

# Identification of thymidylate synthase as a potential therapeutic target for lung cancer

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**BACKGROUND:** Thymidylate synthase (TS), a key enzyme in the *de novo* synthesis of thymidine, is an important chemotherapeutic target for malignant tumours including lung cancer. Although inhibition of TS has an antiproliferative effect in cancer cells, the precise mechanism of this effect has remained unclear.

**METHODS:** We examined the effects of TS inhibition with an RNA interference-based approach. The effect of TS depletion on the growth of lung cancer cells was examined using colorimetric assay and flow cytometry.

**RESULTS:** Measurement of the enzymatic activity of TS in 30 human lung cancer cell lines revealed that such activity differs among tumour histotypes. Almost complete elimination of TS activity by RNA interference resulted in inhibition of cell proliferation in all tested cell lines, suggestive of a pivotal role for TS in cell proliferation independent of the original level of enzyme activity. The antiproliferative effect of TS depletion was accompanied by arrest of cells in S phase of the cell cycle and the induction of caspase-dependent apoptosis as well as by changes in the expression levels of cyclin E and c-Myc. Moreover, TS depletion induced downregulation of the antiapoptotic protein X-linked inhibitor of apoptosis (XIAP), and it seemed to activate the mitochondrial pathway of apoptosis.

**CONCLUSION:** Our data provide insight into the biological relevance of TS as well as a basis for clinical development of TS-targeted therapy for lung cancer.

*British Journal of Cancer* (2010) **103**, 354–361. doi:10.1038/sj.bjc.6605793 www.bjcancer.com

Published online 13 July 2010

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**Keywords:** thymidylate synthase; lung cancer; RNA interference; apoptosis; cell cycle

Thymidylate synthase (TS) is an essential enzyme that catalyses the transfer of a methyl group from methylenetetrahydrofolate to dUMP to generate dTMP (Carreras and Santi, 1995). The subsequent phosphorylation of dTMP to dTTP provides a direct precursor for DNA synthesis. The level of TS expression is increased in highly proliferative cells, and an increased abundance of TS in a broad range of tumours is associated with a poor treatment response and prognosis (Costi *et al*, 2005). Transfection of nontransformed cells with an expression vector for TS has also been found to confer transformed-like behaviour, suggesting that TS itself is a potential oncoprotein (Rahman *et al*, 2004). These findings have led to TS being considered as a molecular target for cancer therapy. To date, the antiproliferative effect of TS inhibition has been examined mostly with the use of drugs such as 5-fluorouracil and its active metabolite 5-fluoro-dUMP, the former of which is used in cancer chemotherapy. Although studies with antisense oligodeoxynucleotides have also shown that depletion of TS results in growth inhibition in human tumour cells (Ferguson *et al*, 1999; Lin *et al*, 2001; Flynn *et al*, 2006), the underlying mechanism of the antiproliferative effect of specific TS inhibition has remained largely unknown.

Lung cancer is one of the most common forms of cancer worldwide. Advanced lung cancer is treated by combination chemotherapy, although further improvement in such therapy is warranted. High levels of TS in tumours have been associated with a poor response to TS-targeting agents in individuals with advanced lung cancer (Oguri *et al*, 2005; Kubota *et al*, 2009; Ozasa *et al*, 2009), although the biological relevance of TS in lung cancer has remained to be well established. We have now abrogated both the expression and activity of TS in lung cancer cells by the application of RNA interference (RNAi). With this approach, we investigated the precise mechanism of the antiproliferative effect of TS depletion in lung cancer cells and further examined the potential role of TS as a target for chemotherapeutic agents in these cells. Our results provide a basis for the further development of TS-targeted therapy in lung cancer patients.

## MATERIALS AND METHODS

### Cell culture and reagents

The human lung cancer cell lines A549, H1975, H1650, H358, SW1573, H460, H1299, H520, Calu-1, H226, H82, H526, and H69 were obtained from American Type Culture Collection (Manassas, VA, USA); PC9 and PC9/ZD were obtained as described previously (Koizumi *et al*, 2005); PC3, LK2, PC1, EBC-1, PC10, HARA, SBC-3,

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Received 8 June 2010; revised 17 June 2010; accepted 24 June 2010; published online 13 July 2010

MS-1, COR-L47, STC-1, SBC-1, and RERF-LC-MA were obtained from Human Science Research Resources Bank (Osaka, Japan); Lu135 and Lu134B were from Riken Cell Bank (Tokyo, Japan); and QG56 was obtained from Immuno-Biological Laboratories (Gunma, Japan). All cells were cultured under a humidified atmosphere of 5% CO<sub>2</sub> at 37°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum. The pan-caspase inhibitor ZVAD-FMK was from Wako (Osaka, Japan).

