

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

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Received July 19, 2007; Accepted October 11, 2007

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 rTS β , a reverse orientation gene of TS, as a 5-FU resistance marker in patients with primary breast cancer. Expression of rTS β was examined in 129 patients with newly diagnosed breast cancer and five breast cancer cell lines by immunohistochemistry, immunocytochemistry and immunoblotting. Clinically, expression of rTS β was found to correlate with survival of the patients ($p=0.023$) when patients received chemotherapeutic regimen containing 5-FU. *In vitro*, rTS β expression was found to correlate with 5-FU resistance in breast cancer cell lines. Notably, in the 5-FU-resistant cells, rTS β was identified in the nucleus, whereas in the 5-FU-sensitive cells, rTS β was found in the cytoplasm. Nuclear localization of rTS β was further found to be associated with protein farnesylation. Therefore, nuclear expression of rTS β could be a novel 5-FU resistance marker in patients with primary breast cancer.

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 N⁵, 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 rTS α or a 47 kDa rTS β protein, of which in the carboxyl end of both proteins a fragment of 340 amino acid residues is identical. Both rTS α

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

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

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

In this study, we investigated the correlation between rTSB expression and clinical outcome of 5-FU-response. We further examined gene expression of TS and rTSB in five breast cancer cell lines. Our results showed that the nuclear level of rTSB 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, Carpinteria, CA). The chromogenic reaction was visualized by peroxidase-conjugated streptavidin (Dako) and aminoethyl carbazole (Sigma). Slides were counterstained 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, 5×10^6 cells were washed with phosphate buffered saline (PBS) (100 mM Na₂HPO₄, 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% β-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 rTSB 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 ($2-5 \times 10^7$) 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 MgCl₂, 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 MgCl₂, 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 MgCl₂, 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 MgCl₂, 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

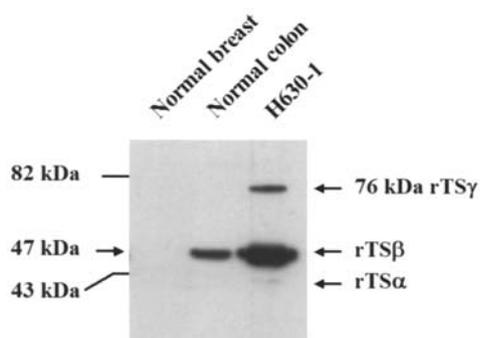


Figure 1. Immunological characterization of monoclonal antibodies to rTS β . Normal breast and colon were resected as non-tumor counterparts from patients with breast cancer or colon cancer. Colon cancer cell, H630-1, extract was used as a positive control. The antibodies also interact with a 43 kDa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

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 rTS β 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 rTS β 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 rTS β . Titer of antibodies was measured by enzyme-linked immunosorbent assay (ELISA). Specificity of the antibodies was determined by immunoblotting 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 rTS β 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 rTS β was mainly detected in the tumor fraction (Fig. 2A). However, no rTS α or rTS γ was detected in these breast cancer specimens. Pathologically, 58 patients (45.0%) were positive for rTS β expression as determined by immunohistochemistry (Fig. 2B). Notably, among these patients, nuclear rTS β 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

