SPANDIDOS PUBLICATIONS

Expression of rTSß as a 5-fluorouracil resistance marker in patients with primary breast cancer

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Abstract. Expression of thymidylate synthase (TS) in tumor cells is frequently suggested as an important prognostic factor for patients scheduled for chemotherapy with 5-fluorouracil (5-FU). However, clinical evidence does not fully support such an anticipation. We studied the expression of rTSß, a reverse orientation gene of TS, as a 5-FU resistance marker in patients with primary breast cancer. Expression of rTSß was examined in 129 patients with newly diagnosed breast cancer and five breast cancer cell lines by immunohistochemistry, immunocytochemistry and immunoblotting. Clinically, expression of rTSß was found to correlate with survival of the patients (p=0.023) when patients received chemotherapeutic regimen containing 5-FU. *In vitro*, rTSß expression was found to correlate with 5-FU resistance in breast cancer cell lines. Notably, in the 5-FU-resistant cells, rTSß was identified in the nucleus, whereas in the 5-FU-sensitive cells, rTSß was found in the cytoplasm. Nuclear localization of rTSß was further found to be associated with protein farnesylation. Therefore, nuclear expression of rTSß could be a novel 5-FU resistance marker in patients with primary breast cancer.

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Abbreviations: 5-FU, 5-fluorouracil; FTI, farnesyltransferase inhibitor; HAS, human serum albumin; rTSα, α form of antisense TS; rTSß, ß form of antisense TS; TS, thymidylate synthase

Key words: drug resistance, 5-fluorouracil, rTSß, nuclear localization, farnesylation, enolase

Introduction

Breast cancer is the second most prevalent malignancy and the fourth leading cause of cancer death in Taiwanese women (1). Although the death rate of uterine cervical cancer has decreased significantly in the past two decades due to the improvement in the early detection of the disease by pap smear among women over 35 years of age; for breast cancer, not only has the incidence increased markedly, but also the age of tumor occurrence has decreased dramatically, which is about ten years younger than that of the Western population (2-4).

Clinically, depending upon the status of estrogen and/or progesterone receptors in cancer cells, some patients receive non-steroidal anti-estrogen tamoxifen for hormonal therapy (5). However, most of the patients could only choose adjuvant chemotherapy containing 5-fluorouracil (5-FU) after surgery. 5-FU is a pro-drug that is converted to fluorouridine to interfere with RNA synthesis, or to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) to inhibit thymidylate synthase (TS) and the consequent DNA synthesis. In the presence of N^5, ^{10}-methylene tetrahydrofolate, FdUMP further forms a stable ternary complex with TS to inhibit synthesis of deoxythymidine monophosphate (dTMP) (reviewed in ref. 6). TS expression in tumor cells is considered as a key prognostic factor for patients receiving chemotherapy containing 5-FU (7).

However, clinical evidence did not fully support this expectation (6,8-13). On the other hand, expression of the *rTS* (ENOSF1) gene (14-16) was found to be closely associated with 5-FU sensitivity besides nucleotide metabolism-related enzymes, e.g., thymidine phosphorylase, ribonucleotide reductase, uridine phosphorylase and thymidine kinase (17-19) that directly affects pyrimidine synthesis in *de novo* or salvage pathways (11).

The *rTS* is located with the *TS* gene on the same stretch of chromosome 18, but in the opposite direction. The two genes partially overlap at 3'-end of each genomic sequence. The *rTS* gene consists of 17 exons, and by alternate splicing, mRNAs can be respectively translated into a 41 kDa rTSα or a 47 kDa rTSß protein, of which in the carboxyl end of both proteins a fragment of 340 amino acid residues is identical. Both rTSα

and rTSß induce the down-regulation of TS in cultured cells (20).

In this study, we investigated the correlation between rTSß expression and clinical outcome of 5 FU-response. We further examined gene expression of TS and rTSß in five breast cancer cell lines. Our results showed that the nuclear level of rTSß was correlated not only with poor prognosis in patients with primary breast cancer, but also with increased drug resistance to 5-FU *in vitro*.

Materials and methods

Materials, cell culture and drug treatment. Culture media and fetal calf serum (FCS) were from Gibco Laboratories (Grand Island, NY). All other materials were reagent grade from Sigma (St. Louis, MO) and Merck (Darmstadt, Germany). Antibodies were provided by Cashmere (Taipei, Taiwan). Breast cancer cells, 60055, BT-20, MCF-1, MCF-7 and T47D, were grown as a monolayer in RPMI-1640 plus 10% FCS. H630 and H630-1 cells were grown as previously described (14-16). Except as noted otherwise, cultured cells were incubated at 37˚C and all media were supplemented with penicillin (100 IU/ ml) and streptomycin (100 μg/ml).