### Assay of TS activity

Thymidylate synthase activity was quantified using tritiated 5-fluoro-dUMP binding assay (Spears *et al*, 1984). Cells were harvested, diluted in 0.2 M Tris-HCl (pH 7.4) containing 20 mM 2-mercaptoethanol, 15 mM CMP, and 100 mM NaF, and disrupted by ultrasonic treatment. The cell lysate was centrifuged at 1600 g for 15 min at 4°C, and the resulting supernatant was centrifuged at 105 000 g for 1 h at 4°C. A portion (50 µl) of the final supernatant was mixed consecutively with 50 µl of Buffer A (600 mM NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 8.0), 100 mM 2-mercaptoethanol, 100 mM NaF, and 15 mM CMP) and with 50 µl of [6-<sup>3</sup>H]5-fluoro-dUMP (7.8 pmol) plus 25 µl of cofactor solution (50 mM potassium phosphate buffer (pH 7.4), 20 mM 2-mercaptoethanol, 100 mM NaF, 15 mM CMP, 2% bovine serum albumin, 2 mM tetrahydrofolic acid, 16 mM sodium ascorbate, and 9 mM formaldehyde). The resulting mixture was incubated at 30°C for 20 min, after which the reaction was terminated by the addition of 100 µl of 2% bovine serum albumin and 275 µl of 1 M HClO<sub>4</sub> and by centrifugation at 1630 g for 15 min at 4°C. The resulting precipitate was suspended in 2 ml of 0.5 M HClO<sub>4</sub>, and the mixture was subjected to ultrasonic treatment followed by centrifugation at 1600 g for 15 min at 4°C. The final precipitate was solubilised with 0.5 ml of 98% formic acid, mixed with 10 ml of ACS II scintillation fluid, and assayed for radioactivity.

### RNAi

Cells were plated at 50–60% confluence in six-well plates or 25 cm<sup>2</sup> flasks and then incubated for 24 h before transient transfection for the indicated times with small interfering RNAs (siRNAs) mixed with the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA). The siRNAs specific for TS mRNA (TS-1, 5'-CAAUCCGCAUCCA ACUAAU-3'; TS-2, 5'-GCUCAGGAUUCUUCGAAAA-3'; and TS-3, 5'-AGCUCAGGAUUCUUCGAAA-3') and a nonspecific siRNA (5'-GUUGAGAGAAUUAAGAGUU-3') were obtained from Nippon EGT (Toyama, Japan). The cells were then subjected to immunoblot analysis, flow cytometry, or assay of TS or caspase-3 activity.

### Immunoblot analysis

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (1 µg ml<sup>-1</sup>). The protein concentration of cell lysates was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA), and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis on a 7.5 or 12% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C either with rabbit polyclonal antibodies to TS (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-actin (1:500 dilution, Sigma), survivin (1:1000 dilution; R&D Systems, Minneapolis, MN, USA), or c-Myc, poly(ADP-ribose) polymerase (PARP), Bcl-2, Bcl-x<sub>L</sub>, Bax, Bak, X-linked inhibitor of apoptosis (XIAP), or Omi/HtrA2 (all in a 1:1000 dilution and from Cell Signaling Technology, Danvers, MA, USA) or with mouse monoclonal antibodies to cyclin E (1:1000 dilution, Santa Cruz Biotechnology),

cytochrome *c* (1:1000 dilution, Cell Signaling Technology), to Smac/Diablo (1:1000 dilution, Cell Signaling Technology). The membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat antibodies to rabbit (Sigma) or mouse (Santa Cruz Biotechnology) immunoglobulin G. Immune complexes were finally detected with chemiluminescence reagents (GE Healthcare, Little Chalfont, UK).

### Growth inhibition assay *in vitro* (MTT assay)

Cells were plated at 50–60% confluence in 25 cm<sup>2</sup> flasks and then incubated for 24 h before transient transfection with an siRNA specific for TS mRNA or a control siRNA as described above. The cells were then isolated by exposure to trypsin, transferred to 96-well flat-bottom plates, and cultured for 72 h before the addition of TetraColor One (5 mM tetrazolium monosodium salt and 0.2 mM 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku, Tokyo, Japan) to each well and incubation for an additional 3 h at 37°C. The absorbance at 490 nm of each well was measured using Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA, USA), and absorbance values were expressed as a percentage of that for nontransfected control cells.

### Cell cycle analysis

Cells were harvested, washed with PBS, fixed with 70% methanol, washed again with PBS, and stained with propidium iodide (0.05 mg ml<sup>-1</sup>) in a solution containing 0.1% Triton X-100, 0.1 mM EDTA, and RNase A (0.05 mg ml<sup>-1</sup>). The stained cells (~1 × 10<sup>6</sup>) were then analysed for DNA content using flow cytometer (FACS Caliber; Becton Dickinson, Franklin Lakes, NJ, USA) and Modfit software (Verity Software House, Topsham, ME, USA).

### Assay of caspase-3 activity

The activity of caspase-3 in cell lysates was measured using CCP32/Caspase-3 Fluometric Protease Assay kit (MBL, Woburn, MA, USA). Fluorescence attributable to cleavage of the Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD-AFC) substrate was measured at excitation and emission wavelengths of 390 and 460 nm, respectively.

### Subcellular fractionation

A cytosolic fraction was isolated from cells by centrifugation. In brief, cells were washed, resuspended in homogenisation buffer (0.25 M sucrose, 10 mM HEPES-NaOH (pH 7.4), and 1 mM EGTA), and homogenised by 50 strokes in a Dounce homogeniser. The homogenate was centrifuged at 1000 g for 15 min at 4°C to remove nuclei and intact cells, and the resulting supernatant was centrifuged at 10 000 g for 15 min at 4°C. The final supernatant (cytosolic fraction) was subjected to immunoblot analysis.

### Statistical analysis

Data were analysed using Student's two-tailed *t*-test. A *P*-value of <0.05 was considered statistically significant.

