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ORIGINAL ARTICLE

Suppression of thymidine phosphorylase expression by promoter methylation in human cancer cells lacking enzyme activity

Vincenzo Guarcello · Carmelo Blanquicett · Fardos N. M. Naguib · Mahmoud H. el Kouni

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Abstract
Purpose Thymidine phosphorylase (TP, EC 2.4.2.4) activity varies in different human cancer cell lines. Nevertheless, little is known about the regulatory mechanisms of TP expression in such cancers. Promoter methylation of dinucleotide cytosine–guanine (CpG) sites is a known mechanism of reversible gene expression silencing.
Methods TP promoter methylation was investigated in five cancer cell lines (SKBR-3, 786-O, HT-29, MDA-231, DLD-1). TP mRNA levels were determined by real-time quantitative PCR. The degree of methylation was identified by bisulfite sequencing. Minimal TP promoter activity was determined by Luciferase reporter assays. DNA–protein interactions were evaluated by electrophoretic mobility shift assays.
Results SKBR-3 cells exhibited the highest TP expression, 786-O, HT-29, and MDA-231 cells exhibited intermediate TP expression, while DLD-1 cells did not express TP as demonstrated by TP mRNA, protein, and enzyme activity levels. SKBR-3 lacked methylation in the TP promoter, intron 1 and exon 1 regions, while DLD-1 showed extensive methylation. Treatment of DLD-1 and SKBR-3 with the methylation-inhibitor, 5-aza-2′-deoxycytidine (5-aza-2dC), resulted in a concentration-dependent increase in TP mRNA and protein levels in DLD-1 but not SKBR-3 cells. Trichostatin-A treatment, a histone deacetylase inhibitor, improved the 5-aza-2dC-induced TP reactivation. Electrophoretic mobility shift assays demonstrated that methylation significantly inhibits transcription factor binding. Supershift analyses suggest that the Sp1 and Sp3 (to a lesser degree) transcription factors have a role in the regulation of TP expression.
Conclusions These findings suggest that TP promoter methylation is a mechanism for down-regulation of TP expression in cancer cells and may have implications in modulating prognosis of cancer patients.

Keywords Thymidine phosphorylase · Promoter methylation · Expression · Regulation · Cancer cells

Abbreviations

5-Aza-2dC	5-Aza-2′-deoxycytidine
CpG	Dinucleotide cytosine–guanine motif
EMSA	Electrophoretic mobility shift assays
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
RT-Q-PCR	Real-time quantitative PCR
PD-ECGF	Platelet derived endothelial cell growth factor
TP	Thymidine phosphorylase (EC 2.4.2.4)
TSA	Trichostatin-A

Introduction

Thymidine phosphorylase (TP; EC 2.4.2.4) is a key enzyme of the pyrimidine salvage pathways. It catalyzes the reversible phosphorolysis of thymidine, deoxyuridine (but

V. Guarcello and C. Blanquicett contributed equally to this work.

V. Guarcello · C. Blanquicett · F. N. M. Naguib · M. H. el Kouni (✉)
Department of Pharmacology and Toxicology, Comprehensive Cancer Center, Center for AIDS Research, University of Alabama at Birmingham, Birmingham, AL 35294, USA
e-mail: m.elkouni@ccc.uab.edu

Present Address:
C. Blanquicett
Department of Medicine, School of Medicine, Emory University, Atlanta, GA 30322, USA

not deoxycytidine) and their analogues, to their respective bases and 2-deoxyribose-1-phosphate as follows:

$$\text{Pyrimidine } 2'\text{-deoxyriboside} + P_i \rightleftharpoons \text{Pyrimidine nucleobase} + \text{2-Deoxyribose-1-phosphate}.$$

As a result, TP regulates the intra- and extracellular thymidine pools as well as thymidine homeostasis in mammalian cells. In addition, TP is proposed to regulate angiogenesis [13, 19, 34]. Indeed, inhibition of TP activity was shown to correlate with cessation of angiogenic action in vivo [5, 39]. TP was also identified as platelet derived endothelial cell growth factor (PD-ECGF) [12, 41], and was originally thought to be a classic polypeptide growth factor that would bind to a cognate cell-surface receptor to elicit a cellular response [5]. Interestingly, TP is not actually an endothelial-cell growth factor; however, TP has been shown to stimulate chemotaxis of endothelial cells in vitro, thereby representing a mechanism for its involvement in angiogenesis [26].

More recently it was discovered that mutations in the TP gene are associated with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive human disease resulting from multiple deletions of skeletal muscle mitochondrial DNA [29]. TP has also received greater attention as the rate-limiting enzyme involved in the activation of the oral fluoropyrimidine anti-cancer drug capecitabine [33, 38]. Thus, cancers which express high activity of TP would be more susceptible to chemotherapy with capecitabine.

Overexpression of TP has been found in many primary and metastatic tumors, relative to the surrounding normal tissue [10, 16, 17, 24, 47], and has been shown to correlate with neovascularization, which is necessary for tumor growth and metastasis [9]. This led to the suggestion that TP may be important in pathological, but not normal, vascularization or angiogenic pathways [11]. As a result, TP has been used as a marker for differentiation [27] and prognosis of some cancers. For instance, high TP expression appears to be an important parameter indicative of a shorter disease-free survival or overall survival in colorectal [17, 36, 42], renal cell carcinoma [18], node-negative non-small cell lung cancer [21], and node-positive primary breast carcinoma [11, 37]. Conversely, the expression of TP has also been demonstrated to correlate significantly with favorable prognosis, particularly in those cancer patients treated with 5-fluorouracil (5-FU) prodrugs such as tegafur and capecitabine [32].

It should be noted, however, that several cancers have low or no TP activity at all [1, 20, 27, 28, 44, 47]. Furthermore, TP activity in some human colon carcinomas xenografts is markedly higher than that found in the same cells when grown in culture [1]. It has been proposed that the high TP activity in solid tumors or xenografts may be due to non-cancerous cells particularly stromal cells which are removed in the process of passaging tumors in culture [1, 3, 23, 25].