Patients. From January 1996 to October 2003, tissue specimens from 129 patients with newly diagnosed breast cancer were collected. All patients were pathologically confirmed with breast cancer. Both the primary tumor and the corresponding non-tumor tissue were obtained from patients who had undergone surgical resection. The stage of disease progression was classified according to the Tumor-node-metastasis system. For patients at stage III or beyond, post-operative adjuvant chemotherapy, which contained cyclophosphamide, methotrexate and 5-fluorouracil commenced within one month if the patient's condition was suitable. After treatment, all patients were followed as routine. Written informed consent was obtained from every patient.

Immunocytochemical, immunohistochemical and immunoblotting analyses. Immunological staining was performed according to the immunoperoxidase method reported by Chiou *et al* (21). Briefly, immunohistochemistry was performed on paraffin sections of the biopsy using the LSAB method (Dako, Carpenteria, CA). The chromogenic reaction was visualized by peroxidase-conjugated streptavidin (Dako) and aminoethyl carbazole (Sigma). Slides were counter-stained with Mayor's hematoxylin, and the positive staining was recognized as crimson-red granules under a light microscope. For immunofluorescence staining, cellular uptake of MitoTracker® green FM (Molecular Probes, Inc., Eugene, OR) was used to label mitochondria. The cells were then fixed with 4% formaldehyde at room temperature for 15 min. After washing three times with PBS, the cells were incubated with the primary antibodies for 90 min, and washed three times with PBS. The secondary antibodies used were rhodamine (TRITC)-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The slide was examined and the photo images were captured on a laser scanning confocal microscope (LSM510, Zeiss, Chicago, IL).

The method for immunoblotting analysis was described previously (22). Briefly, $5x10^6$ cells were washed with phosphate buffered saline (PBS) (100 mM Na_2HPO_4, pH 7.4, 136 mM NaCl) twice and lysed in a loading buffer [50 mM Tris (pH 6.8), 150 mM NaCl, 1 mM disodium EDTA, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.01% bromophenol blue, 5% ß-mercaptomethanol and 1% SDS supplemented with trypsin inhibitor (10 μg/ml)]. Electrophoresis was carried out using 10% polyacrylamide gels with 4.5% stacking gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membrane was then probed with antibodies specific to rTSß protein or farnesylated human serum albumin (HSA). The signal was amplified by biotin-labeled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. The presence of protein was visualized by exposing the membrane to X-Omat film (Eastman Kodak, Rochester, NY) with enhanced chemiluminescent reagent (Pierce, Rockford, IL). In each case, normal breast tissue served as an internal negative control.

Slide evaluation. Slides were read by three independent pathologists without prior clinicopathological knowledge. A specimen was considered positive if >10% of the cancer cells were positively stained; and negative if <10% of the cells were positive (23).

Cytotoxicity assay. Cytotoxicity was determined by a modified MTT method, in which the activity of mitochondrial dehydrogenase was used as a measure. Cells were seeded at 1000, 2500, 5000, and 10000 cells/well 18 h prior to drug challenge. The cells were then continuously treated with various concentrations of 5-FU (ranging from 1.6 to 1.0 mM) for 72 h. The control group was treated with PBS only. Following drug challenge, 10 μl of WST-1 (BioVision, Mountain View, CA) was added and incubation was continued for two hours. The percentage survival of cells was quantified by being compared to the control group. All procedures were performed in triplicate.

Preparation of nuclear extract. The procedure for preparing nuclear extracts was described previously (24). Briefly, monolayer cells ($2\text{-}5x10^7$) grown to 70% confluence were detached with trypsin-EDTA. Following washes with PBS and solution A [20 mM Hepes, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), and 0.2 mM sucrose], cell pellets were re-suspended into 2 ml of ice cold hypotonic solution B [20 mM Hepes (pH 7.5), 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, and 1 mM PMSF] and incubated on ice for 10 min. The cell suspension was then placed in a Dounce homogenizer and subjected to 10 strokes with pestle B, followed by centrifugation at 4˚C, 2,000 x g for 10 min. The supernatant (cytoplasmic extract) was collected and the pellet was re-suspended into 3 ml of solution C [20 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 0.25 mM sucrose]. The nuclear suspension was layered over 3 ml of solution D [20 mM Hepes (pH 7.5), 2 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF and 0.6 mM sucrose] before being centrifuged at 4˚C, 15,000 x g for 30 min. The nuclear pellet was re-suspended into 0.75 ml of solution E (50 mM Hepes, pH 7.5 and 10% sucrose), and the nuclear

