Chronic Exposure of Colorectal Cancer Cells in Culture to Fluoropyrimidine Analogs Induces Thymidylate Synthase and Suppresses p53. A Molecular Explanation for the Mechanism of 5-FU Resistance

POCHI R. SUBBARAYAN[1], MALANCHA SARKAR[2], GRODONOFF NELSON[2], EDILBERTO BENITEZ[2], SHEBANI SINGHAL[3] and BACH ARDALAN[1]

[1]*Department of Medicine, University of Miami Miller School of Medicine, and*
[2]*Department of Biology, University of Miami, Miami, FL – 33136, U.S.A.;*
[3]*Department of Biology, Florida International University, Miami, FL - 33146, U.S.A.*

Abstract. *Resistance to chemotherapy is a major issue in treating malignant diseases. 5-Fluorouracil (5-FU) is the drug of choice in managing colorectal cancer (CRC) patients. However, 5-FU resistance leads to eventual treatment failure. Therefore, delaying or reversing the onset of 5-FU resistance will benefit these terminally ill patient populations. A metabolite of 5-FU irreversibly binds thymidylate synthase (TS) thus inhibiting its activity. Many studies demonstrated that these resistant patients had an increased intratumoral TS level. We used TS-siRNA to reduce TS and resensitize HT29FU CRC cells back to this uracil analogue. We exposed the CRC cell line HT29 to an increasing concentration of 5-FU or 5-fluorouridine (FUR) and established a derivative cell line (HT29FU and HT29FUR). Using real-time polymerase chain reaction (PCR) and Western immunodetection assays, we analyzed the expression of TS and p53 mRNA and protein in control and experimental groups. Cytotoxicity to 5-FU was determined by reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT assay) or trypan blue dye exclusion assay. The HT29FU and HT29FUR cells have a distinct morphology: they are generally asteroid shaped. The half maximal inhibitory concentration (IC_{50}) values for the resistant cell line for 5-FU is over 148 μM compared to 5 μM for the sensitive parental cell line. The resistant cell lines expressed more of TS and less of p53. TS-siRNA suppressed TS only. Other pathways were not significantly altered. It also marginally (20%) re-sensitized resistant cells to 5-FU. Restoration of partial sensitivity to 5-FU by TS-siRNA reiterates the primacy of the DNA synthesis pathway in 5-FU mode of action. We speculate that the short half-life of the transiently transfected siRNA may contribute to the marginal restoration of sensitivity. By integrating TS-siRNA expression vector into the genome and regulating its expression, we may be able to reverse 5-FU resistance and make the cells as sensitive as the parental cell line.*

Correspondence to: P.R. Subbarayan, Department of Medicine (D8-4), University of Miami Miller School of Medicine, 1550 NW 10th Avenue, FOX 431A, Miami, FL-33136, U.S.A. Fax: +1 3052437722, e-mail: spochi@med.miami.edu

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Development of resistance to chemotherapy is a stark reality in treating many neoplastic diseases. A variety of mechanisms such as mutation, gene amplification, induction or suppression of gene expression *etc*. are responsible for this phenomenon (1). Irrespective of the source of resistance there are two approaches to this problem: to stop, or at least delay the onset of chemoresistance; and to develop new drugs or methods to treat refractive tumors. Ideally the first method is the best method. However, in spite of our understanding of chemotherapy resistance, there are no available options to delay or halt its onset. Therefore, we are challenged to develop alternate methods to treat refractory tumors.

For nearly five decades, 5-fluorouracil (5-FU) has been the drug of choice in treating colorectal cancer (CRC) and many other solid tumors (2). 5-FU has a success rate of about 10-30% (2). In current clinical practices, it is used in combination therapy with other added antitumor agents such as CPT-11 and oxaliplatin. As a single agent, these drugs have limited clinical efficacy. Whereas when combined with 5-FU these agents show synergism (3). In addition resistance to 5-FU also renders several combination chemotherapies ineffective. Therefore, it is commonly believed that 5-FU resistance is a major cause of treatment failure.

