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5-Fluorouracil: Identification of Novel Downstream Mediators of Tumour Response

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Abstract. *Background: 5-Fluorouracil (5-FU) is routinely used in the treatment of gastrointestinal, breast and head and neck cancers. A major limitation to the use of this drug is acquired or inherent resistance. Materials and Methods: To examine the downstream molecular signals activated in response to 5-FU, we used DNA microarray technology to examine global transcriptional changes in 5-FU-treated MCF-7 breast cancer cells. Results: We identified several novel 5-FU-inducible target genes that have not previously been linked to 5-FU response, including spermine/spermidine acetyl transferase (SSAT) and annexin II. Treatment of MCF-7 cells with the antifolate tomudex (TDX) and the DNA damaging agent oxaliplatin also caused up-regulation of each target gene. Inactivation of wild-type p53 abrogated the 5-FU-mediated induction of SSAT and annexin II. Inducible expression of thymidylate synthase completely abrogated TDX-, but not 5-FU-mediated induction of each gene. Furthermore, basal expression of SSAT and annexin II was elevated in cells resistant to 5-FU. Conclusion: These data demonstrate the potential of microarray analysis to identify novel genes associated with response or resistance to chemotherapeutic agents.*

The antimetabolite 5-FU is routinely used in the treatment of patients with gastrointestinal, breast and head and neck cancers (1). 5-FU rapidly enters the cell using the same facilitated transport mechanism as uracil (2) and is rapidly converted into three key active metabolites: fluorouridine triphosphate (FUTP), which is mis-incorporated into RNA causing alteration of its processing and function; fluorodeoxyuridine triphosphate (FdUTP), which is mis-incorporated into DNA resulting in DNA damage; and fluorodeoxyuridine monophosphate (FdUMP), which together with the reduced folate 5, 10-methylene tetrahydrofolate (CH_2THF), forms a stable ternary complex with thymidylate synthase (TS) resulting in enzyme inhibition (3). TS catalyses the reductive methylation of deoxyuridine monophosphate to deoxythymidine monophosphate, with CH_2THF as the methyl donor. This reaction provides the sole *de novo* source of thymidylate, which is necessary for DNA replication and repair. Inhibition of TS results in an imbalance in nucleotide pools, which in turn causes DNA damage through false nucleotide incorporation during DNA replication and repair (4). A major limitation to the use of 5-FU is acquired or inherent resistance. A number of *in vitro* and *in vivo* studies have demonstrated that increased TS expression correlates with decreased sensitivity to 5-FU (5-7). In addition, the 5-FU catabolizing enzyme dihydropyrimidine dehydrogenase (DPD) and the 5-FU anabolizing enzymes orotate phosphoribosyltransferase (OPRT) and uridine phosphorylase (UP) have been implicated in modulating resistance to 5-FU (8, 9). Furthermore, a number of pre-clinical and clinical studies have reported that p53 plays an important role in tumour cell response to 5-FU (10-12). It is clearly important that the downstream molecular signaling pathways that link the cellular damage caused by 5-FU to cell survival and cell death are fully elucidated, as dysregulation of molecules in these pathways is likely to modulate the response to this agent. To this end, we used DNA microarray technology to examine global transcriptional changes in MCF-7 breast cancer cells following treatment with an $IC_{60(72h)}$ (60% inhibitory concentration at 72h) dose of 5-FU (13). We have subsequently identified and validated several 5-FU-inducible target genes that have not previously been linked to 5-FU response. In addition, we have demonstrated inducible expression of these genes following treatment with the TS-targeted antifolate TDX and the third generation platinum compound oxaliplatin. Furthermore, a subset of these genes were up-regulated following treatment with the topoisomerase I inhibitor CPT-11 (Irinotecan), suggesting a general role for these target molecules as downstream mediators of tumour cell response to chemotherapy.

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Key Words: 5-Fluorouracil, tomudex, oxaliplatin, CPT-11, cDNA microarray.