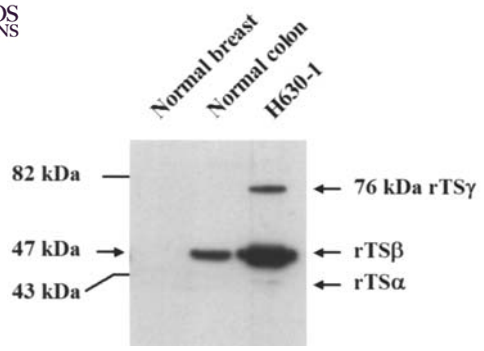

Figure 1. Immunological characterization of monoclonal antibodies to rTSß. Normal breast and colon were resected as non-tumor counterparts from patients with breast cancer or colon cancer. Colon cancer cell, H630-1, extract was used as a positive control. The antibodies also interact with a 43 kDa protein (rTSα) and a 76 kDa protein (rTSγ). However, the signals of rTSα and rTSγ proteins are much weaker than that of rTSß.

preparation was incubated with 0.3 M of NaCl on ice for 30 min. After centrifugation at 4˚C, 15,000 x g for 30 min, the supernatant was saved as a nuclear extract.

Statistical analysis. Relations between rTSß expression and clinicopathological parameters were analyzed using the Chi-square test. When the expected number of any analysis was smaller than or equal to five cases, Fisher's exact test was used. Coefficient of rank correlation between rTSß expression and other factors were analyzed by Spearman rank correlation, in which the rank correlation coefficient ranges between -1 and 1. Survival curves were plotted with the Kaplan-Meier method (25), and the statistical difference of survival between different groups was compared by the log-rank test (26). Statistical analysis was performed using GraphPad Prism4 statistical software (San Diego, CA). A P-value of <0.05 was considered as statistically significant.

Results

Characterization of antibodies to rTSß. Titer of antibodies was measured by enzyme-linked immunosorbent assay (ELISA). Specificity of the antibodies was determined by immuno-blotting in order to recognize a 47 kDa band in a whole cell lysate of cancer cells (Fig. 1). A 76 kDa protein band was frequently detected in drug-resistant H630-1 colon cancer cells (16).

Expression of rTSß in breast cancer cells and the correlation with survival in patients with primary breast cancer. Expression of *rTS* was first examined by immunoblot analysis in the primary breast cancers, and rTSß was mainly detected in the tumor fraction (Fig. 2A). However, no rTS*α* or rTSγ was detected in these breast cancer specimens. Pathologically, 58 patients (45.0%) were positive for rTSß expression as determined by immunohistochemistry (Fig. 2B). Notably, among these patients, nuclear rTSß was detected in 35 (27.1%) of the specimens (Fig. 2B, right panel). Clinically, the median follow-up period for the patients was 30 months, ranging from 2.0 to 84 months. Mean age of the patients was 52.0 years (24 - 67 years). Statistical differences between

