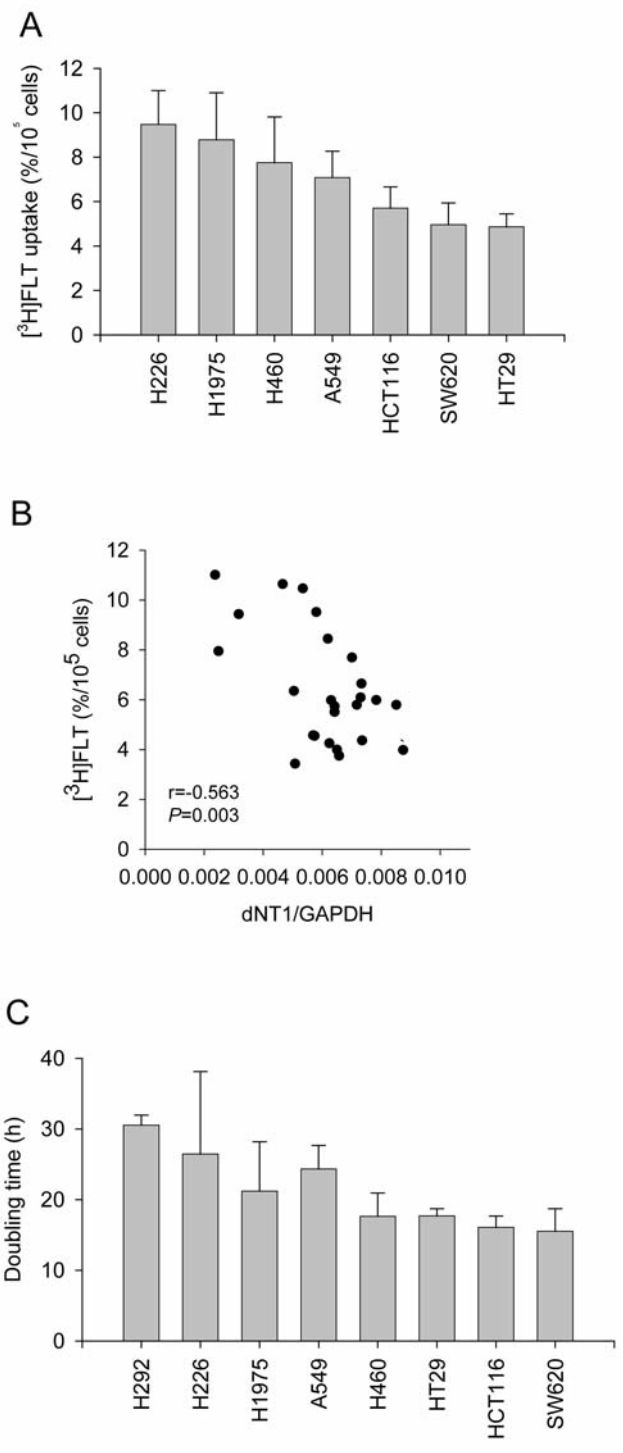

Figure 2. *[3H]FLT uptake and doubling time in lung and colon cancer cell lines. A: Asynchronously proliferating cells with 60% confluency were exposed to [3H]FLT for 2 h and used for measuring [18F]FLT uptake and cell number. B:Plots of dNT1/GAPDH values versus [3H]FLT from 25 pairs of data. The Pearson correlation coefficient and P values are shown. C: Doubling time was calculated from the linear increase in cell number for 3 days after seeding at a confluency of 1×10^5/6-well plate.*

and lymphoblastoid cells, since dNT is implicated in complications of anti-HIV agents that induce mitochondrial toxicity in these tissues (15, 16). Investigation of dNT1 expression in cancer cell lines is essential in view of its suggested role in [^{18}F]FLT metabolism. In this study, we have shown that the mRNA expression patterns of TK1 and dNT1 are strongly correlated in 20 different cancer cell lines. The magnitude of difference in terms of expression in cell lines for dNT1 was 27.2-fold, which was greater than that for TK1 (7.90-fold), indicating that dNT1 expression is a distinct parameter among cancer cells. Moreover, cells expressing higher levels of dNT1 accumulated lower levels of [^{3}H]FLT, suggesting that basal dNT1 expression is a key determinant for FLT uptake.