Table I. *List of the primers used for semi-quantitative RT-PCR.*

Primer	Primer length	Sequence 5'-3'	Product length
*TS*5' Primer A	24 mer	GGGCAGATCCAACACATCCTCCGC	294 bp
TS Rev. Primer	20 mer	GCCCAAGTCCCCTTCTTCTC	
p53 (Sense)	23 mer	CATTCTGGGACAGCCAAGTCTGT	609 bp
p53 (Antisense)	23 mer	CTGGGGAGAGGAGCTGGTGTTGT	
β-Actin F	25 mer	TCACCCACACTGTGCCCATCTACGA	295 bp
β-Actin R	25 mer	CAGCGGAACCGCTCATTGCCAATGG	

These primers span the exon-intron junction so that genomic DNA is not amplified under the given conditions.

Thymidylate synthase (TS) is the pivotal enzyme that methylates deoxyuridylate (dUMP) to deoxythymidylate (dTMP) (4). Interference with TS activity, prevents the formation of dTMP, inhibits DNA synthesis and thereby cell division. Uracil analog 5-FU is converted to FdUMP. It tightly binds TS and blocks the formation of dTMP. Compared to normal tissues, TS levels have been noted to be elevated in tumors. An increase in cellular TS overcomes FdUMP-mediated blockage. This phenomenon is correlated to 5-FU resistance and eventual treatment failure (5-7).

Apart from its role in DNA synthesis, TS autoregulates its own translation by a negative feedback loop (8). It also negatively regulates the translation of *c-Myc* and *p53* genes at the translational level (9). Consequently, overexpression of TS shuts off the synthesis of p53 protein, a sentinel in cell cycle transition. Upon DNA damage, p53 stops the cells from proliferation, activates DNA repair machinery and directs cells beyond repair to apoptosis (10). Therefore, we hypothesize that down-regulation of *p53* and an increased TS level allows the incorporation of fluorinated analogs into nascent DNA. Thus, p53 deficiency results in unchecked proliferation of replication-defective cells. Since the expression of TS and p53 are inversely correlated (8), there is an opportunity for them to be regulated and the resistant cells to be re-sensitized to 5-FU. To test this hypothesis, we selected 5-FU-resistant colorectal cancer cell line HT29 (referred to as HT29FU and HT29FUR) that overexpressed TS and suppressed p53 levels. Using siRNA methodology, we down-regulated the *TS* mRNA level and monitored 5-FU toxicity using MTT assay. In this communication, we report the results of our study.

Materials and Methods

Cell lines and culture conditions. The human colorectal carcinoma cell line HT29 was purchased from American Type Culture Collection (ATCC, USA). It was cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and maintained at 37˚C in a 5% CO_2 humid atmosphere. All experiments were conducted with exponentially growing cells 24 hours after seeding 2×10^5 cells in 6-well plates.

Development of 5-FU-resistant cells. To mimic *in vivo* development of 5-FU-resistant colorectal tumors and to characterize such refractive cells, a 5-FU-resistant HT29 cell line was developed by continuous exposure of sensitive cells to step-wise increasing concentrations of 5-FU or fluorouridine (FUR). We selected the surviving cells and characterized them as described below. The 5-FU and FUR-resistant derivatives were designated HT29FU and HT29FUR, respectively, to distinguish their origin.

Cytotoxicity assay. The half maximal inhibitory concentration (IC_{50}) values for HT29FU and HT29FUR to 5-FU were determined by trypan blue dye exclusion assay. Briefly, 5×10^4 HT29FU, and HT29FUR cells were plated in 24-well plates. Twenty-four hours later an increasing concentration of 5-FU (0-300 μM) was added. At 72 hours post 5-FU addition, cells were trypsinized, stained with trypan blue and counted using hemocytometer. The experiments were carried out in duplicate and repeated three times.

Cytotoxicity assays following siRNA transfection. The effect of siRNA on the cytotoxicity of 5-FU was determined by MTT assay. In preparation for this assay, the cells were cultured in RPMI-1460 instead of McCoys 5A medium. Approximately 5000 cells/well were plated in a 96-well plate and 48 h later, 100 nM *TS* or non-specific (NS) siRNA were added in a total volume of 100 μl. After 24 h, the transfection medium was replaced with fresh medium with or without 5-FU (5 μM) and incubation continued for an additional 72 h. At the end of the treatment, 50 μl of MTT (2 mg/ml) were added to each well and cells were further incubated for 4 h at 37˚C. The reduction of MTT by viable cells was measured at 570 nm using an ELISA reader. The experiment was set up in eight wells and repeated twice.