## RESULTS

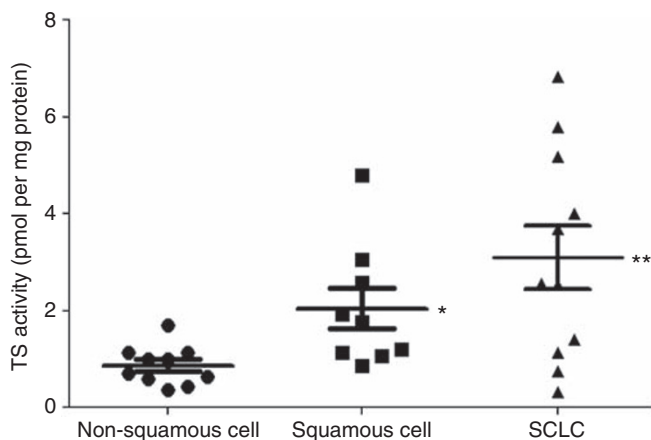
### TS activity varies among histotypes of lung cancer cells

We first examined the enzymatic activity of TS in 30 human lung cancer cell lines (Table 1). The median TS activity in small cell lung cancer (SCLC) lines was significantly higher than that in non-SCLC (NSCLC) lines. Among NSCLC cell lines, the values for squamous cell carcinoma were higher than those for non-squamous cell

**Table 1** TS activity of lung cancer cell lines classified according to histology

Cell line	Histology	TS activity (pmol per mg protein)
A549	Adeno	1.003 ± 0.142
HI975	Adeno	1.005 ± 0.276
HI1650	Adeno	0.705 ± 0.177
PC9	Adeno	0.370 ± 0.042
PC9/ZD	Adeno	0.635 ± 0.148
H358	Adeno	1.140 ± 0.127
PC3	Adeno	0.591 ± 0.325
SW1573	Adeno	1.695 ± 0.544
H460	Large cell	0.420 ± 0.184
HI299	Large cell	1.121 ± 0.594
H520	Squamous	1.755 ± 0.813
Calu-1	Squamous	4.805 ± 3.061
H226	Squamous	1.930 ± 0.820
LK2	Squamous	1.121 ± 0.042
PCI	Squamous	3.055 ± 0.997
EBC-1	Squamous	1.055 ± 0.078
PC10	Squamous	1.204 ± 0.052
QG56	Squamous	0.870 ± 0.030
HARA	Squamous	2.590 ± 0.340
SBC-3	SCLC	5.795 ± 0.247
H82	SCLC	5.170 ± 0.641
H526	SCLC	1.125 ± 0.092
H69	SCLC	4.005 ± 0.078
MS-1	SCLC	2.555 ± 0.474
COR-L47	SCLC	3.760 ± 0.560
STC-1	SCLC	6.832 ± 0.490
SBC-1	SCLC	0.753 ± 0.023
Lu135	SCLC	3.698 ± 0.190
Lu134B	SCLC	0.310 ± 0.100
REFR-LC-MA	SCLC	1.413 ± 0.183

Abbreviations: SCLC = small cell lung cancer; TS = thymidylate synthase. Data are means ± s.d. from three independent experiments.



**Figure 1** Thymidylate synthase (TS) activity in lung cancer cell lines stratified according to histotype. Central horizontal lines represent median values, with the upper and lower bars representing the 95% confidence interval. \* $P < 0.05$  for squamous cell carcinoma vs non-squamous cell carcinoma; \*\* $P < 0.05$  for SCLC vs either squamous cell or non-squamous cell carcinoma.

carcinoma (Figure 1). There was no significant correlation between TS activity and cell proliferation rate among these lung cancer cell lines (data not shown). These data thus suggested that TS activity varies according to histotype among lung cancer cell lines.

## TS depletion induces growth inhibition regardless of original TS activity level in lung cancer cells

We next examined the effect of TS depletion by RNAi on the growth of lung cancer cell lines. The abundance of TS was markedly decreased as a result of cell transfection with either of three different siRNAs targeted to TS mRNA (Figure 2A). Given that the TS-1 siRNA induced the most pronounced downregulation of TS expression, we selected this siRNA for use in subsequent experiments. In all tested lung cancer cells, transfection with TS-1 resulted in marked depletion of TS, whereas no such effect was observed in cells transfected with a nonspecific siRNA (Figure 2B). Moreover, transfection of cells with TS-1 resulted in a >90% decrease in TS activity compared with that in corresponding cells transfected with a nonspecific siRNA or in untreated cells (Figure 2C), regardless of the original levels of TS expression and activity. The antiproliferative effect of TS depletion was evaluated using the MTT assay. Depletion of TS resulted in pronounced inhibition of proliferation in all tested cells compared with the corresponding cells transfected with a nonspecific siRNA or untreated cells (Figure 2D), and this antiproliferative effect was found to be time dependent (Figure 2E). These data thus suggested that the almost complete elimination of TS activity resulted in marked inhibition of the proliferation of lung cancer cells regardless of the original level of such activity.