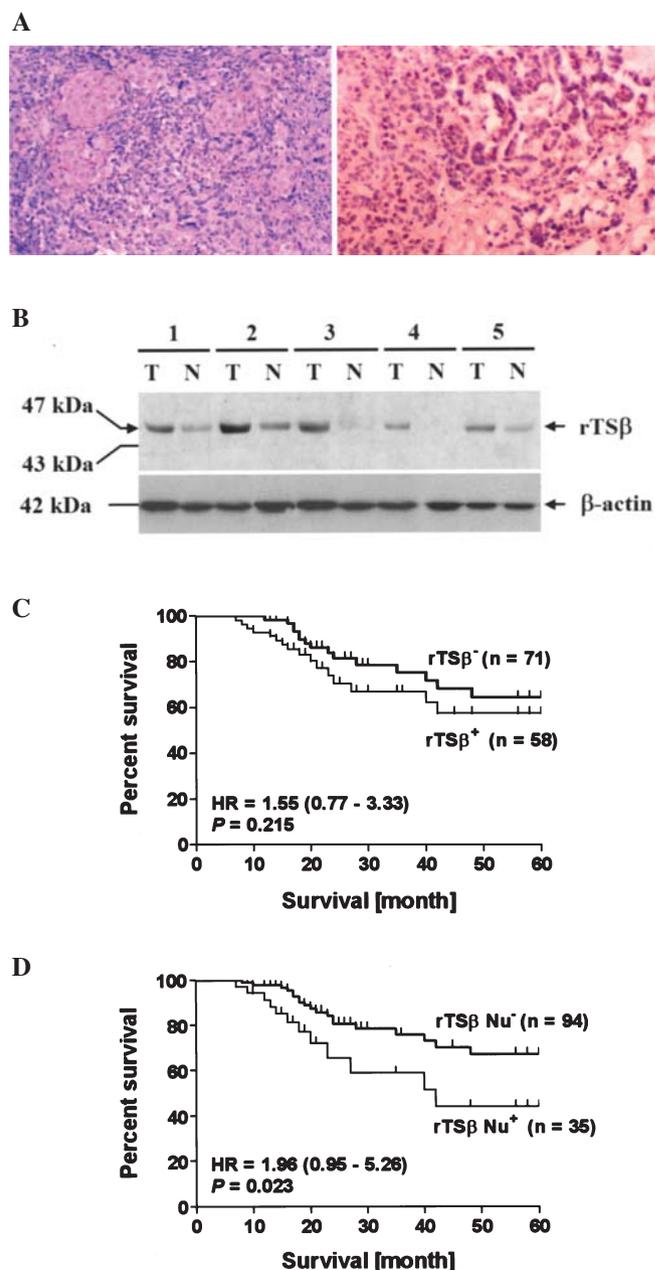


Figure 2. Expression of rTS β and patients' survival. (A) Immunohistochemical staining. Left panel: rTS β in cytoplasm. Right panel: rTS β in nuclei (original magnification x200). (B) Detection of rTS β by immunoblotting. T: tumor fraction, N: non-tumor counterpart. Survival difference between patient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

patients with and those without nuclear rTS β 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 rTS β expression, 12 (34.3%) patients had tumor recurrence. Among the 94 patients who had only cytoplasmic rTS β expression, 14 (14.9%) patients had tumor recurrence. The difference was

Table I. Correlation of rTS β expression with clinicopathological parameters.

Clinicopathological parameter	Nuclear rTS β expression		P-value
	No (n=94)	Yes (n=35)	
Age (yr) (n=129)	53.4 \pm 7.9	48.2 \pm 7.3	0.001 ^a
\geq 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	
\geq 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	

^aTwo-sided p-value determined by the t-test. ^bP-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. ^cCopies of HER-2/neu gene were determined by fluorescent *in situ* hybridization using probes from Vysis, Inc. (Downers Grove, IL).