Suppression of TS by siRNA. SMARTpool reagent containing a mix of four siRNA designed for different target regions of *TS* transcript was purchased from Dharmacon, USA. 1×10^5 HT29FU cells were plated in six well plates and incubated overnight. A mixture of one hundred nM SMARTpool reagent and Lipofectamine 2000 (Invitrogen, USA) was added to the culture and continued incubation for 24 h. The effect of *TS* siRNA on *TS* and *p53* messages following 48 h of transfection was determined by real-time PCR. The effect of *TS* siRNA post 72 h treatment on TS and p53 protein was monitored by Western immunoblot. Non-specific siRNA (NS) containing a mixture of scrambled siRNA was used as control. The experiments were repeated three times.

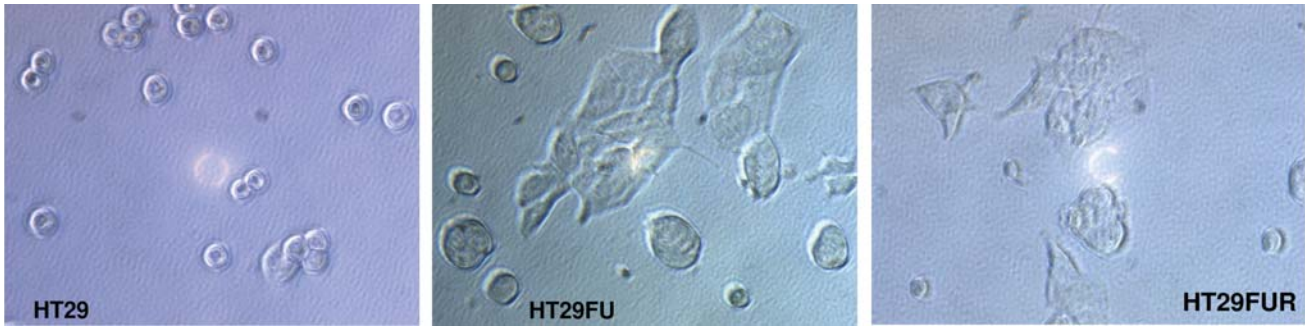

Figure 1. *Photomicrograph of 5-FU-sensitive HT29 and two different 5-FU-resistant derivative cell lines. The cells were photographed under phase-contrast microscope at ×400 magnification.*

Quantitative gene expression analysis by real-time Polymerase Chain Reaction (PCR). Pre and post experimental mRNA levels of *TS* and *p53* were quantified by real-time PCR using the primers shown in Table I. Oligonucleotides for cDNA synthesis and real time PCR were prepared in the University of Miami Miller School of Medicine DNA core facility.

Total RNA from the cells in control and experimental groups were prepared using Tri-Reagent (Sigma, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase. Each reaction mix contained 0.5 μg of oligo dT16 primer per μg of the total RNA samples used. It was heated to 70˚C for 5 min and chilled immediately on ice. The final reaction volume of 25 μl contained 0.5 mM each of dNTP mix (Promega, USA), 25 U of RNase inhibitor (Applied Biosystems, USA) and 200 U of M-MLV reverse transcriptase (Promega, USA). cDNA was synthesized at 42°C for 60 minutes and the reaction was stopped by chilling on ice.

Real-time quantitative PCR was carried out using 5 μl of the first-strand synthesis mixture. The reaction cocktail contained 200 nM each of the gene-specific primers and 25 μl of QuantiTect SYBR Green PCR Master Mix (Qiagen, USA) in a final volume of 50 μl. RT PCR was performed in a 96-well plate on a GeneAmp Sequence Detection System 5700 (Applied Biosystems, USA). β-Actin gene was used as the internal reference. Each template was analyzed at two different dilutions and in triplicate. The mean normalized expression (MNE) was calculated using the formula (11):

$$\text{MNE} = \frac{\dfrac{(E_{ref})^{Ctref}\ \text{Well 1}}{(E_{target})^{Cttarget}\ \text{Well 1}} + \dfrac{(E_{ref})^{Ctref}\ \text{Well 2}}{(E_{target})^{Cttarget}\ \text{Well 2}} + \dfrac{(E_{ref})^{Ctref}\ \text{Well 3}}{(E_{target})^{Cttarget}\ \text{Well 3}}}{3}$$

Where E=efficiency of PCR reaction, ref=reference gene (β-actin), target = target gene(s).