## TS depletion induces S-phase arrest in lung cancer cells

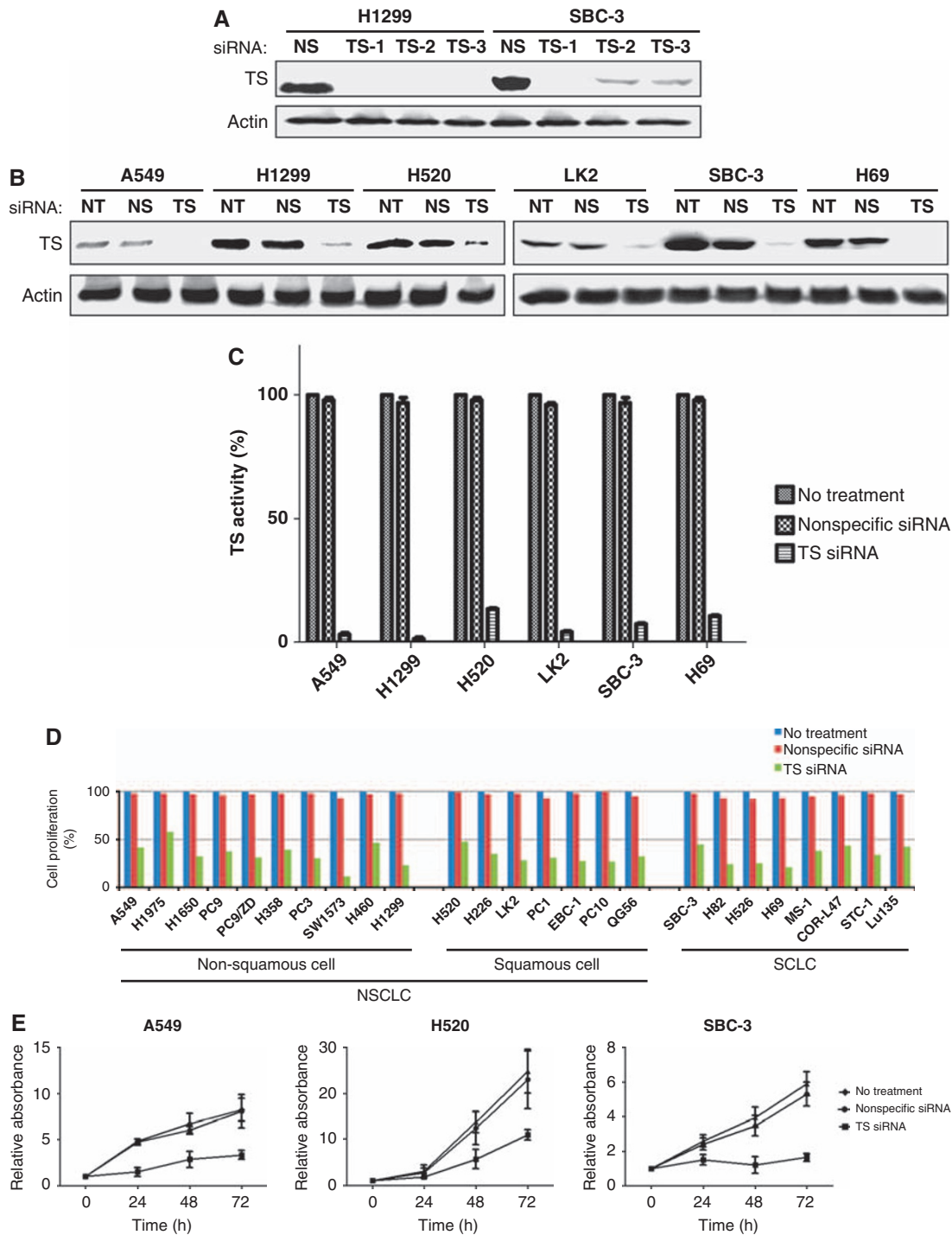
To investigate the mechanism by which TS depletion inhibits lung cancer cell growth, we examined the cell cycle profile by flow cytometry. Transfection with TS-1 siRNA increased the proportion of cells in S phase of the cell cycle and reduced that of cells in  $G_1$  or  $G_2$ -M phases in all tested cell lines regardless of histotype (Figure 3A). Immunoblot analysis of proteins implicated in regulation of the  $G_1$ -S transition revealed that TS depletion increased the abundance of cyclin E in all tested cell lines (Figure 3B) without affecting that of cyclins D or A (data not shown). In addition, TS depletion induced downregulation of c-Myc (Figure 3B), a transcription factor that activates the expression of several cell cycle-related genes. However, expression of c-Myc was not detected in H69 cells, consistent with previous observations (Plummer *et al*, 1993). These results thus suggested that the S-phase arrest induced by TS depletion in lung cancer cells was related to upregulation of cyclin E and downregulation of c-Myc.

## TS depletion induces caspase-dependent apoptosis in lung cancer cells

We next examined the effect of TS depletion on apoptosis in lung cancer cells. Flow cytometric analysis revealed that TS depletion induced a time-dependent increase in the size of the sub- $G_1$  (apoptotic) cell population (Figure 4A). Depletion of TS also induced the cleavage of PARP (Figure 4B), a characteristic of apoptosis, in the cell lines examined. Furthermore, the activity of caspase-3 in cell lysates was increased as a consequence of TS depletion (Figure 4C), and previous exposure of lung cancer cells to the pan-caspase inhibitor ZVAD-FMK significantly inhibited the increase in the size of the sub- $G_1$  cell population induced by depletion of TS (Figure 4D). These data thus indicated that TS depletion induces caspase-dependent apoptosis in lung cancer cells.