Materials and Methods

Materials. 5-FU was purchased from Sigma Chemical Co. (St. Louis, MO, USA). TDX was obtained from AstraZeneca (Macclesfield, England). CPT-11 and oxaliplatin were obtained from Pharmacia and Upjohn (Kalamazoo, MI, USA) and Sanofi-Synthelabo (Malvern, PA, USA) respectively. 1mM stock solutions were prepared in sterile 1xPBS, with the exception of oxaliplatin which was prepared in injection water, and stored at 4˚C prior to use.

Tissue culture. All cells were maintained in 5% CO_2 at 37˚C. H630, H630-R10 and RKO colorectal cancer cells, and MCF-7 and MDA435 breast cancer cells were maintained in DMEM with 10% dialysed foetal calf serum (FCS) supplemented with 1mM sodium pyruvate, 2 mM L-glutamine and 50 μg/ml penicillin-streptomycin (all from GIBCO BRL/Life technologies, Paisley, Scotland). M7TS90 cells (11) were maintained in MCF-7 medium supplemented with 1 μg/ml puromycin, 1 μg/ml tetracycline (both from Sigma, Poole, Dorset, England) and 100 μg/ml G418 (GIBCO BRL/Life technologies). M7TS90-E6 cells (11) were maintained in M7TS90 medium supplemented with 200 μg/ml hygromycin (GIBCO BRL/Life technologies). HCT116 p53 wild-type ($p53^{+/+}$) and null ($p53^{-/-}$) isogenic human colon cancer cells were kindly provided by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD, USA). Drug-resistant HCT116 $p53^{+/+}$ sub-lines were developed in our laboratory by repeated exposure to stepwise increasing concentrations of 5-FU, CPT-11 or oxaliplatin over a period of approximately ten months. Parental and drug-resistant HCT116 cell lines were grown in McCoy's 5A medium supplemented with 10% dialysed FCS, 50 μg/ml penicillin-streptomycin, 2 mM L-glutamine and 1mM sodium pyruvate. HCT116 5-FU-resistant and CPT-11-resistant cells were maintained in the presence of 2 μM 5-FU and 1 μM CPT-11, respectively. HCT116 oxaliplatin-resistant cells were found to be stably resistant and were therefore maintained in oxaliplatin-free medium that was spiked every 4 weeks with 8 μM oxaliplatin. Prior to each experiment, resistant sub-lines were cultured in the absence of drug for 48 hours.

cDNA microarray expression profiling. RNA was isolated from untreated (control) and 5-FU-treated MCF-7 cells after 6, 12, 24 and 48 hours. Ten micrograms of RNA from each timepoint were combined for 5-FU-treated and control samples. Biotin and dinitrophenol (DNP)-labeled cDNA probes were prepared from 2 μg of control and 5-FU-treated samples, respectively. Biotin and DNP-labeled cDNAs were mixed and simultaneously hybridised to the Micromax Human cDNA Microarray (NEN Lifesciences, Boston, MA, USA) for 16 hours in a humid incubator at 65˚C. Hybridized cDNA probes were visualised using the cyanine-5 (control) and cyanine-3 (5-FU-treated) reporter dyes, according to the Tyramide Signal Amplification process. Cyanine-3 and cyanine-5 fluorescence was detected using a Scanarray confocal laser scanner (GSI Lumonics, Inc., Billerica, MA, USA). Differential scanning data was processed using ImaGene analysis software (Biodiscovery, Inc., Marina Del Ray, CA, USA).

Immunoblotting. Cells were washed twice in ice-cold 1xPBS, harvested and resuspended in 200 μl of 1xRIPA buffer (20mM TRIS pH7.4, 150mM NaCl, 1mM EDTA pH8.0, 1% Triton X-100, 0.1% SDS). Cells were then lysed and centrifuged at 15,000rpm/4˚C for 15 minutes to remove cell debris. Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA). Thirty micrograms of each protein sample were resolved by SDS-PAGE and transferred to a PVDF membrane by electroblotting. Immunodetection was performed using a TS sheep monoclonal antibody (Rockland, Gilbertsville, PA, USA) and a 1/2000 dilution of a horseradish peroxidase-conjugated donkey anti-sheep secondary antibody (Serotec, Oxford, England). Equal lane loading was assessed using a mouse monoclonal β-tubulin antibody (Sigma). The fluorescent signal was detected using the Super Signal chemiluminescent detection system according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Table I. *Oligonucleotide primers for generation of cDNA Northern probes.*