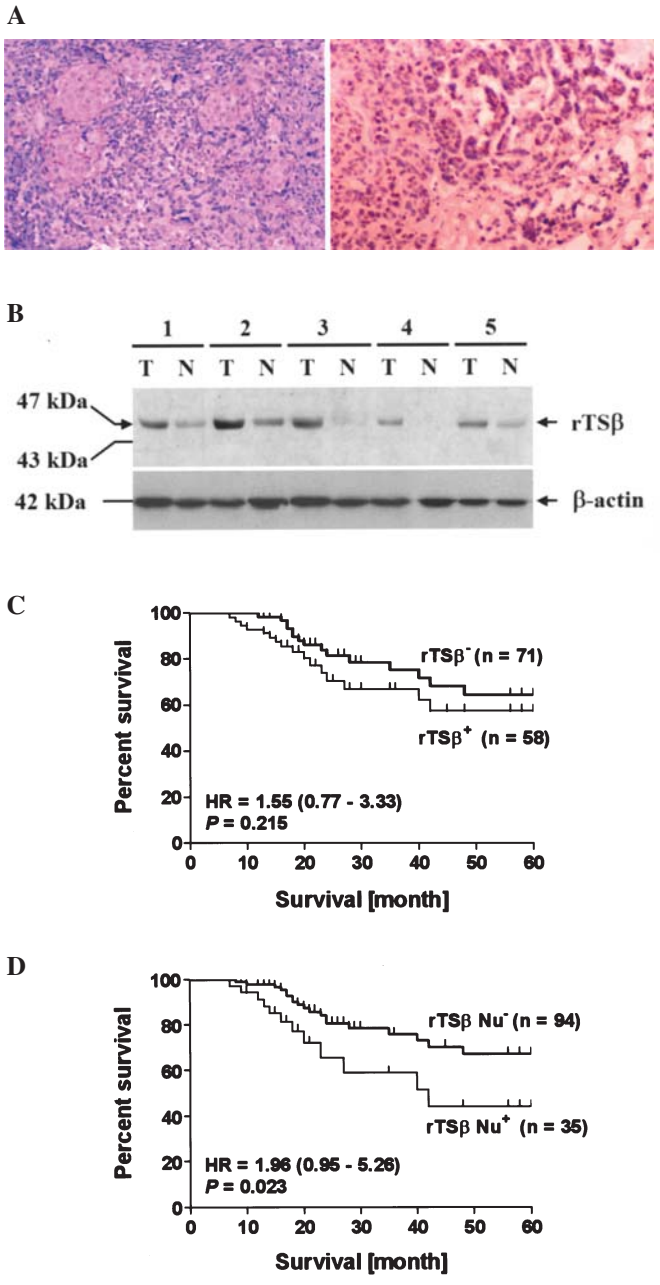

Figure 2. Expression of rTSß and patients' survival. (A) Immunohistochemical staining. Left panel: rTSß in cytoplasm. Right panel: rTSß in nuclei (original magnification x200). (B) Detection of rTSß by immunoblotting. T: tumor fraction, N: non-tumor counterpart. Survival difference between patient groups that were divided by (C) expression of rTSß or not (P=0.215), or (D) the presence of nuclear rTSß (P=0.023) or not. rTSß Nu$^+$: the presence of nuclear rTSß in cancer cells, rTSß Nu$^-$: rTSß was present in the cytoplasm of the cancer cells.

patients with and those without nuclear rTSß expression were found to be significant with respect to patients' age (p=0.029), tumor stage (p=0.01), lymph node involvement (p=0.034), presence of estrogen and/or progesterone receptor (p=0.03), and status of Her-2/neu gene amplification (p<0.001) (Table I). Following surgery, 26 patients had tumor recurrence within 36 months. Among the 35 patients who had nuclear rTSß expression, 12 (34.3%) patients had tumor recurrence. Among the 94 patients who had only cytoplasmic rTSß expression, 14 (14.9%) patients had tumor recurrence. The difference was

Table I. Correlation of rTSß expression with clinicopathological parameters.

Clinicopathological parameter	Nuclear rTSß expression		P-value
	No (n=94)	Yes (n=35)	
Age (yr) (n=129)	53.4±7.9	48.2±7.3	0.001[a]
≥45 (n=57)	47	10	0.029[b]
<45 (n=72)	47	25	
Tumor size			
T1 (n=17)	15	2	0.01[b]
T2 (n=68)	54	14	
≥T3 (n=44)	25	19	
Lymph node involvement			
N0 (n=43)	38	5	<0.005[b]
N1-3 (n=86)	56	30	
Nuclear grading			
1 (n=9)	8	1	0.056[b]
2 (n=46)	38	8	
3 (n=74)	48	26	
ER/PR			
ER^+/PR^+ (n=78)	52	26	0.03[b]
ER^+/PR^- or ER^-/PR^+ (n=14)	9	5	
ER^-/PR^- (n=37)	33	4	
Copies of HER-2/neu gene[c]			
3-5 or >5 (n=45)	23	22	<0.001[b]
2 (n=84)	71	13	

[a]Two-sided p-value determined by the t-test. [b]P-value determined by the χ^2 test. When the expected number of any analysis was smaller than or equal to five cases, the Fisher's exact test was used. [c]Copies of HER-2/neu gene were determined by fluorescent *in situ* hybridization using probes from Vysis, Inc. (Downers Grove, IL).

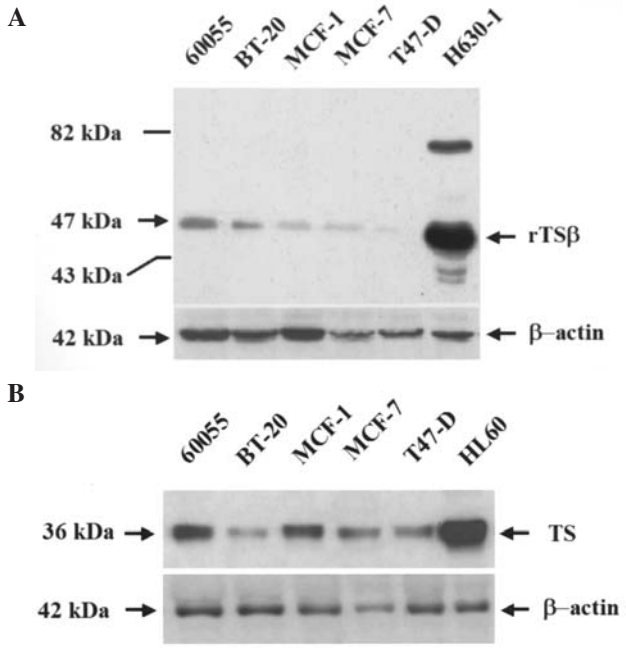

Figure 3. Expression levels of rTSß and TS proteins in breast cancer cell lines. (A) Protein (40 μg) from the indicated cell lines was separated by gel electrophoresis and blotted as described. H630-1 was used as a positive standard. (B) Expression of TS protein; HL-60 cells were used as a positive standard to show the 36 kDa TS protein.