## TS depletion activates the mitochondrial pathway of apoptosis and induces downregulation of XIAP

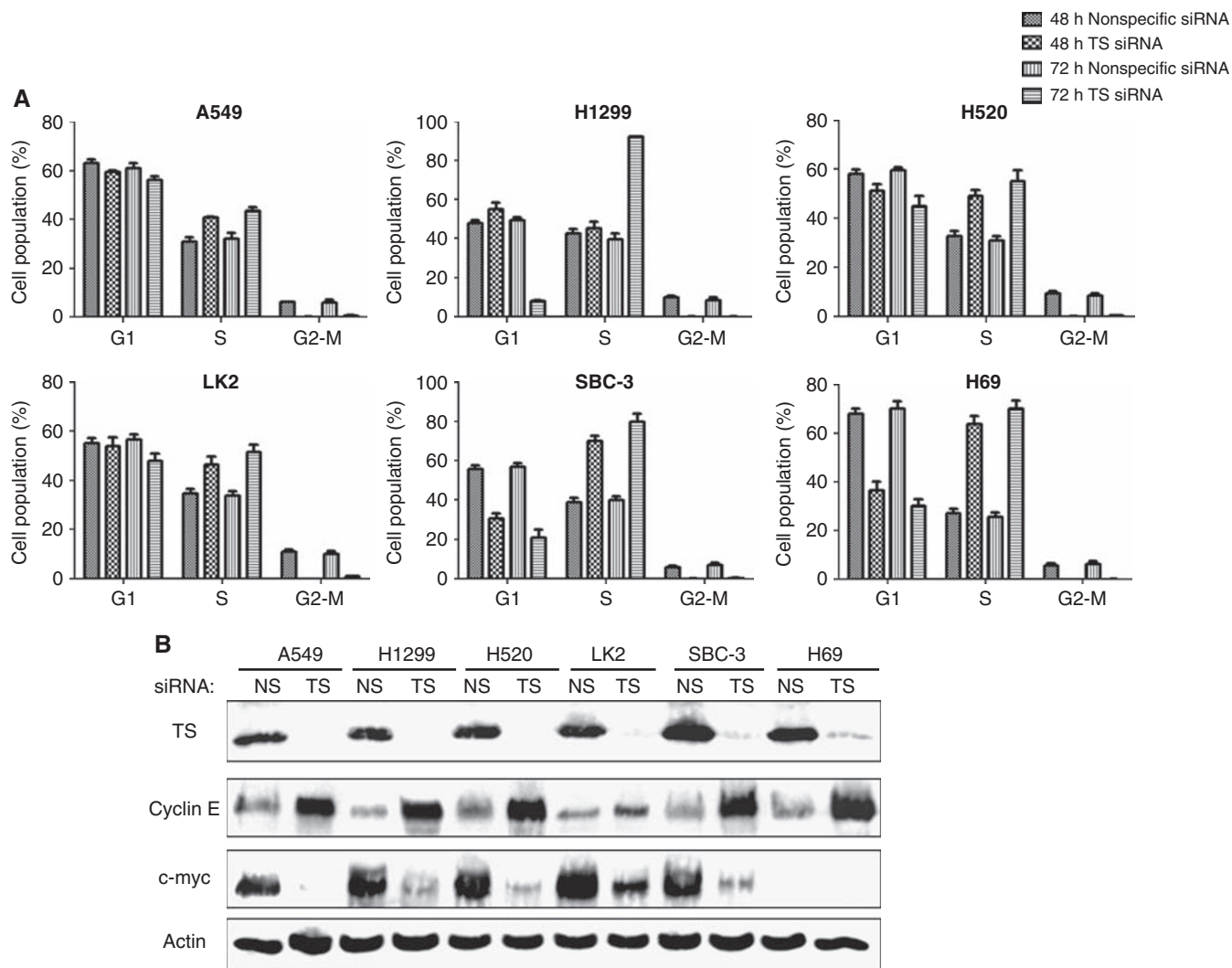
To elucidate further the mechanism of apoptosis induced by TS depletion, we examined the expression of members of the Bcl-2



**Figure 2** Effects of transient depletion of TS on TS activity and the proliferation of lung cancer cell lines. **(A)** The indicated cell lines were transfected with a nonspecific (NS) siRNA or with either of three different siRNAs specific for TS mRNA (TS-1, TS-2, and TS-3) for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS and  $\beta$ -actin (loading control). **(B)** The indicated cell lines were left untreated (NT) or were transfected with nonspecific or TS-1 siRNAs for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS and  $\beta$ -actin. **(C)** Cells were left untreated or were transfected with NS or TS-1 siRNAs for 72 h, after which cell lysates were prepared and assayed for TS activity. Data are expressed as a percentage of the value for untreated cells and are means  $\pm$  s.d. of triplicates from experiments that were repeated on at least one additional occasion with similar results. **(D)** Cells were left untreated or were transfected with NS or TS-1 siRNAs for 72 h, after which cell viability was assessed with the MTT assay. Data are expressed as a percentage of the value for untreated cells and are means of triplicates from experiments that were repeated on two additional occasions with similar results. **(E)** Cells were left untreated or were transfected with NS or TS-1 siRNAs for the indicated times, after which cell viability was assessed with the MTT assay. Data are means  $\pm$  s.d. of triplicates from experiments that were repeated on two additional occasions with similar results.