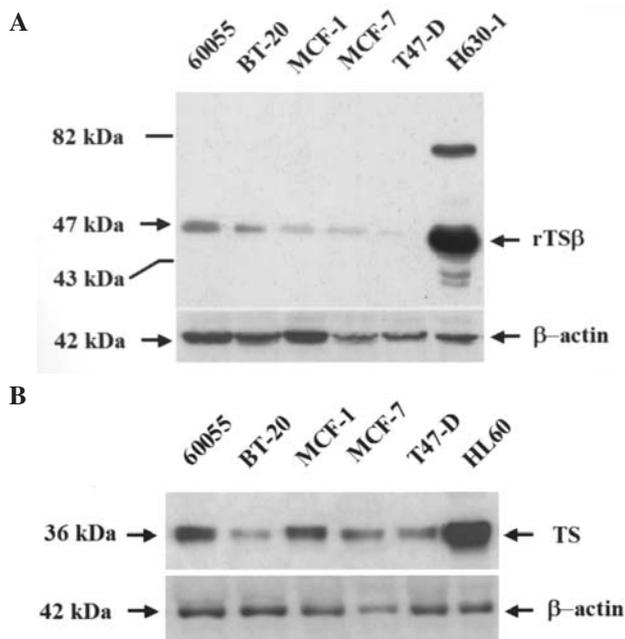


Figure 3. Expression levels of rTS β and TS proteins in breast cancer cell lines. (A) Protein (40 μ g) from the indicated cell lines was separated by gel electrophoresis and blotted as described. H630-1 was used as a positive standard. (B) Expression of TS protein; HL-60 cells were used as a positive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

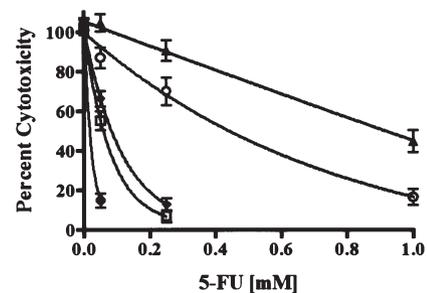


Figure 4. Sensitivity of breast cancer cells to 5-fluorouracil. Percent survival is plotted as a function of 5-FU concentration. Survival curves are the mean of triplicate experiments. \blacktriangle : BT-20, \circ : 60055, \blacklozenge : MCF-7, \square : MCF-1, \bullet : T47D.

significant ($p=0.015$). These results clearly showed that in addition to age, expression of nuclear rTS β 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 rTS β was not significant in survival analysis (Fig. 2C, $p=0.215$), patients with nuclear rTS β had poorer survival, and the statistical difference was significant (Fig. 2D, $p=0.023$). The median survival of patients with nuclear rTS β was 42 months. These data suggest that expression of nuclear rTS β is

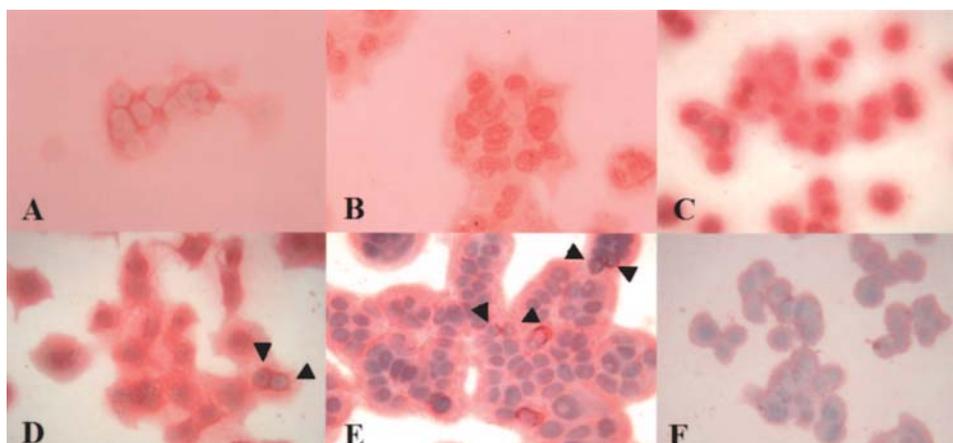


Figure 5. Differential localization of rTSB. (A) Expression of rTSB was detected in the cytoplasm of 5-FU-sensitive colon cancer cells H630. (B) rTSB protein was detected in the nuclei of 5-FU-resistant cells H630-1. (C) rTSB was found in the nuclei of 5-FU-resistant breast cancer cells H60055. (D) rTSB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