Immunodetection of TS and p53. M-PER reagent (Pierce, USA) was used to prepare cell lysates and the protein content was quantified by Lowry's method using Bio-Rad Protein Assay reagent (BioRad, USA) and bovine serum albumin (BSA) as the standard. A total of 30 μg of solubilized protein was electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Some of the reagents used and their sources are: Horseradish peroxidase (HRP) conjugated anti-rabbit IgG (Promega, USA), anti-p53 and HRP conjugated anti-mouse IgG antibodies (Santa Cruz Biotechnology, USA), anti-actin (Sigma, USA) and anti-TS (NeoMarkers, USA).

Following transfer to nylon membrane, antibody-positive bands were detected with Enhanced Chemiluminescence (ECL) reagent (Amersham, USA). Pre-stained protein molecular weight marker (BioRad, USA) was used as size standards. The TS- and p53-positive bands were quantified using UN-SCAN-IT gel 6.1 (Silk Scientific, Inc, USA). The ratio of TS and p53 to the β-actin or non-specific bands were calculated. These values were used to evaluate the differential expression between samples. For siRNA experiments, the mean band intensities of mock and NS siRNA control were utilized to determine the degree of suppression of *TS* and induction of *p53*.

Results

Morphology of the resistant derivative cells. The fluoropyrimidine-resistant derivative cells have distinct morphology that differed from the parental cell type. Typically they are elongated and asteroid shaped unlike the roundness of the parental type (Figure 1). Sensitive and resistant cell lines had a similar doubling time of approximately 22 to 24 hours. A high rate of pH change was noted in the medium of the resistant cells, necessitating its replacement every alternate day. On the other hand, wild-type (sensitive) cells did not need such frequent attention. We speculate that for the fluoropyrimidine-resistant cells, the doubling and apoptotic rate might be equal. The IC_{50} value of 5-FU for HT29FU was >128 μM and for HT29 FUR cells was ~14 μM. This is in contrast to 5 μM needed to kill 50% of the parental HT29 cells.

Molecular characterization of resistant cells. The amount of TS in 5-FU-resistant cells was found to be increased at both mRNA (12-fold; Figure 2A) and protein levels (Figure 3; Table II). Our aim was to determine the effect of constitutively overexpressed TS on *p53* translation under normal physiological conditions. Quantification of *p53* mRNA in

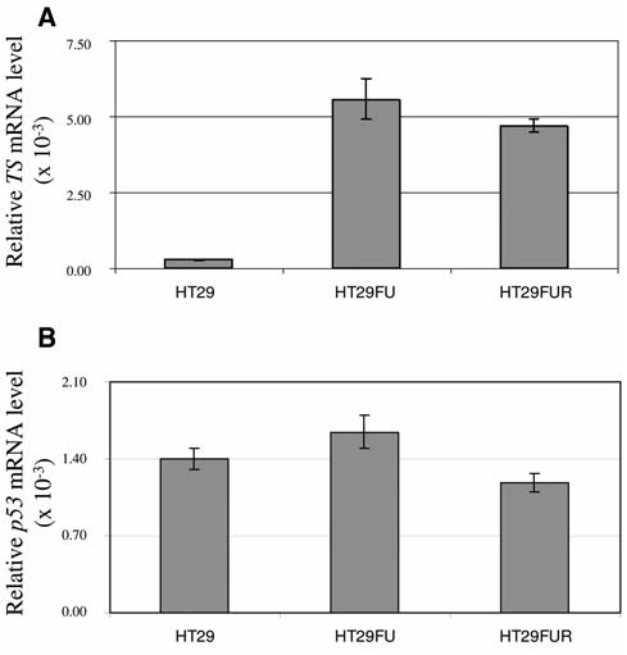

Figure 2. *Mean normalized expression of TS and p53 mRNA in 5-FU-sensitive and -resistant derivative HT29 colorectal cancer cells. A: The 5-FU-resistant derivative cell lines express ~12-fold more TS mRNA than the sensitive wild-type HT29 cells. B: Relatively very little or negligible difference in p53 mRNA level was observed between the sensitive and resistant cell lines. Mean normalized expression (arbitrary units) of three independent assays is presented here.*