In spite of the importance of TP and its diverse roles, the regulatory mechanisms involved in the variability of its expression in various tissues and tumors at different stages of differentiation remain unclear. The currently characterized molecular mechanisms of genomic instability do not fully explain the range of pathological features and clinical outcomes observed in different tumors. This is further complicated by contradictory studies on TP's function in cancer prognosis. Investigation of the mechanisms regulating TP expression would clarify certain discrepancies related to TP's function and could potentially aid in the optimization of capecitabine-based therapy.

Methylation of dinucleotide cytosine–guanine motifs (CpG), especially CpG islands located in promoter regions, is one known mechanism of gene regulation in mammalian cells and a common event of reversible gene silencing in human neoplasia [22, 35]. In contrast to normal cells, hypermethylation of CpG islands is a frequently observed phenomenon in nearly every cancer type, particularly with relation to tumor suppressor, apoptosis, and DNA repair genes. Consequently, de novo gene methylation has been implicated in tumorigenesis [7, 30]. In the current study, we investigated whether methylation of the TP promoter could be involved in the regulation of TP expression, thus accounting for a mechanism responsible for TP suppression. Knowledge of the regulatory mechanisms of TP expression could clarify the basis for TP variation among different cancer cells. In addition, this knowledge may have implications for altering the prognosis of cancer patients who could potentially be treated with recently developed demethylating agents in combination with TP-activated chemotherapeutic agents.

Materials and methods

Chemicals

RPMI 1640 medium, Trypsin–EDTA and Penicillin–streptomycin were obtained from Gibco Laboratories, Life Technologies Inc. (Grand Island, NY). DMEM/F12 medium was from Mediatech (Herndon, VA). Fetal calf serum was purchased from HyClone Laboratories Inc. (Logan, UT). HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). MAX Efficiency DH5α Competent bacteria were from Invitrogen (Carlsbad, CA). Rabbit monoclonal human actin-antibodies were from Sigma Chemical Co. (St Louis, MO). Mouse monoclonal TP-antibodies were from R & D systems Inc. (Minneapolis, MN). Anti-mouse horseradish peroxidase-conjugated IgG, as well as Sp1 and Ap2 antibodies were

from Santa Cruz Biotechnology (Santa Cruz, CA). Sp3 was from Active Motif (Carlsbad, CA), and pre-immune rabbit IgG came from DAKO (Carpinteria, CA). Goat anti-rabbit horseradish peroxidase-conjugated IgG, molecular weight markers, non-fat milk as well as bovine γ-globulin and dye reagent for protein assays were from Bio-Rad Laboratories (Richmond, CA). [2-^{14}C]Thymidine (56 Ci/mol) was purchased from Moraveck Biochemical Inc. (Brea, CA). Silica Gel G/UV_{254} thin layer chromatography (TLC) polygram plates were acquired from Fisher Scientific (Pittsburgh, PA). Unless mentioned, all other chemicals were purchased from the Sigma Chemical Co. (St Louis, MO).

Tumor cell lines and culture

Five cell lines were used: DLD-1 and HT29 are colorectal carcinoma in origin, MDA-MB-231 and SK-BR-3 are breast tumor cells, and 786-O (CRL-1932) is a renal carcinoma cell line. All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD), with the exception of the 786-0 cells which were kindly provided by Dr. Jeffrey Smith, from the University of Alabama at Birmingham. Cell lines were selected based on the level of their TP activity. For example, the SKBR-3 has high TP expression, while the DLD-1 does not express TP.

All cells were maintained at 37°C in growth medium (RPMI 1640 medium containing 10% fetal calf serum), in plastic tissue culture flasks (75 cm^2), in a humidified incubator, under 5% CO_2 and 95% air. Cells were recovered from exponentially growing cultures by using a solution of trypsin (0.05 g/L) and EDTA (0.02 mg/L) in phosphate buffered salt solution (PBS) free of calcium, magnesium and carbonate.

Preparation of cell extracts

Exponentially growing cells were harvested from monolayer culture by trypsinization. The cell suspension was centrifuged at 228×*g* for 10 min, and the cell pellet was washed three times in 10 mL volumes of ice-cold 20 mM PBS (pH 8). Cells were next homogenized by sonication in three volumes of the same buffer at 4°C, using a Fisher Sonic Dismembrator 300 (Fisher Scientific, Pittsburgh, PA), and the homogenate was centrifuged at 105,000×*g* for 1 h at 4°C. The supernatant fluid (cytosol) was collected and used as the enzyme source.

Enzyme assay

TP activity was measured radioisotopically by monitoring the formation of [2-^{14}C]thymine from [2-^{14}C]thymidine as described previously [8]. The standard assay mixture contained 20 mM potassium phosphate (pH 8.0), 1 mM DTT, 1 mM EDTA (ethylenediamine tetraacetic acid) 0.25 mM [2-^{14}C]thymidine (2.24 Ci/mol) and 25 μL enzyme extract, in a final volume of 50 μL. The reaction was initiated by addition of the enzyme, incubated at 37°C for 20 min, unless otherwise noted. The reaction was terminated by boiling in a water bath for 2 min followed by freezing for at least 20 min. Precipitated proteins were pelleted by centrifugation, and 10 μL of supernatant fluids were spotted on Silica Gel G/UV_{254} TLC plates. The plates were developed in a mobile phase of chloroform, methanol, and acetic acid mixture (90:5:5, v/v/v) until the mobile phase approached the top of the plates. The amount of radioactivity in the substrate and product were detected and quantified on a percentage basis using a Berthold LB-2821 Automatic TLC Linear Analyzer. This method was cross-validated with a commercially available TP enzyme-linked immunosorbent assay (TP ELISA) which was used as directed by the manufacturer (Roche Diagnostics, Nutley, NJ).

Protein determination

Protein concentrations were determined spectrophotometrically by the Bradford method [4] using bovine γ-globulin as a standard.