Gene	Primer Sequence (5'-3')	
SSAT	Sense:	GCTAAATTCGTGATCCGC
	Antisense:	CAATGCTGTGTCCTTCCG
Annexin II	Sense:	GGGTGATCACTCTACACC
	Antisense:	CAGTGCTGATGCAGGTTC
Thymosin β-10	Sense:	TCGGAACGAGACTGCACG
	Antisense:	CTCTTCCTCCACATCACG
MAT-8	Sense:	GCTCTGACATGCAGAAGG
	Antisense:	CCTCCACCCAATTTCAGC
Chaperonin-10	Sense:	GTAATGGCAGGACAAGCG
	Antisense:	GGGCAGCATGTTGATGC
18S	Sense:	CAGTGAAACTGCGAATGG
	Antisense:	CCAAGATCCAACTACCAG

Northern blot analysis. Northern blots were performed as previously described (14). cDNA probes for Northern blotting were generated by PCR using 1 μg of MCF-7 total RNA as a template. Primer sequences are listed in Table I.

Results

cDNA microarray expression profiling. To elucidate novel mechanisms of response to 5-FU, we carried out cDNA microarray analysis of transcriptional changes in MCF-7 breast cancer cells following treatment with an $\sim IC_{60(72h)}$ dose of 5-FU. Using the 2,400 gene Micromax Human cDNA Microarray, we detected >619 genes that were up-regulated by >3-fold and 16 genes that were down-regulated by >3-fold. Candidate target genes were initially grouped according to function using the DRAGON database (Database Referencing of Array Genes ONline). In order to further refine our gene list we selected a higher cut-off value of >6-fold induction. Since our samples had been pooled over several timepoints, the fold-change in gene expression represented a cumulative increase. We therefore decided a higher cut-off value was prudent. In addition to fold-change, genes were also selected on the basis of signal intensity, with intensities of >3000

Table II. *Functional grouping of genes identified by cDNA microarray analysis as being up-regulated by 5-FU in MCF-7 cells. Potential target genes were grouped according to their function using the DRAGON database (Database Referencing of Array Genes ONline, http://pevsnerlab.kennedykrieger.org/dragon.htm).*

Family	Examples	Fold Induction	Signal Intensity
Signal transduction	Raf	3.9	8686
	K-ras	4.8	9662
Apoptosis	APO-1	4.2	4453
	FLIP protein	3.7	5793
Cell cycle	Cyclin G	8.5	13789
	CDC2	3.1	1779
Structural	Thymosin β-10	8.5	27041
	Thymosin β-4	4.3	46355
Polyamine metabolism	SSAT	13.0	3662
	Spermidine aminopropyltransferase	5.0	6633
Cell surface	MAT-8 protein	10.1	6522
	Annexin II	12.3	24463
Mitochondrial	Chaperonin 10	11.6	8478
	Enoyl-CoA hydratase	3.4	2512
Ribosomal proteins	Ribosomal protein S28	10.9	24039
	L23 mRNA for putative ribosomal protein	4.6	12662