Considering the opposite physiological roles of TK1 and dNT1, the correlated expression patterns of TK1 and dNT1 in cancer cell lines is an unexpected finding. During adipocyte and myocyte differentiation, TK1 and dNT activities are cooperatively regulated to reduce proliferative capacity (15). 3T3-L1 mouse fibroblasts and L6 rat myoblasts in the resting (100% confluent) or differentiating states show lower TK1 and higher dNT activities than in the proliferating state. In their study, the dNT1 mRNA level was not changed during cell differentiation (15). Our correlative result between TK1 and dNT1 expression in cancer cell lines may be attributed to adaptation of these cells for immortalization or the need to maintain a constant dNTP pool level. Further studies are essential to determine the mechanisms underlying their expression patterns. Nevertheless, as information on dNT1 in cancer cell lines islimited, our results contribute to the understanding of the cooperative regulation between TK1 and dNT1 and the selection of experimental models for *in vitro* analyses.

The substrate specificity of dNT1 for endogenous ligands has been thoroughly examined. Substrate efficiency (V_{max}/K_m) is the highest for dUMP-5', followed by UMP-3', dTMP-5', and dGMP-5' (9). The issue of whether dNT1 can dephosphorylate FLT-monophosphate has not been directly established, but is highly possible because AZT-monophosphate, a structural analog, is a superior substrate to dTMP-5' (10, 14). Accordingly, we aimed to determine the role of dNT1 in FLT metabolism indirectly by measuring [^{3}H]FLT uptake in cancer cell lines expressing various levels of TK1 and dNT1. Basal expression of TK1 was not significantly related to [3H]FLT uptake. This phenomenon differs from the observation that TK1 is a determinant of radiolabeled FLT uptake for proliferation monitoring (11). The previous studies demonstrated that the correlations between TK1 activity, S phase fraction and FLT uptake were strong (18-20), but intercellular correlation between TK1 activity and FLT uptake was cell line-dependent (19). We thought that TK1 activity would reflect the intracellular changes in proliferation capacity, but would not be a sole determinant for basal FLT uptake of various cancer cells. The reason that TK1 mRNA level did not reflect [^{3}H]FLT uptake among various cell lines may be independent regulation of TK1 mRNA at translational and post-translational levels (21). This study revealed that [^{3}H]FLT uptake exhibited moderate negative correlation with dNT1 expression, implying that [^{3}H]FLT monophosphate is dephosphorylated by dNT1. The high magnitude of difference in dNT1 expression in cancer cells underlies its role as a determinant for basal FLT uptake of cancer cells.

A major limitation of this study is that protein expression was not addressed in association with mRNA expression. Commercial antibodies for dNT1 are unavailable, and we are thus currently attempting to generate a specific antibody. Moreover, the physiological role of dNT1 has not been fully elucidated. TK1 and dNT1 expression patterns do not appear to be related to cellular doubling time, which is potentially important in tumor aggressiveness and sensitivity to cytotoxic drugs (17). This result is in keeping with a previous NCI-60 panel study showing no significant differences in TK1 activities between the two groups stratified above and below the median doubling time (17). Further studies on the functional roles of TK1 and dNT1 in cancer can be performed using cancer tissue or gene-inducible models.

TK1 and dNT1 are involved in deoxyribonucleotide and FLT metabolism (1, 2, 14). In the present investigation, correlative expression of TK1 and dNT1 in lung and colon cancer cell lines was comprehensively profiled and our results further showed that dNT1 expression determines the lower uptake of [^{3}H]FLT. These findings may aid in clarifying how TK1 and dNT1 genes are regulated in cancer cell lines and their effects on the metabolism of deoxyribonucleotides and FLT.

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