TP western blotting

The expression levels of TP in total cell lysates were determined by western blotting. Cultured cells were harvested and dissolved in lysis buffer containing 0.1% Nonidet P-40 and 1:100 protease inhibitors cocktail (Sigma Aldrich, St Louis, MO). Aliquots containing equal amounts of total protein along with molecular weight markers were loaded onto 10% SDS–PAGE (sodium dodecyl-sulfate–polyacrylamide gels), electrophoresed, and transferred onto nitrocellulose membranes. After blocking non-specific binding with 5% non-fat milk, in Tris-buffered saline at 4°C overnight, the blotted membranes were incubated with anti-TP (1:2,000 dilution) mouse monoclonal antibody for 1 h at room temperature, followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (1:10,000 dilution). The membranes were incubated with enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences, Piscataway, NJ) and exposed to X-ray films (Fujifilm Medical Systems, Stamford, CT). For control, the membranes were later stripped from the anti-TP antibodies and reprobed with rabbit anti-human actin (1:15,000 dilution) followed by incubation with goat anti-rabbit horseradish peroxidase conjugated secondary antibody (1:3,000 dilution) to detect

actin levels in the loaded samples. The membranes were then incubated with enhanced chemiluminescence western blotting detection reagents and exposed to X-ray films.

DNA extraction and bisulfite modification

Genomic DNA was extracted using guanidinium thiocyanate. Briefly, 1 mL of guanidinium thiocyanate was added directly to the culture, and the cells were scraped off the flask. About 500 μL of ice-cold 7.5 M ammonium acetate were added and allowed to stand for 10 min on ice. The solution was then extracted first with phenol and then with phenol–chloroform (1:1) and finally, with chloroform–isoamyl alcohol (24:1). The upper aqueous phase was recovered, and 0.5 volume of isopropanol was added and the tube gently inverted several times until the DNA precipitated. The visible DNA was collected using a glass rod, allowed to stand in air until the alcohol evaporated and resolubilized in 10 mM Tris–Cl/1 mM EDTA overnight at 4°C.

For the bisulfite modification, genomic DNA was denatured by the treatment with 50 μL of 0.2 M NaOH for 10 min at 37°C. About 30 μL of 10 mM hydroquinone and 520 μL of 3 M sodium bisulfite at pH 5, both freshly prepared, were added and mixed, and samples were incubated under 200 μL of mineral oil, at 50°C for 16 h. After the removal of the mineral oil, the modified DNA was purified using the Promega wizard DNA purification (Promega, Madison, WI) according to the manufacturer's protocol. Subsequently, it was eluted into 50 μL of DNAse/RNAse free water and treated with 0.3 M NaOH for 5 min, at room temperature, followed by ethanol precipitation. DNA was resuspended in water and was used either immediately or stored at –20°C. Genomic DNA isolated from DLD-1 and SKBR-3 cell lines was incubated with the isoschizomers MspI and HpaII (New England Bio-Labs, Beverly, MA) for 4 h at 37°C to determine whether DNA from these cell lines was methylated.

Delineation of the critical promoter region of the TP gene: transient transfection

To delineate the limit of the TP minimal promoter region, the full length TP promoter and a series of 5′-unidirectional deletions terminated at +76 with respect to the transcription start site, were generated by PCR (template was derived from PD-ECGF clone PL8 [15] generously provided by Dr. C.-H. Heldin). These constructs were subcloned into the linker region of the basic promoterless pGL3 vector (Promega) upstream of the firefly luciferase gene and the internal control vector (pRL-CMV, 0.5 μg/well). The use of dual reporters enables the normalization of the experimental gene transcription with respect to the control reporter transcription. Plasmids were purified using the Maxi-prep (Plasmid Maxi Kit; QIAGEN) method, and sequenced to exclude errors in the sequence. These constructs were transiently transfected into HeLa cells. HeLa cells were plated in DMEM/F12 media at a density of 1×10^5/well in a 24-well culture plate. After 24 h, transient transfection was performed using 3 μL of LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Cells were co-transfected with either the pGL3 Luciferase reporter vector containing a full-length TP promoter cDNA (pGL3-TP, 1 μg/well), a deletion construct (pGL3-TPn, 1 μg/well), or the internal control vector containing the *Renilla* luciferase gene (pRL-CMV, 0.5 μg/well) as a normalizer according to the manufacturer's recommendations (Promega, Madison, WI). The following day, cells were lysed using 100 μL of passive lysis buffer, and luciferase activity was measured and normalized to the internal control (pRL, *Renilla* luciferase), according to the Dual-Luciferase Assay Protocol (Promega, Madison, WI). Luciferase activity was measured on a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA).

Genomic sequencing

Sodium bisulfite-modified DNA (100 ng) was amplified with TP gene specific primers 5′-TAGGAGAGTGGGG AGGGTTT-3′ (sense) and 5′-AAACCCCAATTCCCAA AATC-3′ (antisense). The PCR reaction contained 1× PCR buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer, 0.2 mM dNTPs and 0.4 U of Taq polymerase (Roche Diagnostics, Indianapolis, IN). PCR conditions were as follows: 95°C for 4 min to denature the DNA, then 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min (annealing and amplification); and finally, 72°C for 7 min.

To obtain the PCR product, a second round of PCR with nested primers was performed. PCR conditions were the same as above with 10 μL of the first PCR product for 32 cycles. The PCR product was run on a 1% agarose gel and the band was cut and purified with a gel extraction kit (Qiagen, Valencia, CA). The amplified product was cloned in pCR II-TOPO TA cloned vector (Invitrogen) and transformed in MAX Efficiency DH5α Competent bacterial strain. The plasmid was purified with the use of a Qiagen miniprep kit and sequenced with the use of T7 and Sp6 sequence primers (Midland, Lubbock, TX) which lie outside the inserted DNA fragment.