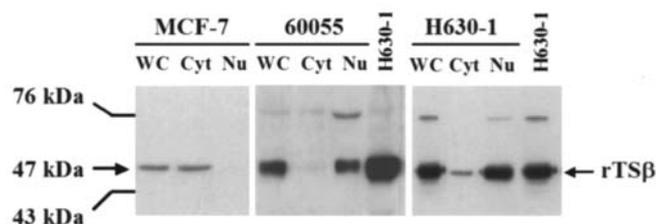


Figure 6. Subcellular distribution of rTSB. Following hypotonic treatment, cells were dounced to break the plasma membrane. Cytoplasmic and nuclear extracts were prepared as described and equal amounts (40 μ g) of protein were subjected to immunoblot analysis. WC: whole cell lysate, Cyt: cytoplasmic 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 rTSB is associated with 5-FU resistance in breast cancer cells. As shown in Fig. 3A, protein level of rTSB varied among the breast cancer cell lines as determined by immunoblot analysis. Expression of rTSB 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 rTSB, 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 μ M for MCF-7, 52 μ M for MCF-1 and 8.3 μ M for T47D. Compared to T47D, the IC_{50} value of the other four cell lines is 6-110-fold higher.

Moreover, rTSB 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), rTSB was detected in the cytoplasm (Fig. 5A, E and F). In some BT-20 and MCF-1 cells, rTSB 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 rTSB 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 rTSB 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, rTSB 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, rTSB was indeed located in the nuclei.

Farnesylation of rTSB 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 rTSB 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 rTSB. 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 rTSB, which was precipitated from a nuclear extract of 60055 cells by monoclonal antibodies specific to rTSB (lane 4, Fig. 7A). Antibodies specific to farnesylated HAS did not react with rTSB (lane 3, Fig. 7A) from T47D, in which rTSB 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