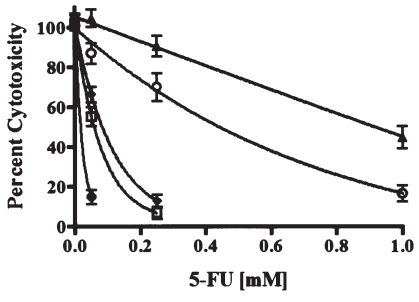

Figure 4. Sensitivity of breast cancer cells to 5-fluorouracil. Percent survival is plotted as a function of 5-FU concentration. Survival curves are the mean of triplicate experiments. ▲: BT-20, ○: 60055, ◆: MCF-7, □ MCF-1, ●: T47D.

significant (p=0.015). These results clearly showed that in addition to age, expression of nuclear rTSß was correlated with advanced tumor stage, lymph node involvement, ER and/or PR expression and Her2-neu gene amplification in tumor cells. Although the difference between patient groups divided by total rTSß was not significant in survival analysis (Fig. 2C, p=0.215), patients with nuclear rTSß had poorer survival, and the statistical difference was significant (Fig. 2D, p=0.023). The median survival of patients with nuclear rTSß was 42 months. These data suggest that expression of nuclear rTSß is

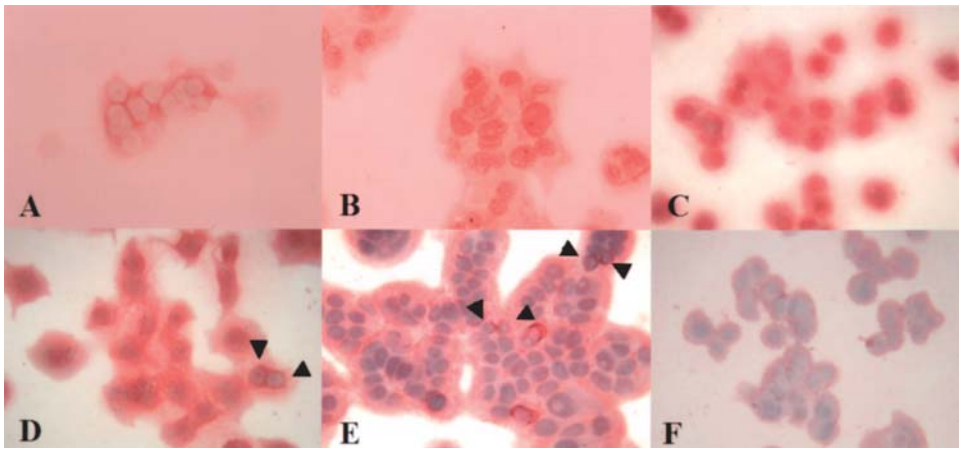

Figure 5. Differential localization of rTSß. (A) Expression of rTSß was detected in the cytoplasm of 5-FU-sensitive colon cancer cells H630. (B) rTSß protein was detected in the nuclei of 5-FU-resistant cells H630-1. (C) rTSß was found in the nuclei of 5-FU-resistant breast cancer cells H60055. (D) rTSß protein was located in the perinuclear region of the possibly post-mitotic BT-20 cells (arrows). (E) Perinuclear rTSß protein was also detected in the possibly post-mitotic MCF-1 cells (arrows). (F) Expression of rTSß was barely detected in 5-FU-sensitive breast cancer cells T47D. Original magnification x200.

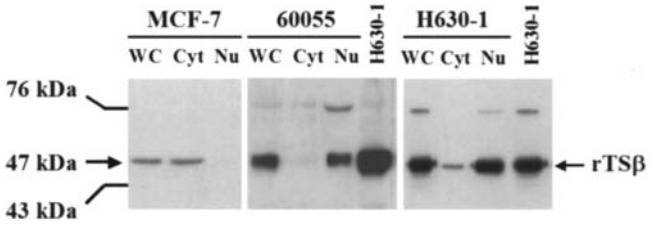

Figure 6. Subcellular distribution of rTSß. Following hypotonic treatment, cells were dounced to break the plasma membrane. Cytoplasmic and nuclear extracts were prepared as described and equal amounts (40 μg) of protein were subjected to immunoblot analysis. WC: whole cell lysate, Cyt: cytoplasmic fraction, Nu: Nuclear extract, and H630-1 extract were used as a positive control.

associated with poorer prognosis, which could be a result of drug resistance and early tumor recurrence.