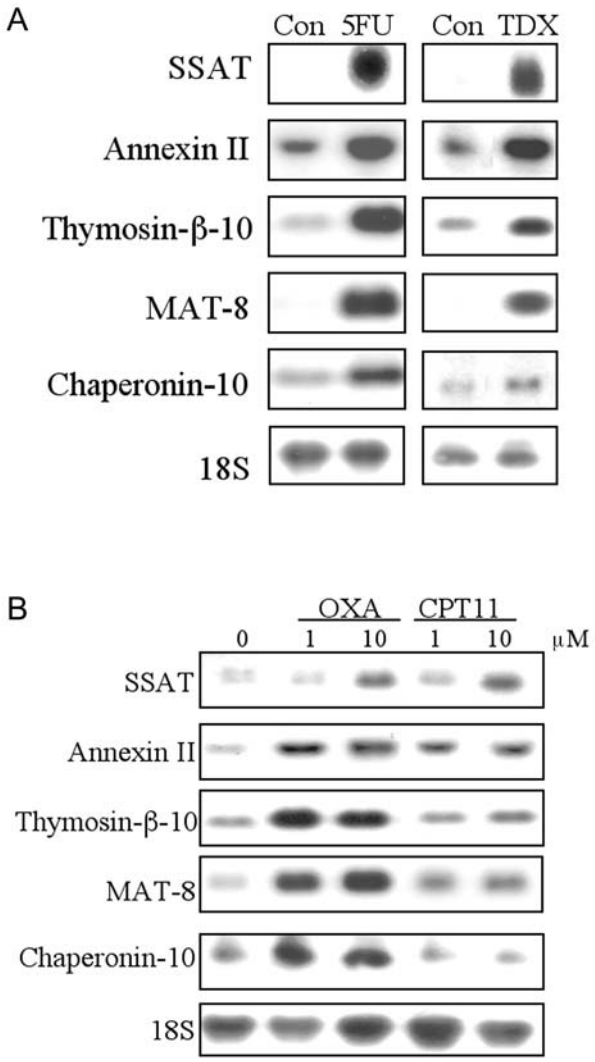

Figure 1. *A. Northern blot analysis of SSAT, annexin II, thymosin β-10, MAT-8 and chaperonin-10 mRNA expression in MCF-7 cells treated for 72 hours with no drug (Con), 10 μM 5-FU (5-FU) or 10nM TDX (TDX). 18S rRNA expression was assessed as a loading control. B. Northern blot analysis of SSAT, annexin II, thymosin β-10, MAT-8 and chaperonin-10 mRNA expression in MCF-7 cells treated for 72 hours with no drug, 1 μM and 10μM oxaliplatin (OXA) or 1 μM and 10 μM CPT-11. 18S rRNA expression was assessed as a loading control.*

considered to be sufficiently higher than background. The biological functions of genes identified by the cDNA microarray screen were diverse and included cell cycle regulators; structural, ribosomal and apoptotic genes; as well as genes involved in signaling pathways and polyamine metabolism (Table II).

Northern blot analysis of gene expression following treatment with TS inhibitors. Using Northern blot analysis we validated several novel genes which were consistently up-regulated following exposure to both 5-FU and the TS-targeted antifolate TDX, namely SSAT, annexin II, thymosin β-10, MAT-8 and chaperonin-10 (Figure 1A). SSAT is the enzyme responsible for catalysing the rate limiting step in the N1-acetylation of the polyamines spermine and spermidine (15). We demonstrated that expression of SSAT mRNA was induced after treatment with 10 μM 5-FU and 10nM TDX for 72 hours by ~15- and ~13-fold, respectively. Annexin II has been reported to regulate cell proliferation and apoptosis (16). We demonstrated an approximate 5-fold increase in the levels of annexin II in response to 5-FU and TDX after 72 hours. Thymosin β-10 has also been implicated in regulating apoptotic mechanisms (17). We found that thymosin β-10 mRNA levels increased by ~8- and ~5-fold following treatment with 5-FU and TDX, respectively. MAT-8 is a transmembrane protein that regulates chloride ion transport (18). We found that MAT-8 mRNA expression was up-regulated by ~11-fold following exposure to 5-FU for 72 hours, while treatment with TDX resulted in an ~6-fold increase in MAT-8 mRNA levels. Chaperonin-10 is a mitochondrial heat shock protein (19) and was up-regulated 72 hours post-treatment with 5-FU, with levels ~4-fold higher than control. However, treatment with TDX only resulted in a moderate ~1.5-fold induction of chaperonin-10.