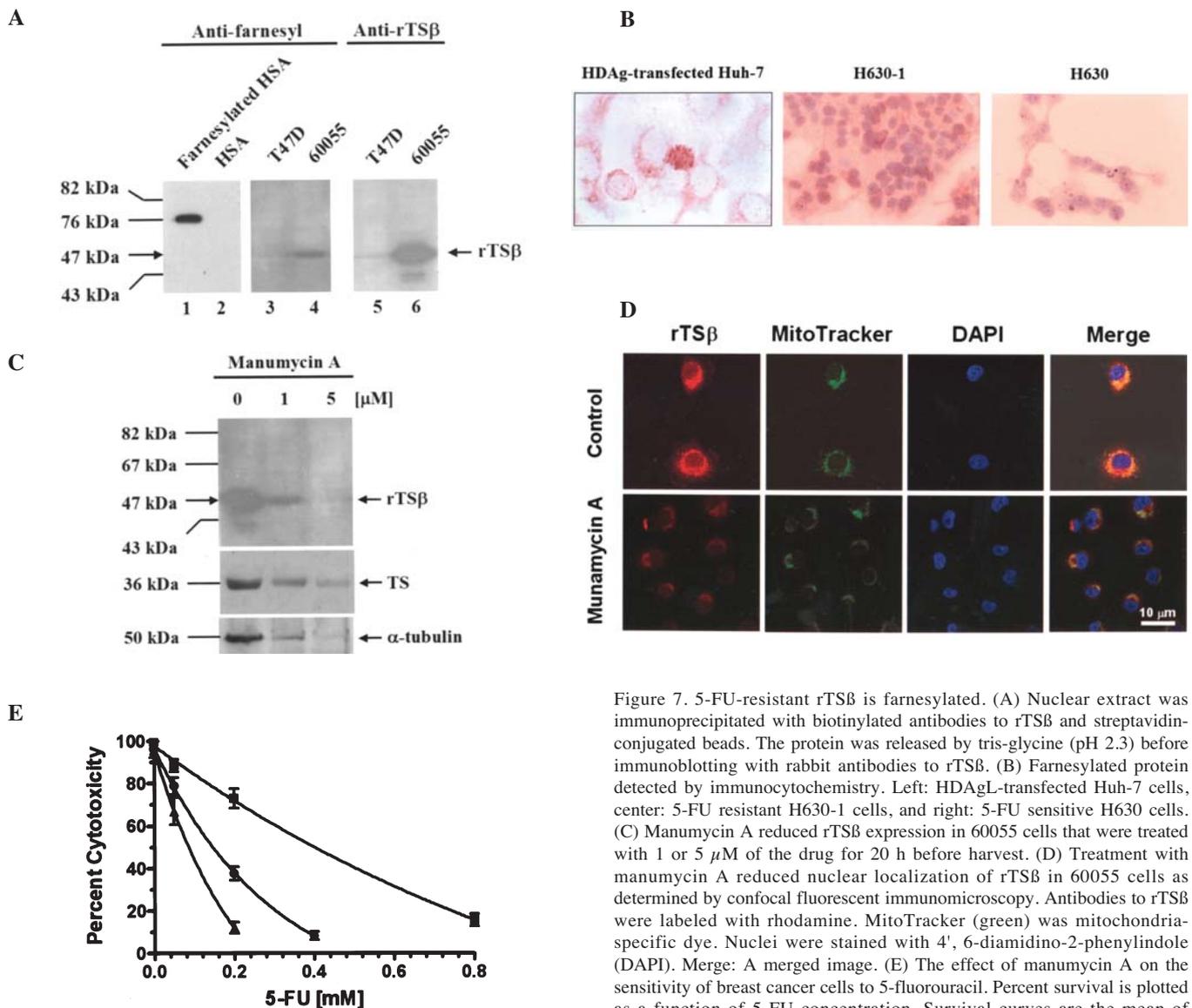


Figure 7. 5-FU-resistant rTS β is farnesylated. (A) Nuclear extract was immunoprecipitated with biotinylated antibodies to rTS β and streptavidin-conjugated beads. The protein was released by tris-glycine (pH 2.3) before immunoblotting with rabbit antibodies to rTS β . (B) Farnesylated protein detected 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 rTS β expression in 60055 cells that were treated with 1 or 5 μ M of the drug for 20 h before harvest. (D) Treatment with manumycin A reduced nuclear localization of rTS β in 60055 cells as determined by confocal fluorescent immunomicroscopy. Antibodies to rTS β 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 μ M manumycin A, ▲: 60055 cells treated with 5 μ M 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 rTS β (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 rTS β in breast cancer correlated with patient survival, and patients with nuclear expression of rTS β 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 rTS β 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 rTS β 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 rTS β 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 rTS β overexpression correlated with 5-FU resistance in both breast and colon cancer cells. Moreover, results of the immunocytochemistry and immunoblotting showed that rTS β was frequently detected in the nuclei and the nuclear level of rTS β was associated with 5-FU resistance. These results, however, did not fulfill the expected enzyme function. On the other hand, when

considered together with clinical outcomes, our work suggested that the nuclear level of rTSS β 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 rTSS β 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 rTSS β motif resembles enolase (28), it is therefore reasonable to suggest that rTSS β 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 rTSS β .

The detection of farnesylated rTSS β in the nucleus indicated that isoprenylation of the protein might be responsible for nuclear transportation of the protein. The presence of rTSS β 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 rTSS β . The addition of manumycin A also inhibits the protein level of TS and α -tubulin, and such results suggest that farnesylation of rTSS β 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 rTSS β . The nucleus-translocated rTSS β 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 rTSS β , we are less certain that nuclear rTSS β 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 rTSS β expression was frequently associated with poor prognosis in patients with primary breast cancer, especially in those with nuclear rTSS β . *In vitro*, higher expression level of nuclear rTSS β also correlated with lower 5-FU cytotoxicity of breast cancer cell lines. Compared to cytoplasmic rTSS β , nuclear rTSS β was positive for farnesylation. These results suggest that expression of nuclear rTSS β could be an important factor in reflecting 5-FU resistance in patients with primary breast cancer, and nuclear localization of rTSS β is farnesylation-dependent.

Acknowledgements

This study was supported by grants from the National Science Council (NSC95-2320-B-005-001, K.-C.C.) and Foundation of Chang-Hua Christian Hospital (95-CCH-KMU-39), Taiwan, R.O.C.