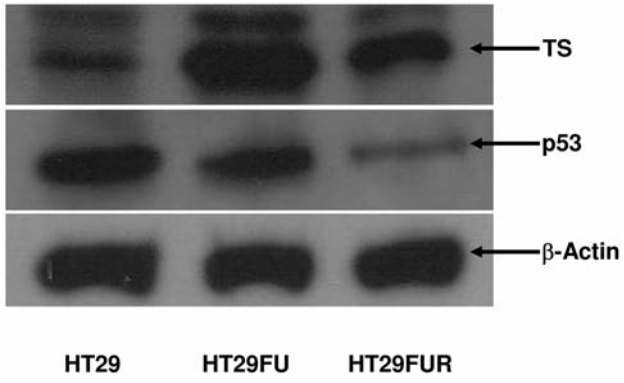

Figure 3. *Western immunoblot analyses of the TS and p53 proteins in the 5-FU-sensitive and -resistant derivative cell lines. There was an increase in thymidylate synthase protein in the 5-FU resistant derivative HT29 cells. A concomitant decrease in p53 translation was also noted in the resistant derivatives. β-Actin was used as the loading control. These results are representative of experiments repeated more than three times.*

these derivative cell lines demonstrated that its mRNA level was not significantly affected by the over-expressed TS (Figure 2B). However, as seen in Figure 3 and Table II (and as described below), TS directly inhibited *p53* translation.

Table II. *Densitometric quantification of TS and p53 bands in 5-FU-sensitive and -resistant derivative cell lines as depicted in Figure 3.*

	TS intensity	p53 intensity
HT29	100%	100%
HT29FU	210%	89%
HT29FUR	122%	21%

The data were normalized to the β-actin band intensity (loading control).

Table III. *Densitometric quantification of TS and p53 bands (as in Figure 5) following TS siRNA transfection.*

		TS	p53
HT29	TS siRNA	29%	123%
HT29FU	TS siRNA	23%	115%
HT29FUR	TS siRNA	11%	170%

The average band intensity of the mock and non-specific siRNA (NS-siRNA) transfected lanes were used. The data were normalized to the non-specific band intensity (loading control).

Table IV. *5-FU resistant cells were sensitized by TS siRNA to 5 μM of 5-FU as determined by MTT assay.*

	(% cells killed) HT29FU	HT29FUR
Mock	22	19
TS siRNA	35	31
NS siRNA	19	15

Silencing TS mRNA synthesis releases repression of p53 translation. In cells overexpressing TS, there is a concomitant reduction in p53 (9), a cell cycle checkpoint protein. Alternately, if we could reduce the quantity of TS, we should be able to restore or increase *p53* translation as found in the parental cell type. To test this hypothesis, we treated TS-overexpressing HT29FU and HT29FUR cells with *TS* siRNA. SiRNA degraded the *TS* transcripts by over 10-fold (Figure 4C, E and 5) and protein (Table III) in the resistant cell lines. Meanwhile, the *p53* mRNA level was not significantly affected (Figure 4D, F), but translation repression was relaxed (Figure 5; Table III). These observations are consistent with the starting amount of *TS* and *p53* transcripts. Our results suggest an inverse relationship between TS and p53 levels in cultured cells and as demonstrated earlier by Dr. Chu and his co-workers (8). In the 5-FU-sensitive parental cell type HT29, the *TS* siRNA