and inhibitor of apoptosis (IAP) families of proteins, both of which are important regulators of apoptotic signalling pathways (Hengartner, 2000). Although depletion of TS did not substantially

affect the expression levels of Bcl-2, Bcl-x<sub>L</sub>, Bax, Bak, and survivin, it resulted in a substantial decrease in the abundance of X-linked inhibitor of apoptosis (XIAP) (Figure 5A). The activity of XIAP is



**Figure 3** Effects of TS depletion on cell cycle distribution and on cyclin E and c-Myc expression in lung cancer cells. **(A)** The indicated cell lines were transfected with nonspecific (NS) or TS-I siRNAs for 48 or 72 h and were then fixed, stained with propidium iodide, and analysed for cell cycle distribution by flow cytometry. Data are means  $\pm$  s.d. of triplicates from experiments that were repeated on two additional occasions with similar results. **(B)** Cells were transfected with NS or TS-I siRNAs for 72 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS, cyclin E, c-Myc, and  $\beta$ -actin. Transfection with the NS siRNA had no substantial effect on cell cycle distribution or on the expression of cyclin E or c-Myc compared with untreated cells.

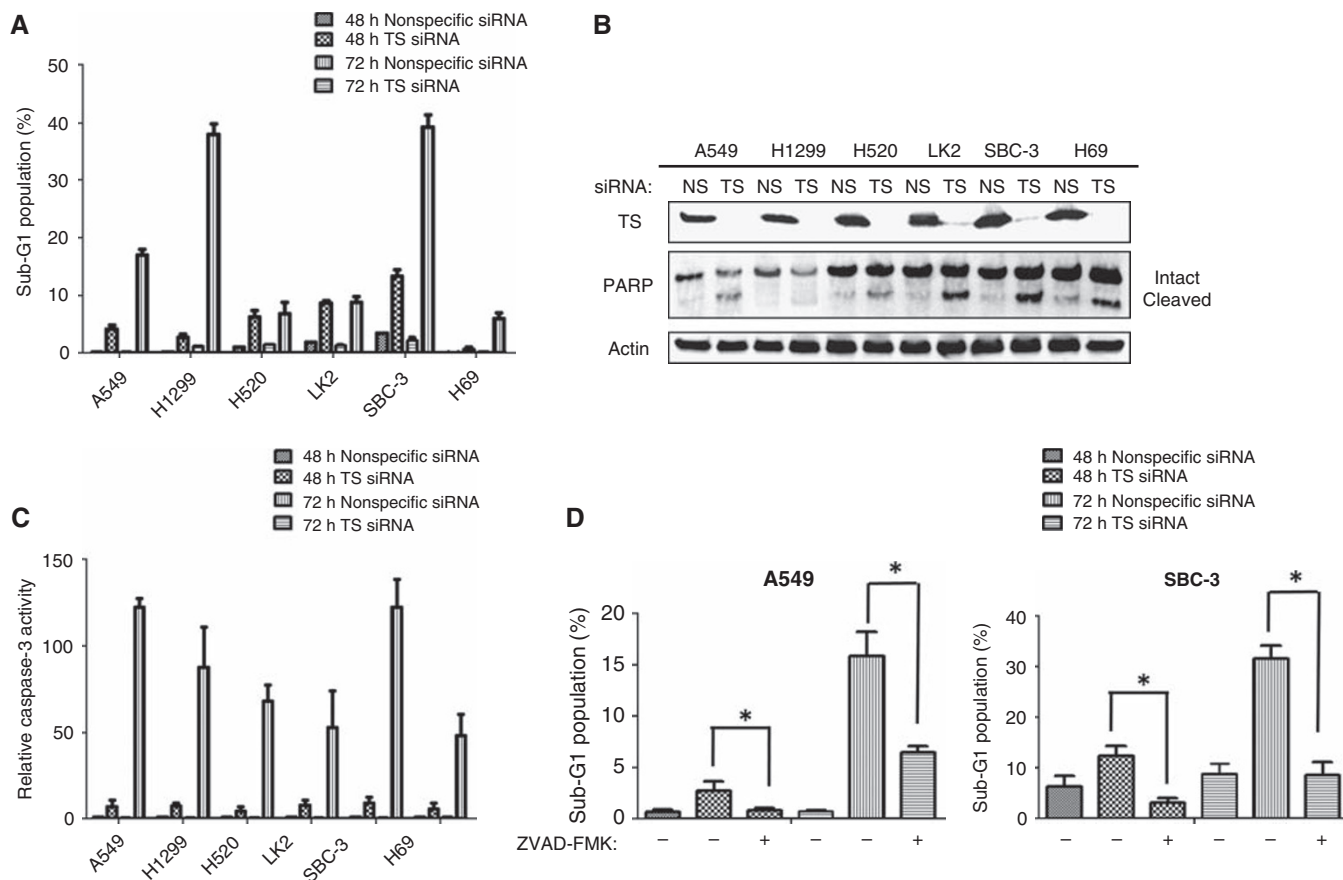
modulated by mitochondrial proteins such as cytochrome *c*, Smac (also known as Diablo), and Omi (also known as HtrA2) (Hengartner, 2000; Srinivasula *et al*, 2003; Martinez-Ruiz *et al*, 2008). To investigate the mechanism of the downregulation of XIAP induced by TS depletion, we examined the release of these mitochondrial proteins into the cytosol. Immunoblot analysis revealed that the amounts of these mitochondrial proteins in the cytosol were increased by TS depletion in a time-dependent manner (Figure 5B). These data thus suggested that TS depletion-induced apoptosis is mediated, at least in part, by the mitochondrial signalling pathway.