Expression of rTSß is associated with 5-FU resistance in breast cancer cells. As shown in Fig. 3A, protein level of rTSß varied among the breast cancer cell lines as determined by immunoblot analysis. Expression of rTSß was high in 60055 and BT-20, intermediate in MCF-1 and MCF-7 cells, and low in T47D cells. Expression of TS, however, was high in 60055 and MCF-1, and intermediate in the other three cell lines (Fig. 3B). Notably, resistance to 5-FU (BT-20> 60055>MCF-1=MCF-7>T47D) corresponded well to the level of rTSß, but not to that of TS in these cells (Fig. 4). The IC_{50} value of 5-FU in these cancer cells is 0.92 mM for BT-20, 0.43 mM for 60055, 87 μM for MCF-7, 52 μM for MCF-1 and 8.3 μM for T47D. Compared to T47D, the IC_{50} value of the other four cell lines is 6-110-fold higher.

Moreover, rTSß was identified in the nuclei of 5-FU-resistant cell lines (H630-1, 60055 and BT20) by immunocytochemistry (Fig. 5B-D). In cells that are sensitive to 5-FU (MCF-1 and H630), rTSß was detected in the cytoplasm (Fig. 5A, E and F). In some BT-20 and MCF-1 cells, rTSß expression is perinuclear. These cells are probably post-mitotic as judged by the conjoining smaller nuclei (indicated by arrows in Fig. 5D and E).

To study the correlation of 5-FU resistance with rTSß location, we selected three cell lines, MCF-7, 60055, and H630-1, that had representative phenotypes with respect to 5-FU resistance. In addition to what is shown in Fig. 5, location of the rTSß was determined by immunoblotting, and the results are shown in Fig. 6. For MCF-7, although the protein was located perinuclearly as determined by immunocytochemistry, it was not detected in the nuclear fraction. On the other hand, in 60055 and H630-1 cells, rTSß was identified in the nuclei by immunocytochemistry, and it was also predominantly detected in the nuclear fraction as determined by immunoblotting (Fig. 6). These data indicated that in 5-FU-resistant breast cancer and colon cancer cells, rTSß was indeed located in the nuclei.

Farnesylation of rTSß in 5-FU-resistant cancer cells. Besides the 76 kDa protein band that was detected in H630-1 cells, no apparent difference in molecular weight of rTSß from either the nucleus or cytoplasm was found. Post-translational modification with a small molecule was anticipated. Moreover, since protein farnesylation has been associated with nuclear translocation of large antigen (AgL) of hepatitis δ (HD) virus, farnesylation was expected as a potential modification of rTSß. Murine antibodies were therefore raised to recognize a 67 kDa farnesylated human serum albumin (HAS) (lane 1, Fig. 7A) but not naked HSA (lane 2, Fig. 7A) (27). Antibodies specific to farnesylated HAS also recognized rTSß, which was precipitated from a nuclear extract of 60055 cells by monoclonal antibodies specific to rTSß (lane 4, Fig. 7A). Antibodies specific to farnesylated HAS did not react with rTSß (lane 3, Fig. 7A) from T47D, in which rTSß was located in the cytoplasm.

By immunocytochemistry, antibodies specific to farnesylated HAS recognized proteins in the nuclei of HDAgL-expressing Huh-7 cell (left panel, Fig. 7B) and in those of 5-FU-resistant H630-1 cells (center panel, Fig. 7B). No immunocytochemical signal was detected in 5-FU