RNA extraction and real-time quantitative PCR (RT-Q-PCR)

Total RNA was isolated using the Qiagen RNA Purification kit according to the manufacturer's instructions (Qiagen,

Valencia, CA). All sample concentrations were determined spectrophotometrically at A_{260} and diluted to a final concentration of 20 ng/μL in RNAse-free water containing 12.5 ng/μL of total yeast RNA (Ambion, Austin, TX) as a carrier. Quantification of TP mRNA was performed by RT-Q-PCR using an ABI PRISM 7700 Sequence Detector, designed to monitor multiple fluorescent signals in a 96 well format. The relative amount of RNA in the samples was calculated by linear extrapolation from a standard curve generated by the use of 0.1, 0.5, 1.0, 5.0 and 10 ng of RNA. A normalized relative expression value was then calculated by division of the target by a validated internal control (S9 ribosomal Housekeeping gene) using the relative standard curve method as previously described [2]. Briefly, known amounts of standard RNA are reverse transcribed in parallel, along with test sample, in a volume of 20 μL of a solution composed of 50 mM Tris–Cl buffer containing 37.5 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, 40 U RNAase and 200 U of Superscript RT II (Invitrogen, Carlsbad, CA). For quantitative PCR, 1/40th of the cDNA obtained from the reverse transcription was used. The PCR reaction was run in a 20 μL containing 2 L of 10× Sybr Green Master Mix 0.16 μL Taq Start antibody, 1 μL uracil DNA glycosylase, 0.25 μL forward 5′-TCCTGCGGACGGAATCC-3′ and reverse primer 5′-TGAGAATGGAGGCTGTGATGAG-3′.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts (Promega, Madison, WI) were stored at –80°C. Gel Shift assays were performed according to the manufacturer; however, nuclear extract, EDTA, glycerol, $MgCl_2$ and poly(dI–dC) optimal concentrations (described below) were obtained by varying the concentrations of each of these components individually, while maintaining the remainder component concentrations fixed during the assay runs. The 233-bp fragment of the TP promoter from the 5′-flanking region, corresponding to the promoter region including nucleotides –1,240 to –1,470, was amplified by PCR (30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s). Following amplification, the PCR product was gel-purified with the Qiaquick gel extraction kit (Qiagen, Valencia, CA), and end labeled with [γ-^{32}P] (Perkin Elmer Life, Wellesley, MA) and Ready-To-Go T4 Polynucleotide kinase (Amersham Biosciences, Piscataway, NJ), according to the manufacturers' instructions. Next, the probe was passed through a G50 Spin column (Roche Applied Science, Indianapolis, IN) to remove the unincorporated label. The individual 30-bp double-stranded DNA oligonucleotides, representing fragments of the TP promoter sequence, are shown in Table 1. These double-stranded DNA oligonucleotides were synthesized by Midland (Lubbock, TX) and gel purified in our laboratory. Oligonucleotides were used in excess and were included in the reactions as specific competitors for transcription factor-binding to the labeled probe (0.033 pmol, 20,000 cpm of ^{32}P-labelled oligonucleotide). The radiolabeled probe was incubated with or without nuclear extract (2.0 μg of protein) and the respective oligonucleotides in a final volume of 15 μL EMSA buffer (10 mM Tris, pH 7.5, 5% glycerol, 0.5 mM EDTA, pH 7.1, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.1 mg/mL poly[dI–dC]), at room temperature for 20 min. For oligonucleotide competition analysis, a 10-, 100-, or 300-fold molar excess of cold competitor oligonucleotide was added to the mixture, incubated at 30°C for 15 min, before adding the probe. The reaction mixtures were electrophoresed on a 3.8% polyacrylamide non-denaturing gel in 0.5× Tris–borate–EDTA (45 mM Tris–borate and 0.5 mM EDTA) at 4°C and 150 V, followed by visualization by autoradiography (Fuji medical X-ray film, Stamford, CT) with intensifying screens at –80°C. To determine the effect of methylation on nuclear protein binding to the TP promoter, methylation of the TP probe was accomplished enzymatically with SssI CpG methylase (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions, except that a 640 μM *S*-adenosylmethionine and 4 U of SssI methylase enzyme were used to account for the abundant GC sequences found in the TP promoter. For super shift analysis, Sp1, Sp3, Ap2 antibodies or pre-immune rabbit IgG were used.

Table 1 DNA duplex oligonucleotide sequences derived from the human thymidine phosphorylase promoter and used as competitors for electrophoretic mobility shift assays

Duplex oligonucleotide	Sequence
1(AP2) –169 to –142	5′-AGGGATCACCCCCCGCCGACGGCCCCGGG-3′
2(AP2/SP1) –78 to –49	5′-CAAGGCTTCCCGGGGGCGGCGACTGCCGAG-3′
3(SP1(–)) –55 to –33	5′-TGCCGAGCTCCGCCCTCCAGGCG-3′
4(SP1 (–)) –11 to +12	5′-GGGCGCCGCCGCCCCGCCGCCG-3′

Only the sense strand is shown, the (–) sign indicates the location of the consensus binding in the antisense strand

Results

Enzymatic activity of TP and correlation with its protein and mRNA levels

The results in Table 2 show that SKBR-3 breast cancer cell line has the highest enzyme activity while the activity was undetectable in the DLD-1 colorectal cancer line. TP activities in the remaining cell lines were intermediate between that of SK-BR-3 and DLD-1 cells (Table 2).

Figure 1a shows the amounts of TP mRNA in the different cell lines as quantified by RT-Q-PCR. Figure 1b

Table 2 Percent conversion of thymidine to thymine by thymidine phosphorylase and the specific activity (pmol/min/mg protein) of the enzyme from five various cancer cell lines after different incubation periods

Cell line	% Conversion at different incubation periods			Specific activity
	20 min	40 min	60 min	
SKBR	12.3 ± 0.63	18.4 ± 1.85	26.7 ± 3.3	126.8 ± 2.1
786-0	1.00 ± 0.03	1.99 ± 0.31	3.12 ± 0.36	28.1 ± 0.69
MDA-231	0.60 ± 0.52	0.65 ± 0.21	0.93 ± 0.15	1.67 ± 0.66
HT-29	0	0.51 ± 0.09	0.56 ± 0.21	1.60 ± 0.37
DLD-1	0	0	0	0

Values are the mean ± SD from at least three replica

shows a representative western blot analysis, demonstrating a concordance with the TP mRNA results. Among the five cultured cell lines tested, TP expression was highest in SKBR-3 cells, followed by 786-0, MDA-MB-231 and HT-29 cells. Expression of TP in HT-29 cells was not detected by Western Blot analysis, while in DLD-1 cells, TP expression was undetectable by either method (Fig. 1a, b).