## DISCUSSION

Studies of TS-targeted therapy as well as the role of TS in DNA synthesis have provided the rationale for consideration of this enzyme as a prime therapeutic target. However, the precise mechanism by which inhibition of TS results in inhibition of tumour cell growth has remained incompletely understood.

The aim of this study was therefore to investigate the underlying mechanism of the antiproliferative effect of specific TS inhibition in lung cancer cells with the use of an siRNA-based approach.

We first examined TS activity in lung cancer cell lines of different histotypes. Thymidylate synthase activity was determined with the use of the well-established 5-fluoro-dUMP binding assay. We found that TS activity was significantly higher in SCLC lines than in NSCLC lines, and that, among the latter, TS activity was higher in squamous cell carcinoma lines than in non-squamous cell carcinoma lines. A previous microarray analysis showed that mRNAs for proliferation-related proteins including TS were more abundant in SCLC lines than in NSCLC lines (Bhattacharjee *et al*, 2001). In addition, recent studies showed that the amount of TS mRNA was higher in resection specimens from patients with squamous cell carcinoma of the lung than in those from individuals with other histotypes of NSCLC (Ceppi *et al*, 2006; Ishihama *et al*, 2009; Monica *et al*, 2009). Given that TS activity in lung cancer cell lines was proportional to the abundance of TS protein in the present study (data not shown), our data showing a differential profile of TS activity among histotypes of lung cancer

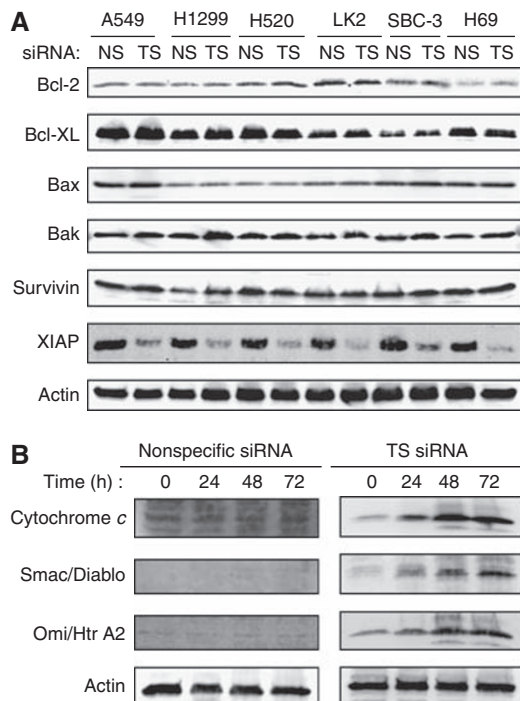


**Figure 4** Effect of TS depletion on apoptosis in lung cancer cells. **(A)** The indicated cell lines were transfected with nonspecific (NS) or TS-I siRNAs for 48 or 72 h and were then fixed, stained with propidium iodide, and subjected to flow cytometry for quantitation of the sub-G<sub>1</sub> population. Data are means  $\pm$  s.d. of triplicates from experiments that were repeated on two additional occasions with similar results. **(B)** Cells were transfected with NS or TS-I siRNAs for 72 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to TS, PARP, and  $\beta$ -actin. The positions of intact and cleaved forms of PARP are indicated. **(C)** Cells were transfected with NS or TS-I siRNAs for 48 or 72 h, lysed, and assayed for caspase-3 activity. Data are expressed relative to the value for cells transfected with NS siRNA and are means  $\pm$  s.d. from three independent experiments. **(D)** Cells were incubated for 2 h with or without ZVAD-FMK (50  $\mu$ M), transfected with NS or TS-I siRNAs for 48 or 72 h (in the continued absence or presence of ZVAD-FMK), and then evaluated for the size of the sub-G<sub>1</sub> population as in **(A)**. Data are means  $\pm$  s.d. of triplicates from experiments that were repeated on two additional occasions with similar results. \* $P < 0.05$  for the indicated comparisons. Transfection with the NS siRNA had no substantial effects on these markers of apoptosis compared with untreated cells.

are consistent with these previous findings. The cell line SCLC differs from NSCLC in terms of its faster growth and earlier spread (Allen and Jahanzeb, 2008), and recent clinical trials in NSCLC patients have revealed a poorer prognosis for squamous cell carcinoma than for adenocarcinoma (Asamura *et al*, 2008). The differential activity of TS among histotypes of lung cancer is thus suggestive of a role for this enzyme in promoting cell proliferation, with TS activity being a potential marker of tumour aggressiveness in lung cancer, although TS activity was not correlated with cell proliferation rate among the lung cancer cell lines examined in this study. We induced downregulation of both TS abundance and enzymatic activity in lung cancer cell lines by RNAi. The almost complete elimination of TS activity was associated with a marked antiproliferative effect in all tested lung cancer cell lines, including those with an original relatively low level of TS activity. These data suggest that TS is important for tumour cell proliferation in a manner independent of the original activity level.