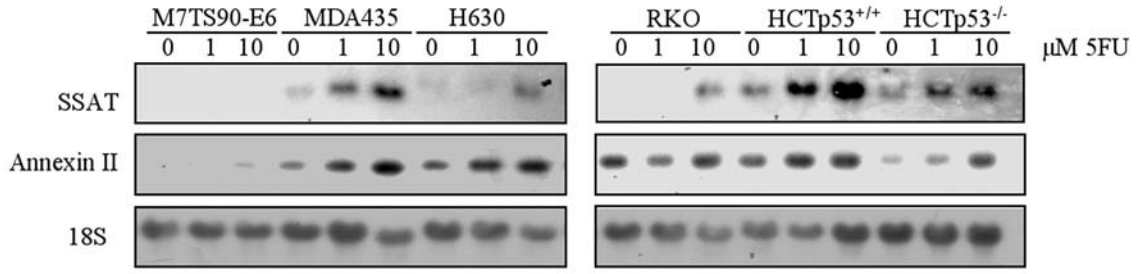

Figure 2. *Northern blot analysis of SSAT and annexin II mRNA expression in p53 null M7TS90-E6 cells, p53 mutant MDA435 and H630 cells, p53 wild-type RKO cells and HCT116 p53$^{+/+}$ and p53$^{-/-}$ cell lines. Cells were treated for 72 hours with no drug, 1 μM 5-FU or 10 μM 5-FU. 18S rRNA expression was assessed as a loading control.*

Northern blot analysis of gene expression following treatment with oxaliplatin and CPT-11. The platinum-based DNA-damaging agent oxaliplatin and the topoisomerase I inhibitor CPT-11 have demonstrated synergistic activity with TS inhibitors in preclinical studies (20, 21). In addition, both agents are currently used in the treatment of advanced colorectal cancer and have demonstrated significantly improved response rates compared to 5-FU alone (22, 23). We examined the expression of the novel 5-FU- (and TDX-) inducible target genes after treatment of MCF-7 cells with 1 μM and 10 μM oxaliplatin and CPT-11 (Figure 1B). We found SSAT mRNA was up-regulated by ~6-fold in response to treatment with 10 μM (~$IC_{60(72h)}$) oxaliplatin and by ~8-fold in response to 10 μM (~$IC_{60(72h)}$) CPT-11. Annexin II mRNA was up-regulated by ~6-fold in response to oxaliplatin and ~4-fold in response to CPT-11. Unlike SSAT, annexin II was up-regulated in response to lower concentrations (1 μM) of each drug, which corresponded to an ~$IC_{40(72h)}$ dose of oxaliplatin and ~$IC_{50(72h)}$ dose of CPT-11. Expression of thymosin β-10 was up-regulated by ~8-fold in response to 1 μM and 10 μM oxaliplatin, however it was not significantly induced following treatment with CPT-11. MAT-8 mRNA expression was also dramatically induced in response to 1 μM and 10 μM oxaliplatin (by ~10-fold), whereas CPT-11-mediated induction was more modest with ~3-fold induction observed in response to 1 μM and 10 μM CPT-11. Finally, chaperonin-10 was significantly up-regulated (by ~4-fold) following treatment with oxaliplatin, but not CPT-11. As SSAT and annexin II were the most strongly up-regulated in response to all 4 drugs analysed, we decided to focus on these two novel target genes in our further analyses.

Expression of SSAT and annexin II in a panel of cell lines following 5-FU treatment. p53 has been previously reported to play an important role in regulating downstream signaling following 5-FU treatment (11). In order to determine whether p53 plays a role in 5-FU-mediated target gene up-regulation, we examined the expression of SSAT and annexin II following treatment with 1 μM and 10 μM 5-FU in HCT116 p53 wild-type (p53$^{+/+}$) and null (p53$^{-/-}$) isogenic colorectal cancer cell lines, as well as the p53 wild-type RKO and p53 mutant H630 colorectal cancer cell lines and p53 mutant MDA435 and p53 null MCF-7-derived M7TS90-E6 (11) breast cancer cell lines. In the M7TS90-E6 cell line there was no evidence of SSAT induction following treatment with 10 μM 5-FU for 72 hours (Figure 2), in contrast to the p53 wild-type parental MCF-7 cells in which SSAT was highly up-regulated (Figure 1A). Similarly, only moderate induction of annexin II expression was observed in the p53 null line following 5-FU exposure. In p53 mutant MDA435 cells, we observed a similar increase in mRNA expression of SSAT and annexin II as that observed in the p53 wild-type MCF-7 cell line. In p53 wild-type RKO colorectal cancer cells, SSAT was up-regulated by ~5-fold compared to the control following treatment with 10 μM 5-FU. Expression of annexin II, however, was unaltered following 5-FU treatment, although basal expression was relatively high in this cell line. In p53 mutant H630 cells we found an ~4-fold induction of SSAT following drug treatment compared to control, whilst annexin II expression was increased by ~3- and ~5-fold following exposure to 1 μM and 10 μM 5-FU, respectively. In HCT116 p53$^{+/+}$ colorectal cancer cells, SSAT expression was dramatically up-regulated by ~8-fold following exposure to 1 μM 5-FU and by ~15-fold in response to 10 μM 5-FU. In the p53$^{-/-}$ isogenic daughter line, SSAT mRNA induction was significantly attenuated with levels of induction reduced to ~3- and ~5-fold in response to 1 μM and 10 μM 5-FU, respectively. Annexin II mRNA was only up-regulated by ~2-fold in HCT116 p53$^{+/+}$ cells in response to 1 μM and 10 μM 5-FU although, similarly to the RKO cell line, the basal expression was relatively high. In the HCT116 p53$^{-/-}$ line, basal expression of annexin II mRNA was lower than in the p53 wild-type line, however, expression was induced by ~5-fold in response to 10 μM 5-FU to levels approaching that observed in p53 wild-type cells.