References

1. Annual reports of the Department of Health, the Executive Yuan, Republic of China, 2003.
2. Taiwan Cancer Organization Group, Cancer practices guideline of the National Health Research Institutes, Republic of China, 2003. (<http://www.nhri.org.tw>).
3. Chie WC, Chen CF, Lee WC, Chen CJ and Lin RS: Age-period-cohort analysis of breast cancer mortality. *Anticancer Res* 15: 511-515, 1995.
4. Chie WC, Chen CF, Chen CJ, Chang CL, Liaw YP and Lin RS: Geographic variation of breast cancer in Taiwan: international and migrant comparison. *Anticancer Res* 15: 2745-2749, 1995.
5. Fritz P, Murdter TE, Eichelbaum M, Siegle I, Weissert M and Zanger UM: Microsomal epoxide hydrolase expression as a predictor of tamoxifen response in primary breast cancer: a retrospective exploratory study with long-term follow-up. *J Clin Oncol* 19: 3-9, 2001.
6. Longley DB, Harkin DP and Johnston PG: 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3: 330-338, 2003.
7. Carreras CW and Santi DV: The catalytic mechanism and structure of thymidylate synthase. *Annu Rev Biochem* 64: 721-762, 1995.
8. Berger SH and Hakala MT: Relationship of dUMP and free FdUMP pools to inhibition of thymidylate synthase by 5-fluorouracil. *Mol Pharmacol* 25: 303-309, 1984.
9. Spears CP, Gustavsson BG, Berne M, Frosing R, Bernstein L and Hayes AA: Mechanisms of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. *Cancer Res* 48: 5894-5900, 1988.
10. Pickard M, Dive C and Kinsella AR: Differences in resistance to 5-fluorouracil as a function of cell cycle delay and not apoptosis. *Br J Cancer* 72: 1389-1396, 1995.
11. Kinsella AR, Smith D and Pickard M: Resistance to chemotherapeutic antimetabolites: a function of salvage pathway involvement and cellular response to DNA damage. *Br J Cancer* 75: 935-945, 1997.