We found that depletion of TS induced S-phase arrest and caspase-dependent apoptosis in lung cancer cells. Previous studies have shown that TS inhibition results in an imbalance between the amounts of dUTP and dTTP and a consequent decrease in the efficiency of DNA synthesis (Curtin *et al*, 1991; Houghton *et al*, 1993). Furthermore, this dUTP–dTTP imbalance results in

misincorporation of dUTP into DNA and consequent DNA damage (Curtin *et al*, 1991; Houghton *et al*, 1993). In this study, we examined the effect of TS depletion on DNA damage as determined by immunofluorescence imaging of histone  $\gamma$ -H2AX foci, a sensitive and specific marker of DNA double-strand breaks (Burma *et al*, 2001; Stiff *et al*, 2004). Such foci were detected in  $\sim 90\%$  of lung cancer cells transfected with TS siRNA (Supplementary Figure S1). Given that DNA damage or a reduced rate of DNA synthesis triggers the S-phase checkpoint mechanism (Sclafani and Holzen, 2007), the observed S-phase arrest induced by TS depletion likely results from activation of the S-phase checkpoint. Cellular responses to DNA damage are important for maintenance of genomic stability and cellular integrity (Bunz *et al*, 1998; Hirao *et al*, 2000). Cells either repair DNA damage or, if it is too severe for repair, initiate the cell death program (Zhao *et al*, 2001). Our data thus suggest that cells that arrest in S phase after TS depletion subsequently undergo apoptosis as a result of the accumulation of unreparable DNA damage. We further showed that TS depletion resulted in upregulation of cyclin E and downregulation of c-Myc. Both cyclin E and c-Myc contribute to the transition of cells from G<sub>1</sub> to S phase (Wang *et al*, 2008; Malumbres and Barbacid, 2009) and have recently been implicated in promotion of caspase-dependent apoptosis subsequent to



**Figure 5** Effects of TS depletion on the expression of Bcl-2 and IAP family members and on the release of mitochondrial proteins into the cytosol in lung cancer cells. **(A)** The indicated cell lines were transfected with nonspecific (NS) or TS-1 siRNAs for 72 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. **(B)** SBC-3 cells were transfected with NS or TS-1 siRNAs for 24, 48, or 72 h, after which a cytosolic fraction was prepared and subjected to immunoblot analysis with antibodies to cytochrome *c*, Smac/Diablo, Omi/HtrA2, and  $\beta$ -actin. Transfection with the NS siRNA had no substantial effects on the abundance of Bcl-2 or IAP family proteins or on the release of mitochondrial proteins into the cytosol, compared with untreated cells.

S-phase arrest induced by DNA damage or inhibition of DNA synthesis in tumour cells (Mazumder *et al*, 2000; Leonce *et al*, 2001; Lu *et al*, 2009; Sankar *et al*, 2009). The effects of TS depletion on the abundance of cyclin E and c-Myc therefore likely contribute to the associated S-phase arrest and caspase-dependent apoptosis in lung cancer cells. Our present data thus suggest that the antiproliferative effect of TS depletion is attributable to S-phase

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arrest and the induction of caspase-dependent apoptosis in these cancer cells.

Our investigation of the mechanism by which TS depletion led to caspase-dependent apoptosis revealed that elimination of TS resulted in downregulation of XIAP, a member of the IAP family of proteins. Activation of the mitochondrial signalling pathway for apoptosis results in inhibition of IAP proteins and consequent promotion of caspase-dependent apoptosis (Hengartner, 2000; Takasawa *et al*, 2005; Yu *et al*, 2007). We also found that TS depletion resulted in the release of mitochondrial proteins, including cytochrome *c*, Smac/Diablo, and Omi/HtrA2, into the cytosol, suggestive of a link between activation of the mitochondrial pathway and downregulation of XIAP in lung cancer cells depleted of TS. Activation of the mitochondrial pathway is induced by a variety of stimuli including DNA damage (Hengartner, 2000). Given that TS depletion induced DNA double-strand breakage, our data suggest that loss of TS may contribute to activation of the mitochondrial pathway of apoptosis. We found that TS depletion did not affect the expression level of the IAP protein survivin. Further study will thus be needed to elucidate the precise mechanism by which XIAP is downregulated specifically in TS-depleted cells.

In conclusion, we have shown that the almost complete elimination of TS activity with an RNAi-based approach resulted in an apparently universal antiproliferative effect in lung cancer cells that was attributable to S-phase arrest and the induction of apoptosis. High levels of TS expression have been suggested to predict resistance to TS-targeted agents such as 5-fluorouracil (Johnston *et al*, 2003; Showalter *et al*, 2008). The new TS-targeted agent pemetrexed was found to have low activity in the treatment of SCLC (Ceppi *et al*, 2006; Socinski *et al*, 2009), possibly as a result of a high level of TS expression in such tumours. Our results now suggest that TS depletion inhibits the growth of lung cancer cells including SCLC cells with a high original activity of TS. This apparent discrepancy may be explained by the fact that 5-fluorouracil and pemetrexed inhibit TS activity by only ~60% (van Triest *et al*, 1997, 1999; Codacci-Pisanelli *et al*, 2002; Giovannetti *et al*, 2008), whereas our siRNA-based method inhibit TS activity almost completely. Our present results thus suggest that novel TS-targeted agents with an increased inhibitory efficacy might prove beneficial for the treatment of lung cancer regardless of histotype. They further provide a rationale for future clinical investigation of the therapeutic efficacy of TS-targeted agents for lung cancer patients.

Supplementary Information accompanies the paper on British Journal of Cancer website (<http://www.nature.com/bjc>)

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