TP activity correlated with TP protein levels determined by ELISA with a high correlation coefficient (r^2) of 0.98 as estimated by linear regression analyses (data not shown), while the correlation of TP activity with mRNA levels had a correlation coefficient r^2 of 0.80 (data not shown).

Methylation of the TP gene and relationship to TP enzyme expression

Since it is known that cytosine methylation modulates the activity of certain genes, methylation of the TP gene was examined by the treatment with the isoschizomers MspI and HpaII in SKBR-3 and DLD-1. Both isoschizomers recognize the same CCGG sequence. However, MspI enzyme can cleave both the methylated and non-methylated sequence. In contrast, the HpaII isoschizomer can only cleave the non-methylated sequence. Figure 1d shows that, after cleavage with the HpaII isoschizomer, the genomic DNA isolated from the DLD-1 cell line generated higher molecular weight products as compared to the SKBR-3 cell line. These results demonstrate that the TP gene in DLD-1 cells, which have no detectable TP activity, is methylated as compared with the TP gene in SKBR-3 cells, which have high TP activity. Since it was highly probable that this methylation occurred in the promoter region of the TP gene, the methylation of the TP promoter was examined.

Delineation of the critical promoter region of the TP gene

To delineate the limit of the TP minimal promoter region, a series of 5′-unidirectional deletion constructs were subcloned into a reporter construct containing a Luciferase reporter gene. These deletion constructs were transiently transfected into HeLa cells, and the amount of luciferase activity was correlated with promoter activity. Figure 2 shows that the longest deletion construct (TP-1326) demonstrated a 113-fold increase in promoter activity compared to the promoterless control vector (TP-0). As also illustrated in Fig. 2, the robust promoter activity remained relatively constant from –1,326 until –191, after which it decreased to 50% (TP-94). These data suggest that full TP promoter activity is contained within the first 191 bp upstream of the transcription start site (Fig. 2).

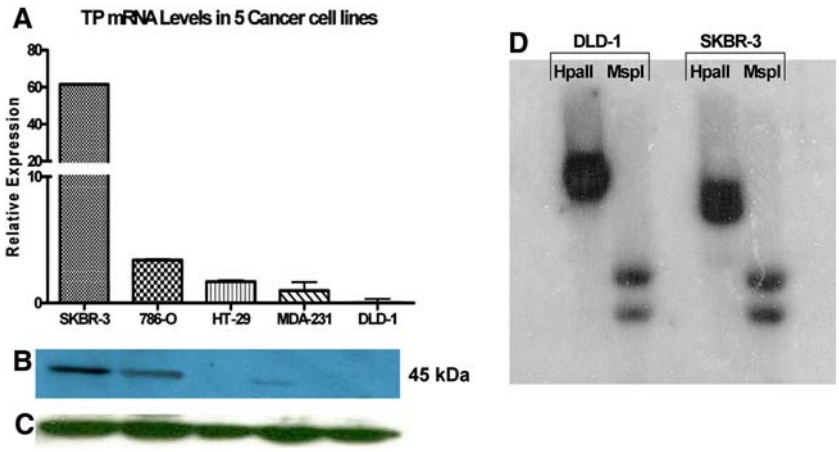

Fig. 1 **a** TP mRNA levels in colon carcinoma cells (DLD-1, HT29), breast carcinoma (MDA-231, SKBR-3) and renal cell carcinoma (786-0). TP mRNA was quantified by real-time quantitative PCR and expressed as a ratio of TP to S9 ribosomal housekeeping gene (internal standard). **b** TP protein levels in the different cell lines and **c** actin protein levels in the loaded samples as determined by Western Blot analysis. **d** Methylation status in DLD-1 and SKBR-3 cell lines using Southern Blot and restriction enzyme analyses

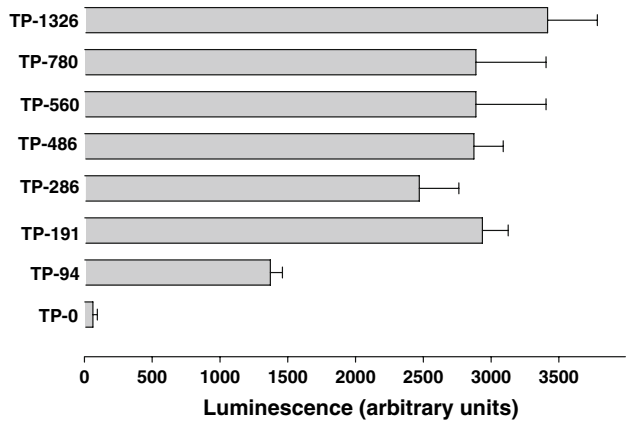

Fig. 2 TP promoter analysis of constitutive expression in HeLa cells. The activity of various 5′-deletions of the TP promoter cloned into a Luciferase reporter vector was analyzed in transiently transfected HeLa cells. Plasmids containing various lengths of the 5′-upstream sequences of the human TP gene were cloned into a luciferase reporter construct and transfected into HeLa cells, as described in "Materials and methods". Luciferase activity was measured and activity normalized to an internal standard (*Renilla luciferase*). Transfections were performed in triplicate, and data are the means and standard deviations from at least three experiments

Pattern and location of individual cytosine methylation sites in the TP 5′-region

Sodium bisulfite genomic sequencing was used to assess the cytosine methylation status of the TP 5′-region containing the highest promoter activity as determined by the Luciferase reporter assays. Figure 3 illustrates that SKBR-3, which express the highest TP levels relative to the other cell lines studied in the present investigation (Table 2), lacked any methylation sites in the region examined. On the other hand, MDA-231 and HT-29 cell lines, both of which express substantially lower levels of TP activity (Table 2), mRNA (Fig. 1a) and protein (Fig. 1b) compared to SKBR-3, showed some degree of methylation, specifically in the intron 1 region of the TP gene (Fig. 3). In sharp contrast, DLD-1 cells, which have no TP activity (Table 2), exhibited dense methylation in nearly every site examined throughout the entire promoter region, as well as the exon 1 and intron 1 regions of the TP gene (Fig. 3).