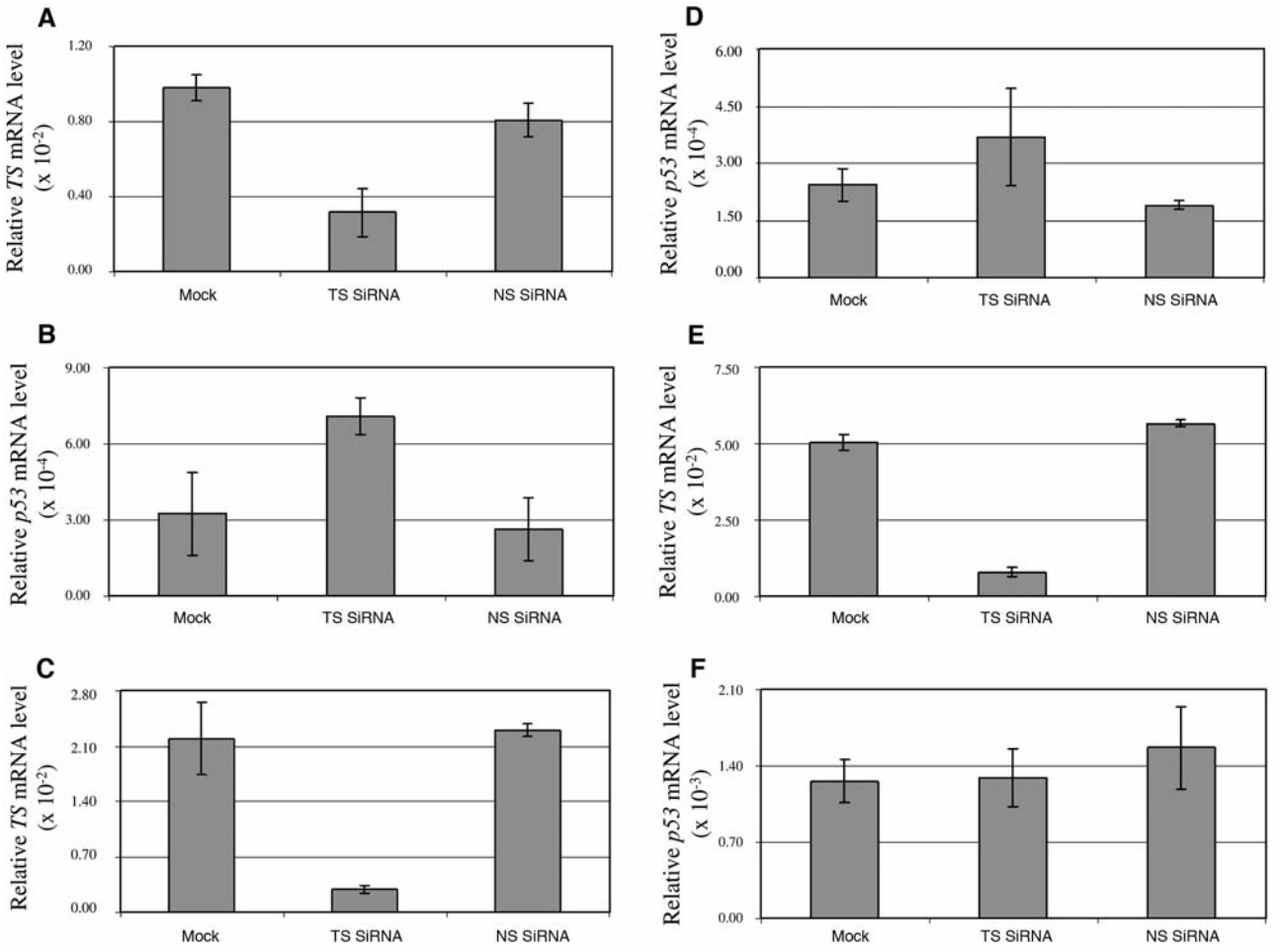

Figure 4. *Silencing of TS mRNA affects the expression of TS message but not of p53. Transfecting the 5-FU-sensitive (A) and -resistant cell lines (C, E) with TS siRNA degraded the TS mRNA within 48 hours. TS siRNA affected p53 transcription marginally in 5-FU-sensitive HT-29 cell line (B) but not in resistant cells (D, F). Cells were transfected with TS-specific siRNA (TS siRNA) or non-specific siRNA (NS siRNA). A parallel set of cells also received Lipofectamine 2000 without any added siRNA and served as mock transfection control (Mock). The experiments were repeated twice.*

down-regulated *TS* mRNA by approximately three-fold (Figure 4A) and very little protein could be detected (Figure 5; Table III). This silencing also stimulated *p53* transcription by about two-fold (Figure 4B). A 23% increase in p53 translation was observed in 5-FU-sensitive HT29 cells (Figure 5; Table III).

Silencing TS expression reduced 5-FU resistance. The ultimate success in elucidating a mechanism of 5-FU resistance is to find a way to treat refractive tumors. We proposed that reducing the TS expression in overexpressing cells may restore sensitivity to 5-FU. Therefore, following *TS* suppression with siRNA we carried out cytotoxicity assays. Reductions in TS led to an increase in the number of cells killed for the same dosage (5 μM) of 5-FU used (Table IV). The result supports our hypothesis that reducing TS expression may re-sensitize the cells to 5-FU. Since our aim is to sensitize the resistant cells, we did not carry out cytotoxicity assays with the sensitive cell line.