Effect of inducible TS expression on drug-induced SSAT and annexin II up-regulation. We next examined the role of TS

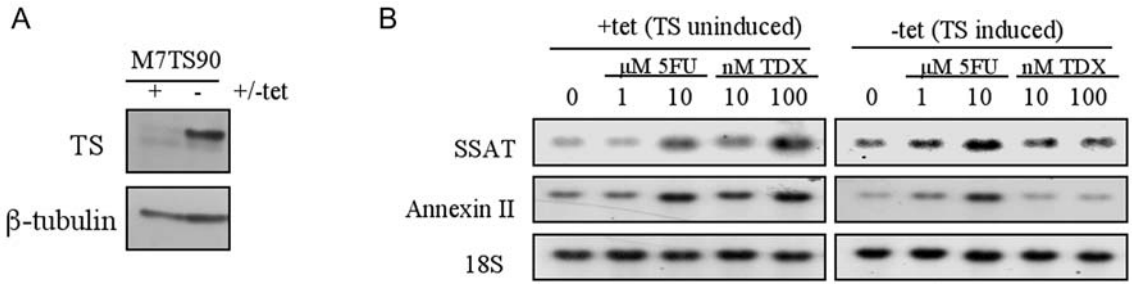

Figure 3. *A. Western blot analysis of TS protein expression in M7TS90 cells in the presence (-tet) and absence (+tet) of tetracycline. β-tubulin protein expression was used as a loading control. B. Northern blot analysis of SSAT and annexin II mRNA expression in TS induced (-tet) and uninduced (+tet) M7TS90 cells treated for 72 hours with no drug, 1 μM and 10 μM 5-FU or 10nM and 100nM TDX. 18S rRNA expression was assessed as a loading control.*

induction in modulating the expression of SSAT and annexin II in MCF-7-derived M7TS90 cells (11). *In vitro* and *in vivo* studies have demonstrated that elevated TS expression correlates with decreased sensitivity to 5-FU (5-7). *In vitro* studies have shown that treatment of cancer cells with 5-FU and TS-targeted antifolate drugs acutely up-regulates TS protein synthesis (24). The molecular basis for this acute up-regulation appears to be inhibition of a TS autoregulatory feedback loop, in which TS binds to and inhibits translation of its own mRNA (24). The extent of inducible TS expression in the M7TS90 cell line is ~6-fold, which is similar to the magnitude of the acute increases in TS expression that have been observed in tumour cells treated with 5-FU and antifolates (25) (Figure 3A). Both SSAT and annexin II mRNA expression were up-regulated in M7TS90 cells following treatment with 10 μM 5-FU (Figure 3B, +tet). Interestingly, the level of 5-FU-mediated induction of each gene was unaltered in the presence of inducible TS expression (Figure 3B, –tet). In contrast, induction of both target genes following treatment with 10nM TDX was completely abrogated in the presence of exogenous TS. These data substantiate previous findings, which suggest that TS inhibition is not the primary mechanism of 5-FU cytotoxicity in MCF-7 cells (11).