Effect of a demethylating agent on TP mRNA levels

To confirm that methylation is a regulatory mechanism of TP transcription, we examined whether or not TP expression in DLD-1 cells could be restored by the demethylating agent, 5-aza-2′-deoxycytidine (5-aza-2dC). 5-Aza-2dC is an irreversible inhibitor of DNA methyltransferase that is known to block de novo methylation in cells. Figure 4 shows that treatment of DLD-1 with 5-aza-2dC for 5 days increased TP mRNA levels in a concentration-dependent manner (TP mRNA increased in parallel with increases in 5-aza-2dC concentrations). In contrast, there was no significant difference in TP expression in SKBR-3 cells when these were exposed to the same treatment (Fig. 4). The decrease of TP mRNA seen at higher concentrations of 5-aza-2dC in SKBR-3 cells (Fig. 4) is most likely due to the adverse effects of high concentrations of this compound on cellular viability. The cytotoxic effect of higher concentrations of 5-aza-2dC may have also taken place in DLD-1 cells; however, it is possible that this effect was obscured by the robust TP induction in these cells.

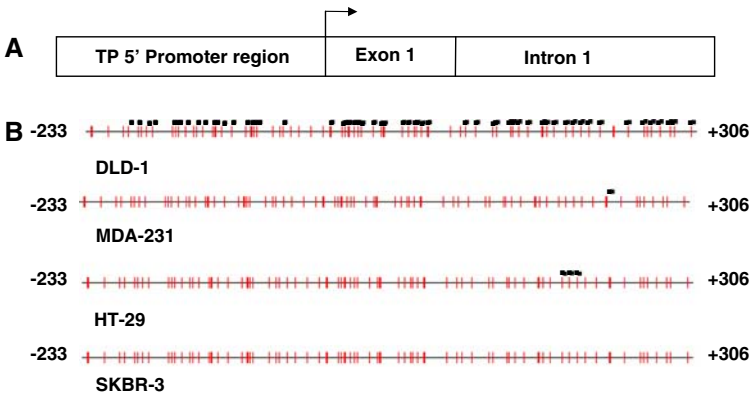

Fig. 3 Sodium bisulfite genomic sequencing of the TP promoter. **a** A schematic representation of the 539 bp region of the TP gene (including the promoter, exon 1 and intron 1 regions) analyzed for methylation, is shown. The *bent arrow* indicates the transcriptional start site. **b** CpG island methylation analysis of selected cancer cells. Bisulfite modification was performed as described in the "Materials and methods". According to this method, genomic DNA treated with bisulfite converts cytosine but not 5-methylcytosine residues into uracil through deamination. Following bisulfite modification of the cells, PCR amplification was performed, and the PCR product was cloned and sequenced as described in "Materials and methods". Each *dot* represents a 5-methyl-cytosine residue in the primary DNA sequence

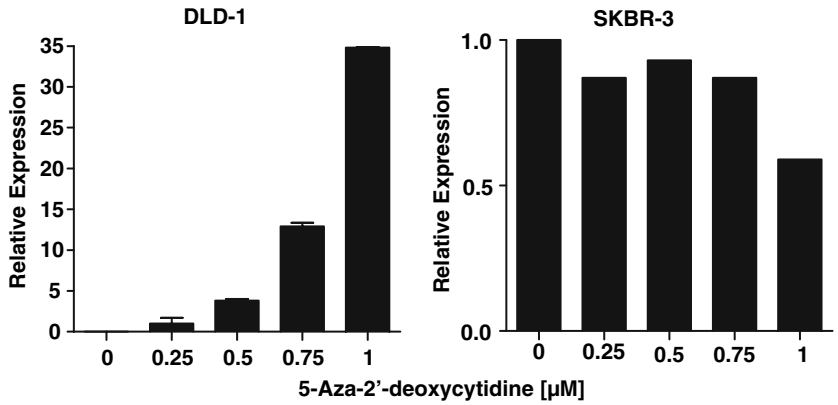

Fig. 4 Effect of demethylation with 5-aza-2dC for 5 consecutive days on the induction of TP mRNA transcription in DLD-1 and SKBR-3 cell lines. TP mRNA expression was quantified by real-time PCR as described in the "Materials and methods". Values of TP mRNA expression were normalized using S9 house keeping gene transcript. Relative expression in DLD-1 cells was calculated as relative to the low activity induced at 0.25 μM 5-aza-2dC. Relative expression in SKBR-3 cells, was calculated as relative to the activity in the absence of 5-aza-2dC. *Error bars* represent the standard deviations from the mean of at least three replica

The effects of the histone deacetylase inhibitor Trichostatin-A (TSA) on TP mRNA and activity in DLD-1 cells were also examined. These studies demonstrated a modest reactivation of the TP gene in DLD-1 cells after 48 h treatment with various concentrations of TSA (data not shown). This reactivation with TSA alone was less than that observed with 5-aza-2dC alone (Fig. 4). However, adding TSA with 5-aza-2dC enhanced the TP expression reactivation induced by 5-aza-2dC alone (data not shown).