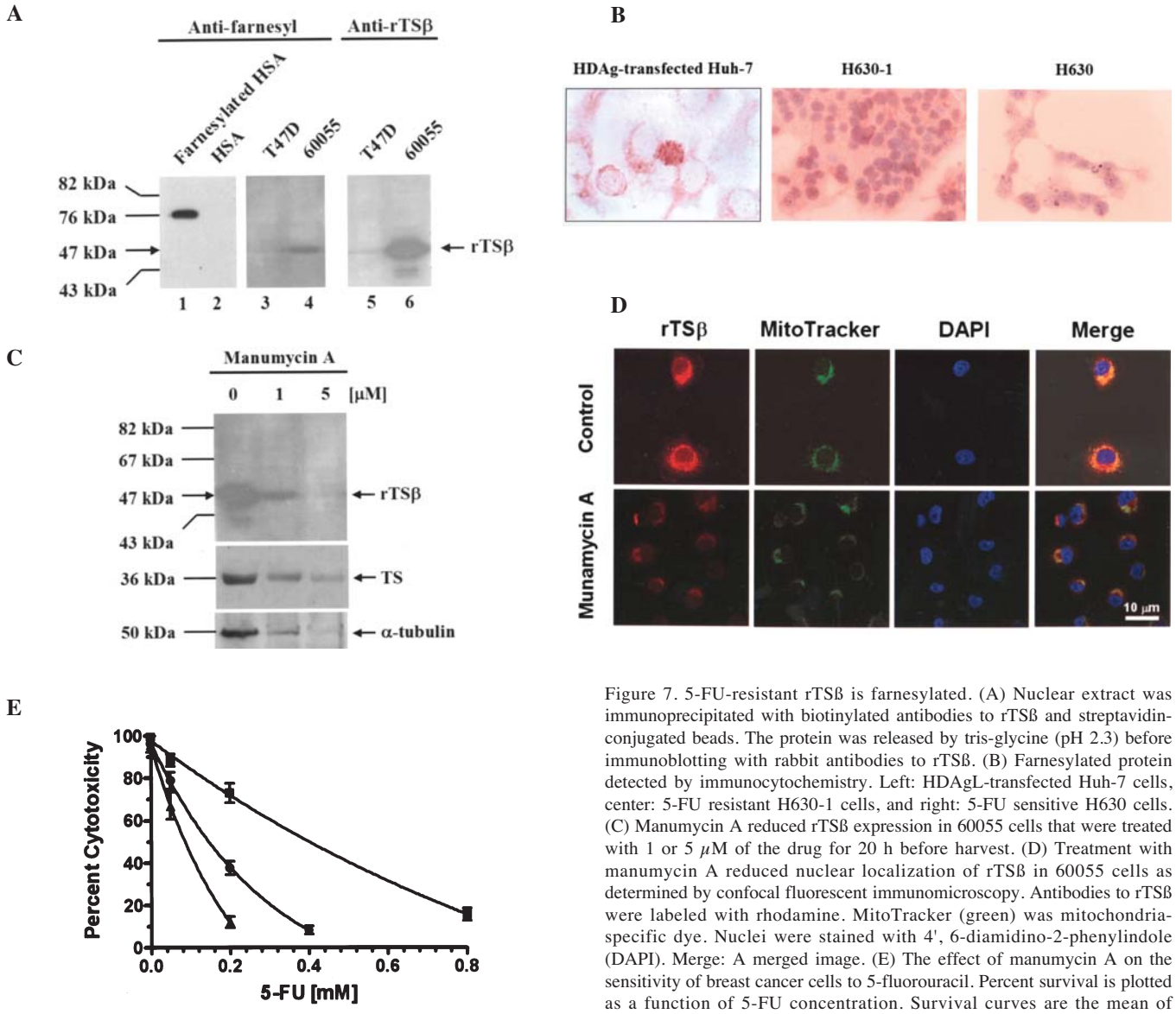

Figure 7. 5-FU-resistant rTSß is farnesylated. (A) Nuclear extract was immunoprecipitated with biotinylated antibodies to rTSß and streptavidin-conjugated beads. The protein was released by tris-glycine (pH 2.3) before immunoblotting with rabbit antibodies to rTSß. (B) Farnesylated protein detected by immunocytochemistry. Left: HDAgL-transfected Huh-7 cells, center: 5-FU resistant H630-1 cells, and right: 5-FU sensitive H630 cells. (C) Manumycin A reduced rTSß expression in 60055 cells that were treated with 1 or 5 μM of the drug for 20 h before harvest. (D) Treatment with manumycin A reduced nuclear localization of rTSß in 60055 cells as determined by confocal fluorescent immunomicroscopy. Antibodies to rTSß were labeled with rhodamine. MitoTracker (green) was mitochondria-specific dye. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI). Merge: A merged image. (E) The effect of manumycin A on the sensitivity of breast cancer cells to 5-fluorouracil. Percent survival is plotted as a function of 5-FU concentration. Survival curves are the mean of triplicate experiments. ■: 60055 cells, ●: 60055 cells treated with 1 μM manumycin A, ▲: 60055 cells treated with 5 μM manumycin A.

sensitive H630 cells (right panel, Fig. 7B). The addition of manumycin A, a farnesyltransferase inhibitor (FTI), significantly reduced expression (Fig. 7C) and nuclear level of rTSß (Fig. 7D). Treatment with manumycin A also reduced 5-FU resistance of the cancer cells (Fig. 7E).

Discussion

The data presented above showed that the presence of nuclear rTSß in breast cancer correlated with patient survival, and patients with nuclear expression of rTSß protein had a significantly higher incidence of tumor recurrence and poorer prognosis. Results of the *in vitro* study further demonstrated that 5-FU-resistant cancer cells had a higher level of rTSß protein in the nucleus.