Expression of SSAT and annexin II in a panel of drug-resistant cell lines. We also compared the basal expression of SSAT and annexin II in the H630 colorectal cancer cell line and a 5-FU-resistant daughter line, H630-R10. Expression of both these target genes was moderately elevated (by ~2-fold) in the resistant line compared to the parental H630 cells (Figure 4A). In addition, we examined the levels of both targets in HCT116 $p53^{+/+}$ colorectal cancer cells which had been made resistant to 5-FU, oxaliplatin and CPT-11. We demonstrated dramatic basal overexpression of SSAT in both 5-FU-resistant (by ~15-fold) and CPT-11-resistant (by ~10-fold) cells compared to sensitive parental cells (Figure 4B). In contrast, we saw no change in SSAT levels in the oxaliplatin-resistant line. With regard to annexin II, we saw no obvious alterations in the level of this gene in any of the HCT116 $p53^{+/+}$ drug-resistant lines relative to parental cells. Thus, the development of resistance in the 5-FU and CPT-11-resistant HCT116 $p53^{+/+}$ cell lines correlated with elevated basal expression of SSAT, whilst in the H630-R10 cells, the 5-FU-resistant phenotype correlated with moderately increased expression of both SSAT and annexin II.

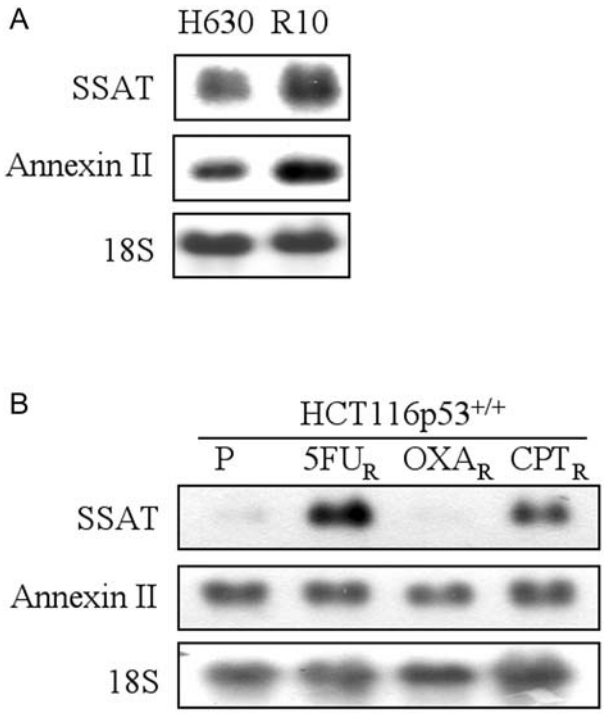

Figure 4. *A. Northern blot analysis of basal SSAT and annexin II mRNA expression in H630 and H630-R10 cells. 18S rRNA expression was assessed as a loading control. B. Northern blot analysis of basal SSAT and annexin II mRNA expression in HCT116 $p53^{+/+}$ parental cells and three daughter lines made resistant to 5-FU ($5\text{-}FU_R$), oxaliplatin (OXA_R) and CPT-11 (CPT_R). 18S rRNA expression was assessed as a loading control.*

Discussion

To attempt to predict the response of tumour cells to chemotherapeutic agents, the downstream signaling pathways that link cellular damage to response (*i.e.* - survival, cell-cycle arrest or apoptosis) must be fully elucidated. The advent of cDNA microarray technology has allowed global changes in transcriptional profiles in response to a defined stimulus (such as drug treatment) to be assessed. Such an approach allows the identification of genes that may be associated with response or resistance to a given agent. To this end, we have used cDNA microarray expression profiling to identify 5-FU-inducible target genes using MCF-7 breast cancer cells as a tumour cell model. Using the Micromax Human cDNA Microarray, we identified five novel target genes that were consistently up-regulated in response to an $IC_{60(72h)}$ dose of 5-FU, namely SSAT, annexin II, thymosin β-10, MAT-8 and chaperonin-10. SSAT is a polyamine catabolic enzyme, which has been reported to positively regulate programmed cell death (15). Annexin II is a phospholipid binding protein, which has been implicated in a number of cellular processes including cell proliferation, differentiation and apoptosis (16). The G-actin binding protein thymosin β-10 plays a role in cellular processes controlling apoptosis (17). MAT-8 is a member of the FXYD family of proteins that regulate chloride ion transport across the cell membrane (18). Chaperonin-10 binds hsp-60 and regulates folding of mitochondrial proteins (19). To our knowledge, none of these genes have previously been linked to 5-FU response.