To confirm that the increase in expression of TP in response to 5-aza-2dC and/or TSA results in the translation of a functional protein, we examined the effect of this treatment on the enzymatic activity of TP in cellular extract of DLD-1. As shown in Fig. 5, TP activity can be increased with the addition of 5-aza-2dC. This increase was enhanced further by combining 5-TSA with aza-2dC. Treatment with 5-aza-2dC for 3 days followed by the combination of 5-aza-2dC and TSA (150 nM) for an additional 48 h, resulted in a significant reactivation of TP transcription in DLD-1 cells. However, at higher concentrations of 5-aza-2dC alone or in combination with TSA, there was a decrease in TP enzymatic activity (Fig. 5). This was most likely a result of the adverse effects of the high concentrations of these compounds on cellular viability. In SKBR-3 cells, there was no increase in TP activity by increasing the concentrations of either 5-aza-2dC alone or in combination with TSA (data not shown).

Electrophoretic mobility shift assays (EMSA)

In order to examine how protein–DNA interactions were affected by methylation, we performed EMSA. Figure 6a, lane 2 shows that one strong DNA–protein complex, as well as two additional weak complexes (one of which migrated faster than the large, strong complex) were detected following the incubation of the un-methylated TP probe with HeLa cells nuclear protein extract, suggesting protein binding to the TP promoter probe. Figure 6a also shows that the formation of such DNA–protein complexes (lane 2) can only be observed with the un-methylated TP probe (lane 2), whereas the methylated probe prevented nuclear protein binding (lane 4).

A candidate Sp1 regulatory site for the TP promoter has been previously identified [46]. Preliminary studies in our laboratory also suggested that the Sp family of transcription factors bind to the TP promoter region. To determine which putative Sp site was involved in the formation of

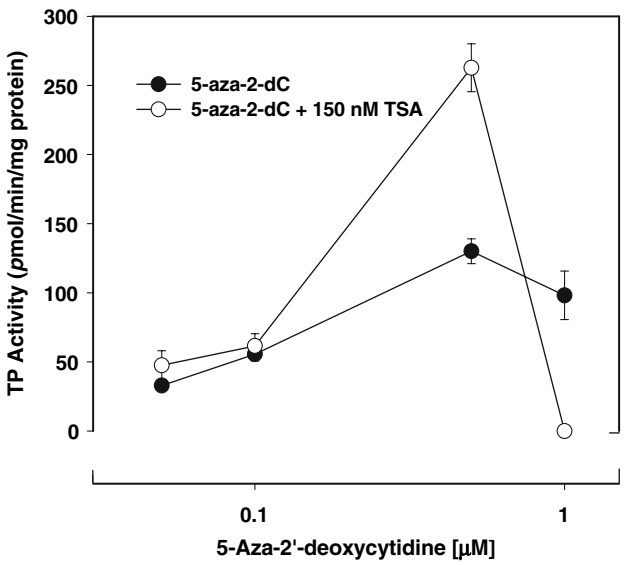

Fig. 5 TP activity in DLD-1 cells after treatment with 5-aza-2dC alone or in combination with Trichostatin-A (TSA). Values represent the mean ± SE from three experiments

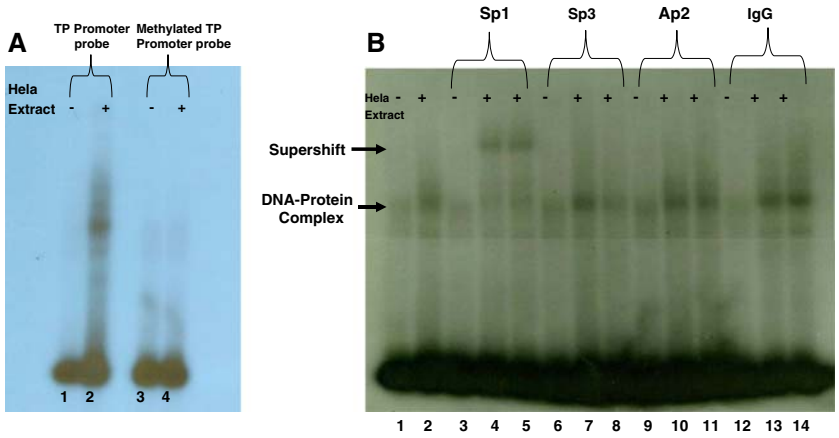

Fig. 6 Determination of the effect of methylation on nuclear protein binding to the TP promoter by electrophoretic mobility shift assays (EMSA). **a** Both the radiolabeled, methylated (using SSSI CpG methylase) and non-methylated TP promoter probes were incubated with (+) or without (–) HeLa nuclear extract (2.0 μg of protein) and electrophoresed on a 3.8% polyacrylamide non-denaturing gel as described in "Materials and methods". **b** Determination of the binding activities of Sp1, Sp3, Ap2 polyclonal rabbit anti-human antibodies or pre-immune rabbit IgG to the TP promoter by Supershift Analysis using 2 g (*lanes 4*, *7*, *10* and *13*) and 4 g (*lanes 5*, *8*, *11* and *14*) of each antibody. Pre-immune rabbit IgG was used as a control to determine the specificity of the super shift assays. The *arrow* (*lanes 4* and *5*) shows the shift in the position of the DNA–protein complex of Sp1 to the TP promoter

these complexes, 10-, 100-, and 300-fold excess of unlabeled oligonucleotides corresponding to various sequences shown in Table 1, including sequences containing Sp1 and Ap2 sites, were used to compete with the complexes. Results indicated that the sequence corresponding to the Sp1 binding site in particular competes the complexes away, possibly by sequestering the available transcription factors present in the nuclear extract (data not shown).