Discussion

The role of TS in DNA synthesis is well established (4). Its increased expression has been recorded in many tumor types, and thus correlated to drug resistance (12). Apparently its auto regulatory property is a prime contributor to the intracellular level of TS. Studies on gene expression regulation revealed that TS autoregulates by binding its own mRNA. The more TS is bound with deoxyuridylate (dUMP), the less it is available for its own translational inhibition. Hence, the substrate concentration determines the rate of TS translation. However, an increased amount of TS appears to be the cause

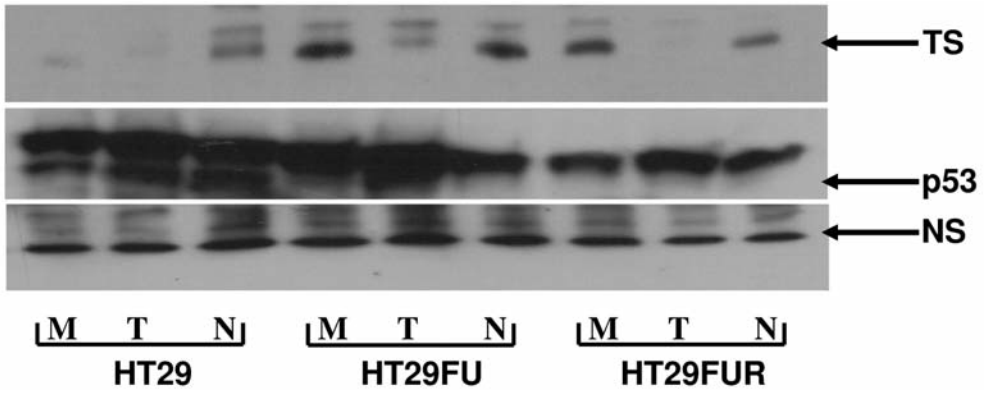

Figure 5. *Transfection of 5-FU-sensitive and -resistant HT29 cells lines with TS siRNA resulted in decreased TS protein and an increase in p53 protein. Immunodetection of TS, p53 and β-actin following siRNA treatment. M, Mock-transfected cells; T, TS siRNA-transfected cells; N, non-specific siRNA-transfected cells; NS, non-specific band.*

of resistance to 5-FU/FUdR. This was confirmed by many independent studies and thus an elevated TS level has been recognized as a reason for treatment failure (12). The unregulated increase in TS, as seen in many tumor types and in 5-FU resistant tumor cell lines, persist even after the removal of excess metabolites. Gene amplification (13) and changes in transcription factors (14, 15) are some of the reasons ascribed to the establishment of continuous overexpression of TS even in the absence of substrates. E2F-1 transcription factor is up-regulated in many resistant tumor types and credited to TS overexpression (14). However, further investigation is needed to conclusively establish the reasons behind TS overexpression in 5-FU-resistant HT29 derivative cells.

In addition to different functional aspects, TS has been demonstrated to be oncogenic (14). Irrespective of the mode of over expression, there exists a fine balance in the regulation of TS. 5-FU disrupts autoregulation and tips this equilibrium towards increased translation. In resistant tumors, overexpressed TS may independently be oncogenic. Down-regulation of TS may abrogate all its functions, as reflected in increased cell kill following siRNA treatment and subsequent 5-FU exposure.

The arginine residue at position 273 is substituted with histidine in the p53 of HT29 cells. Functional analysis of this mutant protein suggested that it is inactive (16). However, overexpression or substitution with wild-type p53 and subsequently induced DNA damage resulted in cell growth arrest at S phase rather than apoptosis. However, under similar experimental conditions, cells with mutant p53 were arrested at the G_2/M phase. An accumulation of cyclin B1 was also noted. We also detected increased expression of cyclin B1 in resistant derivative cell lines (Subbarayan *et al.* unpublished observations). Another group of researchers found that decreased expression of mutant p53 in HT29 cells corresponded to resistance to photodynamic therapy (PDT), but not to therapy with cisplatin (17). Therefore the role of mutant p53 in resistant tumors is not clear and merits further investigation.