We found that an $\sim IC_{60(72h)}$ dose of the TS-targeted antifolate TDX and the DNA damaging agent oxaliplatin also caused up-regulation of each of the target genes. Furthermore, an $\sim IC_{60(72h)}$ dose of the topoisomerase I inhibitor CPT-11 up-regulated expression of all but two of the target molecules (thymosin β-10 and chaperonin-10). These data suggest a potential general role for these molecules as downstream mediators of response to chemotherapy.

In addition, we demonstrated that inactivation of p53 resulted in almost complete loss of 5-FU-mediated induction of SSAT and annexin II in p53 null M7TS90-E6 cells compared to p53 wild-type M7TS90 cells. Similarly, in p53 null $HCT116^{-/-}$ cells induction of both target molecules was significantly reduced following exposure to 10 μM 5-FU, compared to p53 wild-type $HCT116^{+/+}$ cells. These results suggest that p53 may play a role in regulating induction of SSAT and annexin II in response to 5-FU. Interestingly, in the p53 mutant H630 and MDA435 cell lines, both SSAT and annexin II were significantly up-regulated in response to 5-FU. These data suggest that not all mutations in p53 may necessarily abrogate the drug-induced up-regulation of these 5-FU target genes, and/or that these genes may also be regulated by p53 independent pathways in response to 5-FU.

In an effort to define the role of TS in modulating expression of SSAT and annexin II in response to TS inhibitors, we examined induction of both genes by 5-FU and TDX in the presence and absence of exogenous TS. We demonstrated that TDX-, but not 5-FU-, mediated induction of SSAT and annexin II was completely abrogated when TS was overexpressed. These data suggest that expression of both target genes is induced by thymidylate depletion caused by TDX-mediated TS inhibition and also in response to the non-TS-directed cytotoxic effects of 5-FU, namely mis-incorporation of FUTP and FdUTP into RNA and DNA, respectively (3).

We also examined basal expression of SSAT and annexin II in the H630 colorectal cancer cell line and the 5-FU-resistant daughter line H630-R10, in which TS is overexpressed by ~30-fold compared to the parental cell line. We found that expression of both target genes was moderately higher (by ~2-3-fold) in the 5-FU-resistant line. Furthermore, we found that basal levels of SSAT were greatly elevated in 5-FU- and CPT-11-resistant $p53^{+/+}$ HCT116 colorectal cancer cells relative to the parental line, indicating that they may serve as valuable biomarkers of resistance to these agents. Whether these molecules are actively involved in modulating drug resistance, or whether resistant cells have been able to adapt to and tolerate higher levels of each target gene remains to be defined.

In conclusion, we have demonstrated that cDNA microarray analysis is a powerful technology for identifying novel target genes associated with response or resistance to chemotherapeutic agents. Using this technology, we have identified five novel 5-FU-inducible target genes: (a) SSAT; (b) annexin II; (c) thymosin β-10; (d) MAT-8; and (e) chaperonin-10. These genes were also up-regulated by TDX and oxaliplatin, and all but two were also up-regulated by CPT-11. In addition, 5-FU-mediated induction of SSAT and annexin II was significantly reduced in the absence of wild-type p53, suggesting a role for p53 in regulating their expression. We have also shown that induction of SSAT and annexin II by TDX, but not 5-FU, is abrogated when TS is inducibly expressed. Finally, basal expression of SSAT was higher in two 5-FU- and one CPT-11-resistant cell lines, whilst basal expression of annexin II mRNA was elevated in one 5-FU-resistant cell line, suggesting that these genes may serve as useful biomarkers of resistance to 5-FU and CPT-11.

Acknowledgements

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