To determine whether Sp1, along with Sp3 and/or Ap2, were involved in the formation of the protein–DNA complexes detected by EMSA, and to investigate the presence of other potential binding sites on the TP promoter, studies were conducted using polyclonal rabbit anti-human Sp1, Sp3 and Ap2 antibodies. The loss or shift of the complexes was monitored. Figure 6b illustrates that the intensity of the bands corresponding to the protein–DNA complexes decreased in the presence of 2 and 4 μg of Sp1 antibody (lanes 4 and 5, respectively), and there was an appearance of a supershift band with Sp1 antibody incubation, suggesting the binding of Sp1 to the TP promoter. Inclusion of Sp3 antibody at 4 μg (lane 8) but not at 2 μg (lane 7), decreased protein DNA complex formation but to a lesser extent than that observed with Sp1 (lanes 4 and 5). Conversely, incubation with 2 and 4 μg of Ap2 antibody (lanes 10 and 11, respectively), neither caused a loss nor a shift in the migration of the protein DNA complex(es), similar to the controls with 2 and 4 μg of pre-immune rabbit IgG (Fig. 6b, lanes 12 and 13, respectively). These data indicate that the transcription factor Sp1 can bind and that Sp3 may also be involved. Additionally, because neither a migration shift nor a loss of protein–DNA complexes was seen with Ap2 or rabbit IgG incubation, the specificity of the super shift assays for particular transcription factors can be emphasized.

Discussion

The molecular mechanisms by which deregulated gene expression processes occur during cancer progression remain either poorly characterized or relatively unknown [31]. Epigenetic silencing of certain genes, especially tumor suppressor, DNA repair, and anti-metastatic genes, is a common event during carcinogenesis, often involving aberrant DNA methylation and/or histone modifications of gene regulatory regions [45].

In this study, we provide evidence that the expression of TP is regulated at the level of transcription by mechanisms involving epigenetic modifications such as methylation. TP inactivation is associated with hypermethylation of CpG dinucleotides located in the TP promoter, as illustrated with DLD-1 cells (Fig. 3); whereas, high TP expression (Fig. 1) could be associated with complete demethylation, as demonstrated in SKBR-3 cells (Fig. 3b).

We have also shown that TP expression can be reactivated in non-TP-expressing DLD-1 colon cancer cells following demethylation with 5-aza-2dC and to a lesser extent, deacetylation by TSA. The further enhancement of TP re-activation by the combination of 5-aza-2dC and TSA over that achieved by each compound alone is in agreement with other studies on the re-expression of silenced genes [6, 43]. The fact that TSA addition to 5-aza-2dC improved the re-expression of TP genes that had undergone methylation, indicates that histone deacetylation inhibition, in

conjunction with inhibition of methyltransferase activity, constitutes a potent combination in gene reactivation, and further emphasizes that more than one mechanism is often involved in epigenetic TP gene silencing. Nevertheless, the better reactivation of TP expression by 5-aza-2dC, as compared to TSA, suggests that methylation activity is the predominant mechanism in epigenetic TP gene silencing rather than histone deacetylation.

The stronger correlation between protein levels and enzyme activity than that between enzyme activity and mRNA levels also suggest post-transcriptional or post-translational modifications in TP. In this study, the level of TP expression was associated with the degree of methylation of the promoter. DLD-1 cells, which do not express TP, contained the largest number of methylated sites (Fig. 3). DLD-1 also experienced dramatic drug-induced re-expression of TP when treated with a demethylating agent alone (Fig. 4) or in combination with a histone deacetylase inhibitor (Fig. 5). In contrast, the same treatments did not have a significant effect on TP expression in SKBR-3 cells, which do not contain methylated CpG sites (Figs. 3, 4b). MDA-231 and HT 29 cells, both of which express substantially lower levels of TP activity compared to SKBR-3 cells, showed some methylation in the intron 1 region of the TP gene (Fig. 3). Intron regions containing transcriptional enhancer elements have been described in other genes [40].

Overall, the present results suggest an association between DNA methylation and chromatin structure in the loss of TP expression in cancer cells. Our data also indicate that transcriptional deregulation of TP in cancer cells is not uniform across different tumor cell lines. This suggests that other epigenetic mechanisms may be involved in regulating TP expression (e.g., transcription factors, enhancers, etc.). The present results also suggest that the relationships between TP mRNA expression and TP methylation patterns may be reflective of tumor histology and tumor anatomical location, as well as the distinct mechanisms of genetic instability associated with cancer clinical outcome. These findings could represent the basis for utilizing the characterized TP promoter methylation patterns as a tool to assess tumor progression and prognosis, as well as clinical outcome and response to chemotherapy with fluoropyrimidines prodrugs (e.g., capecitabine). In this regard, there also exists the possibility of pharmacologically modulating TP to achieve potential therapeutic results [31], particularly in relation to cancer drugs that are activated by TP (e.g., capecitabine). This drug could be administered in conjunction with methyltransferase inhibitors and/or histone deacetylase inhibitors. Indeed, the response rate to standard care of acute myeloid leukemia patients was increased to 65% by the administration of mehtyltransferase and histone deacetylase inhibitors [14].

Our studies suggest that the Sp1, and possibly Sp3 (but not Ap2), binding sites on the TP promoter are potentially important for transcription, since protein binding at these sites was demonstrated by the formation of protein–DNA complexes. The formation of these protein–DNA complexes was significantly hindered by methylation (Fig. 6a). The ability of Sp1 antibody to resolve complex formation (Fig. 6b) and to cause a supershift in the Sp1 specific band, strongly indicates a role of Sp1-mediated TP transcription and is in agreement with the findings of Zhu et al. [46]. On the other hand, our results do not preclude the possibility that Sp3 may also be involved in regulating TP expression. This is contrary to the findings by Zhu et al. [46] who suggest that Sp3 is not involved in TP transcription. Footprint analyses will be necessary to further confirm or reject this premise.

In summary, the studies presented here indicate that the repression of TP expression in cancer cells is due to some extent to CpG island methylation, and to a lesser degree to histone deacetylase activity, in the promoter region of the TP gene. The present findings also suggest that the Sp1, and possibly Sp3, binding sites may also be involved in the expression of TP. Future studies are required to identify the loci along the TP promoter which are crucial in the transcriptional process. These results may have applications in the treatment of cancer, whereby response rates and drug efficacy could be improved via alteration of critical genes which have undergone epigenetic silencing.

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