12. Mader RM, Sieder AE, Braun J, *et al.*: Transcription and activity of 5-fluorouracil converting enzymes in fluoropyrimidine resistance in colon cancer *in vitro*. *Biochem Pharmacol* 54: 1233-1242, 1997.
13. Mader RM, Muller M and Steger GG: Resistance to 5-fluorouracil. *Gen Pharmacol* 31: 661-666, 1998.
14. Dolnick BJ: Cloning and characterization of a naturally occurring antisense RNA to human thymidylate synthase mRNA. *Nucleic Acids Res* 21: 1747-1752, 1993.
15. Dolnick BJ, Black AR, Winkler PM, Schindler K and Hsueh CT: rTS gene expression is associated with altered cell sensitivity to thymidylate synthase inhibitors. *Adv Enzyme Regul* 36: 165-180, 1996.
16. Dolnick BJ, Angelino NJ, Dolnick R and Sufirin JR: A novel function for the rTS gene. *Cancer Biol Ther* 2: 364-369, 2003.
17. Romain S, Spyrtos F, Descotes F, *et al.*: Prognostic of DNA-synthesizing enzyme activities (thymidine kinase and thymidylate synthase) in 908 T1-T2, N0-N1, M0 breast cancers: a retrospective multicenter study. *Int J Cancer* 87: 860-868, 2000.
18. Romain S, Bendahl PO, Guirou O, Malmstrom P, Martin PM and Ferno M: DNA-synthesizing enzymes in breast cancer (thymidine kinase, thymidylate synthase and thymidylate kinase): association with flow cytometric S-phase fraction and relative prognostic importance in node-negative premenopausal patients. *Int J Cancer* 95: 56-61, 2001.
19. Ogura O, Kanzaki A, Bando H, Ogura T, Toi M and Takebayashi Y: Expression of thymidylate synthase and thymidine phosphorylase in human breast carcinoma: implication for method to detect expression of these molecules in clinic. *Cancer Lett* 190: 97-104, 2003.
20. Dolnick BJ and Black AR: Alternate splicing of the rTS gene product and its overexpression in a 5-fluorouracil-resistant cell line. *Cancer Res* 56: 3207-3210, 1996.
21. Chiou CF, Chow KC, Lin FM, Lin CK, Liu SM and Chen KY: Expression of DNA topoisomerase II α and multidrug resistance p-glycoprotein in acute leukemia - an immunohistochemical study. *Chin Med J (Taipei)* 60: 184-190, 1997.
22. Chow KC and Ross WE: Topoisomerase specific drug sensitivity in relation to cell cycle progression. *Mol Cell Biol* 7: 3119-3123, 1987.
23. Hsu NY, Ho HC, Chow KC, *et al.*: Overexpression of dihydrodiol dehydrogenase as a prognostic marker of non-small cell lung cancer. *Cancer Res* 61: 2727-2731, 2001.
24. Challberg MD and Kelly Jr TJ: Adenovirus DNA replication *in vitro*. *Proc Natl Acad Sci USA* 76: 655-659, 1979.
25. Kaplan EL and Meier P: Nonparametric estimation from incomplete observations. *J Am Stat Assoc* 53: 457-481, 1958.
26. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemother Rep* 50: 163-170, 1966.
27. Lin HP, Hsu SC, Wu JC, Sheen JJ, Yan BS and Syu WJ: Localization of isoprenylated antigen of hepatitis delta virus by anti-farnesyl antibodies. *J Gen Virol* 80: 91-96, 1999.
28. Pagliaro L and Taylor DL: 2-Deoxyglucose and cytochalasin D modulate aldolase mobility in living 3T3 cells. *J Cell Biol* 118: 859-863, 1992.
29. Spence AM, Sheppard PC, Davie JR, *et al.*: Regulation of a bifunctional mRNA results in synthesis of secreted and nuclear probasin. *Proc Natl Acad Sci USA* 86: 7843-7847, 1989.
30. Bugler B, Amalric F and Prats H: Alternative initiation of translation determines cytoplasmic or nuclear localization of basic fibroblast growth factor. *Mol Cell Biol* 11: 573-577, 1991.
31. Feo S, Arcuri D, Piddini E, Passantino R and Giallongo A: ENO1 gene product binds to the c-myc promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1). *FEBS Lett* 473: 47-52, 2000.
32. Michaelson D, Ahearn I, Bergo M, Young S and Philips M: Membrane trafficking of heterotrimeric G proteins via the endoplasmic reticulum and Golgi. *Mol Biol Cell* 13: 3294-3302, 2002.
33. Russo P, Ottoboni C, Malacarne D, Crippa A, Riou JF and O'Connor PM: Nonpeptidomimetic farnesyltransferase inhibitor RPR-115135 increases cytotoxicity of 5-fluorouracil: role of p53. *J Pharmacol Exp Ther* 300: 220-226, 2002.
34. Russo P, Malacarne D, Falugi C, Trombino S and O'Connor PM: RPR-115135, a farnesyltransferase inhibitor, increases 5-FU cytotoxicity in ten human colon cancer cell lines: role of p53. *Int J Cancer* 100: 266-275, 2002.
35. Miyakis S, Sourvinos G and Spandidos DA: Differential expression and mutation of the *ras* family genes in human breast cancer. *Biochem Biophys Res Commun* 251: 609-612, 1998.
36. Hussein D and Taylor SS: Farnesylation of Cenp-F is required for G2/M progression and degradation after mitosis. *J Cell Sci* 115: 3403-3414, 2002.
37. Adamson P, Marshall CJ, Hall A and Tilbrook PA: Post-translational modifications of p21rho proteins. *J Biol Chem* 267: 20033-20038, 1992.
38. Martincic I, Peralta ME and Ngsee JK: Isolation and characterization of a dual prenylated Rab and VAMP2 receptor. *J Biol Chem* 272: 26991-26998, 1997.
39. Fiordalisi JJ, Johnson RL II, Weinbaum CA, *et al.*: High affinity for farnesyltransferase and alternative prenylation contribute individually to K-Ras4B resistance to farnesyltransferase inhibitors. *J Biol Chem* 278: 41718-41727, 2003.