Studies on TS demonstrated that besides its auto-regulatory property and oncogenic potential, TS also represses the translation of *p53* and *c-Myc* by binding to their mRNAs (9). The tumor suppressor p53 checks cell integrity and directs the cell into the proliferative or apoptotic pathway (18). The absence of mutation in *p53* is a major cause of tumor progression (19-22). The translational regulatory property of TS was demonstrated by Liu *et al.* (9). They established that an increased amount of TS directly down-regulated *p53* translation. We extended this study using cells naturally overexpressing TS, which is the case in many 5-FU resistant tumors.

Results of the experiments presented here revealed the regulation of p53 by TS under native conditions also. However, the quantum of decrease is not proportional. For instance, in HT29FU cells, a 110% increase in TS level reduced the p53 level by 11%. On the other hand, in HT29FUR cells, the TS level increased by 22% but that of p53 decreased by 79%. A reason for this discrepancy may lie in the mode of development of these two derivative cell lines. HT29FUR cells also developed cross resistance to 5-FU. FUR has been demonstrated to work primarily through the RNA pathway. Some of the FUDP may be converted to FdUMP and serve as a TS inhibitor (23). This may explain the difference in TS overexpression following exposure to two different fluoropyrimidine analogs. There was a very marginal increase in the *p53* mRNA level in HT29FU cells, and a decrease in HT29FUR cells. Although the difference was small, this may also contribute to the large difference in the suppression of *p53* translation between HT29FU and HT29FUR derivative cell lines. Under normal physiological

conditions, a fine balance exists between various molecules in the cell. *p53* gene expression is regulated by various factors. There would appear to be a threshold level for TS to function as a translation suppressor of *p53*. We do not know that threshold value. Data from our laboratory and others (8) demonstrate that when the TS level is elevated compared to a 'normal' level, it down-regulates *p53* translation. Whether permanent silencing of TS leads to unregulated translation of *p53* needs further analysis.

Suppression of p53 by overexpression of TS gives us a new vista of the process of chemoresistance development and uncontrolled tumor progression. Based on the data, we hypothesized that upon DNA damage cells could still progress through the cell cycle and perpetuate when TS is overexpressed and as a result p53 is suppressed. Contrarily, down-regulation of TS may lead to relaxing the repression on *p53* translation, thus enabling better control of cell cycle regulation. Degrading the TS message using gene-specific siRNA sensitized the otherwise 5-FU-resistant cells. Many different studies demonstrated that resistance development to 5-FU mediated chemotherapy may occur at several levels (24). The association of an elevated TS level with resistance is of interest to us. We learned from this study that up-regulation of TS may be responsible for treatment failure in two ways: i) Conversion of more FdUMP to FdTMP and its incorporation into DNA; ii) suppression of *p53* removes the control on DNA damage and the cell cycle. We also infer that an increased level of TS may be oncogenic (14). However, our results demonstrate that 5-FU resistance is at least partially reversible by down-regulating TS. In the current study, following down-regulation of TS and the consequent up-regulation of p53, about 50% more cells were killed than in the controls. However, ideally we would like to see a complete reversal of resistance.

In 5-FU-sensitive HT29 cells, *TS* siRNA suppressed the TS level by three-fold (~67% silencing). A two-fold (50%) increase in *p53* transcription was also observed, but translation did not increase. This contrasts with the data of 5-FU-resistant cells. We hypothesize that a threshold level for TS exists. Any negative alteration affects cell survival and cellular physiology may dictate such cells are directed to an apoptotic pathway.

Using anti-sense technology, another group of researchers also demonstrated that 5-FU resistance could be reversed (25). They also were unable to restore 100% sensitivity. In both instances, the cells were transiently transfected. Therefore, we reason that the transient nature of the siRNA transfection might be a contributor to the marginal increase in cell kill. Constructing a stable transfectant expressing *TS* siRNA might shed light on whether complete reversal of resistance is possible. Under clinical testing, the effective mean plasma concentration achieved was 3.0-10.5 μM using infusional regimen and 3.3 μM by bolus administration of 5-FU. Our primary aim is to test effectiveness at a dosage of the drugs that are clinically relevant. Therefore, instead of the IC_{50} values, we used 5 μM of 5-FU to test the reversal of sensitivity in HT29FU and HT29FUR cells following siRNA treatment. We conclude that collectively, we are at a stage to progress from interfering with TS function to a more active suppression of TS expression as a modality of cancer treatment.

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