The biological presentation of *rTS*, which is located on the same stretch of chromosome, but in the opposite direction to the TS gene, has suggested its characteristics in counteracting TS gene expression (14). An *in vitro* study by Dolnick *et al* showed that cultured tumor cells with elevated *rTS* gene expression are frequently associated with increased resistance to 5-FU and methotrexate (MTX) (15). They further demonstrated that rTSß could down-regulate TS expression, induce cell growth arrest and increase drug resistance *in trans via* the production of lipophilic metabolites of methionine or the effect of antisense RNA to TS (16). Although cell cycle arrest and 5-FU resistance are anticipated, the events that follow the generation of lipophilic metabolites are not well elucidated. Amino acid sequence analysis of rTSß shows that the enzyme may have motifs similar to L-alanine-DL-glutamate epimerase and other enzymes of the enolase superfamily, which are closely associated with the biogenesis of the cell membrane (28). We confirmed their findings by showing that rTSß overexpression correlated with 5-FU resistance in both breast and colon cancer cells. Moreover, results of the immunocytochemistry and immunoblotting showed that rTSß was frequently detected in the nuclei and the nuclear level of rTSß was associated with 5-FU resistance. These results, however, did not fulfill the expected enzyme function. On the other hand, when

ed together with clinical outcomes, our work l that the nuclear level of rTSß may not only be associated with increased 5-FU resistance, but also with a higher incidence of lymph node involvement, ER and/or PR expression, *Her2-neu* gene amplification and advanced tumor stage, events that are intimately associated with the rapid growth of breast cancer cells (14-16), and that rTSß may act as a nucleus-housed glycolytic protein, which may play a role during G2/M phases of cell cycle progression.

Studies by Spence *et al* (29) and Bugler *et al* (30) have shown that proteins, such as prostatic probasin and basic fibroblast growth factor (bFGF), behave differently when proteins are located in the different organelles. Moreover, Feo *et al* (31) demonstrated that α-enolase, a glycolytic enzyme that catalyzes interconversion of 2-phosphoglycerate and phosphoenol-pyruvate in cytoplasm, could bind to the P2 promoter and function as a repressor for the transcription of *c-myc* oncogene. Notably, nuclear prostatic probasin, bFGF and α-enolase are frequently truncated forms of the protein, which could expose the nuclear localization sequence (NLS) that is deeply embedded in the natural protein configuration (29,32), or these proteins could be translated from an alternate initiation site to affix an extra peptide containing a NLS motif to N-terminus of the cognate protein (30). Moreover, because part of the rTSß motif resembles enolase (28), it is therefore reasonable to suggest that rTSß behaves like enolase having dual functions depending upon where the protein is located. However, in addition to organelle localization, no visible difference was found in molecular weight between nuclear and cytoplasmic rTSß.

The detection of farnesylated rTSß in the nucleus indicated that isoprenylation of the protein might be responsible for nuclear transportation of the protein. The presence of rTSß in the nucleus may then affect DNA replication and 5-FU sensitivity. Treatment with manumycin A, on the other hand, decreased expression and nuclear translocation of rTSß. The addition of manumycin A also inhibits the protein level of TS and α-tubulin, and such results suggest that farnesylation of rTSß could prevent protein degradation and maintain protein level in the cells (14-16). Moreover, our data indicated that like fatty acyl modification of the G-proteins, which help in determining the final destination of the protein either to the plasma membrane or to the endoplasmic reticulum to carry out the respective biological functions (32), farnesylation could also help in determining the organelle targeting of rTSß. The nucleus-translocated rTSß is thus an important regulatory element for phenotype expression.

It is worth noting that treatment with manumycin A, which was designed to inhibit farnesylation of *ras* protein, increased 5-FU cytotoxicity. A study by Russo *et al* (33) suggested that FTI mediates an increase of 5-FU cytotoxicity *via* a p53-dependent pathway. They further showed that the inactivation of p53 lowered FTI's effect on 5-FU toxicity (34). However, p53 is not a direct target of manumycin A. Inactivation of ras-GTPase, on the other hand, induces growth arrest and reduces DNA synthesis, which reflects on the decrease of 5-FU cytotoxicity that is cell cycle-dependent (35).

Hussein and Taylor showed that farnesylation of kinetochore protein Cenp-F is required for cell cycle progression through G2/M phases, and the reaction is crucial for preventing rapid protein turnover following mitosis (36). These observations supported our results, and clearly provided an explanation as to how FTIs affect cell growth in addition to p53 (33,34). However, since no canonical CAAX sequence, a protein farnesylation signal (37), was identified at the C-terminus of rTSß, we are less certain that nuclear rTSß follows the traditional farnesylation process (37-39). Therefore, besides a quantitative difference in gene expression, qualitative change and different organelle localization of the individual gene product may also play an important role in determining the pathological activities of malignant cells, which in turn affects drug sensitivity and prognosis of the patients.

In conclusion, our data show that rTSß expression was frequently associated with poor prognosis in patients with primary breast cancer, especially in those with nuclear rTSß. *In vitro*, higher expression level of nuclear rTSß also correlated with lower 5-FU cytotoxicity of breast cancer cell lines. Compared to cytoplasmic rTSß, nuclear rTSß was positive for farnesylation. These results suggest that expression of nuclear rTSß could be an important factor in reflecting 5-FU resistance in patients with primary breast cancer, and nuclear localization of rTSß is farnesylation-dependent.

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