Original Article

rTSβ as a Novel 5-fluorouracil Resistance Marker of Colorectal Cancer: A Preliminary Study

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Abstract

Introduction: Colorectal cancer is the most common form of malignancy in Taiwan and the third leading cause of death from cancer, preceded only by lung and hepatic cancers. Colorectal cancer is typically treated by surgical intervention and/or chemotherapy and radiotherapy, if necessary. To date, 5-fluorouracil (5-FU) is the most commonly used anti-cancer chemotherapy drug. However, patients commonly experience resistance to the drug therefore limiting its efficiency. In this study, we measured the expression of rTSβ in human colon cancer as a novel 5-FU resistance marker. Materials and Methods: We collected 172 colon cancer samples from 4 different hospitals (including 21 pairs of colon cancer biopsies and 151 pathologic slides of colon cancer). In vitro, we measured the cytotoxicity of 5-FU and 5-FU plus leucovorin in H630 and H630-1 colon cancer cell lines. Results: The results revealed that rTSβ was expressed in 115 (66.9 %) pathology samples and that tumour expression was higher than in corresponding normal tissue. Survival rates of up to 5 years following treatment was significantly higher for patients without rTSβ expression than for those with rTSβ expression (P = 0.0023). In vitro, H630-1 (with rTSβ overexpression) had significantly higher IC_{50} of 5-FU than did H630. IC_{50} of 5-FU decreased when leucovorin was added. Conclusions: Results indicate a close relationship between rTSβ expression and resistance to the drug 5-FU in human colorectal cancer. These results provide further evidence for rTSβ expression as a novel 5-FU resistance marker of colorectal cancer.

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Introduction

Although colorectal cancer is common in Western countries, in the past, it has been uncommon in Asian countries. However, its prevalence has gradually been increasing. Since 1982, malignant cancers have been the leading cause of death in Taiwan. Furthermore, as of 2004, colorectal cancer has become the most common form of cancer and is currently the third leading cause of cancer death in Taiwan, preceded only by lung and hepatic cancers.[1] Although the majority of colorectal cancers are found in patients over 50 years old, an increasing number of patients in Taiwan are young with a poor prognosis.[2]

Treatment of colorectal cancer involves surgical intervention and/or chemotherapy and radiotherapy, if necessary. For chemotherapy, 5-fluorouracil (5-FU) is the most commonly used anti-cancer drug. Yet 5-FU has been found to be only 20% effective and patients require several courses of treatment before the most suitable chemotherapeutic agents are found.[3] Therefore, many studies seek to discover the most effective treatment combination.

5-FU is a pro-drug that can be converted to fluorouridine triphosphate (FUTP) to interfere with RNA synthesis, or to 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) to inhibit thymidylate synthase (TS). In addition, in the presence of 5'10-methylene tetrahydrofolate (mTHF), it will combine with FdUMP and TS to form a stable ternary complex and further interfere with the methylation of deoxyuridine monophosphate (dUMP).[4-6] TS expression in tumour cells is therefore expected to be an important

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prognostic factor, particularly for patients scheduled for chemotherapy with 5-FU.[7,8]

Although previous studies have found a correlation between 5-FU sensitivity and the TS protein, several studies reveal that rTS protein can affect the expression of the TS protein.[9-12] Therefore, the correlation between 5-FU sensitivity and rTS might be considered an important factor for patients receiving chemotherapy.

The rTS gene (ENOSF1) and TS gene are both located on the chromosome 18. Nevertheless, the orientations of these 2 genes are opposite, and their 3' ends are complementary to each other over a stretch of approximately 1 kilobase-pair. The rTS gene contains 17 exons and can be translated into a 41 kDa rTSα and a 47 kDa rTSβ via alternate splicing.[13] Although the rTSβ is the main product, both rTSα and rTSβ can induce the down-regulation of TS.[14]

In this study, we use a monoclonal antibody of rTSβ to evaluate the expression of rTSβ in human colon cancer. We then compared the difference of rTSβ expression between cancer and corresponding normal tissue. In vitro, we further evaluated the correlation between rTSβ and 5-FU resistance and the efficiency of 5-FU and 5-FU plus leucovorin.

Patients and Methods

Materials, Cell Culture and Drug Treatment

We obtained the culture media and fetal calf serum (FCS) 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). Colon cancer cell line, H630 and H630-1, were grown as previously described.[13,15,16] Unless otherwise noted, cultured cells were incubated at 37°C and all media were supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Patients

We collected 21 pairs of colon cancer biopsies from the tissue bank of Cheng Ching Hospital (including the primary tumour and the corresponding normal tissue) and the Institutional Review Board (IRB) of Cheng Ching Hospital agreed to the study.

Another 151 pathologic slides of colon cancer were provided by 4 different hospitals. All these 151 patients received routine follow-up and chemotherapy of 5-FU after surgery and the 5-year survival rates were recorded. These 151 samples were collected before the enforcement of IRB of Taiwan.

Immunocytochemical, Immunohistochemical and Immunoblotting Analyses

Immunocytochemical, immunohistochemical and immunoblotting analyses were conducted as previously described.[17,18] Briefly, 5 x 10^6 cells were washed with phosphate buffered saline (PBS) [100 mM Na_2HPO_4, pH 7.4, 136 mM NaCl] twice and lysed in 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 μM/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 rTSβ protein. The signal was amplified by biotin-labelled goat anti-mouse IgG, and peroxidase-conjugated streptavidin. Protein presence was visualised by exposing the membrane to X-Omat film (Eastman Kodak, Rochester, NY) with enhanced chemiluminescent reagent (Pierce, Rockford, IL). In each case, normal colon tissue served as an internal negative control.

Slide Evaluation

Three independent pathologists without clinicopathological knowledge read the slides. A specimen was considered positive if more than 10% of cancer cells were positively stained and negative if less than 10% of the cells were positive.[19]

Cytotoxicity Assay

Cytotoxicity was determined by the modified MTT method, in which the dehydrogenase of mitochondria in live cells could change MTT into bluish MTT formazan.[20] Cells were seeded at 1000, 2500, 5000 and 10000 cells/well 18 hours before drug challenge and then continuously treated with various concentrations of 5-FU (ranged from 1.6 μM to 1.0 mM) for 72 hours. The control group was treated with PBS only. Following drug challenge, 10 μL of WST-1 (BioVision, Mountain View, CA) was added and were incubated for another 2 hours. Another group was treated with 5-FU and leucovorin 200 μM and the same procedure was repeated as previously described. Percent survival of cells was quantified by comparing with the control group. All procedures were performed in triplicate.

Statistical Analysis

Relations between rTSβ expression and clinicopathological parameters were analysed using a chi-square test. When the expected number of any analysed cell was less than or equal to 5 cases, Fisher's Exact test was used. Survival curves were plotted using the Kaplan-Meier method, and the statistical difference of survival between different groups was compared by a log rank test.[21,22] Statistical analysis was performed using GraphPad Prism4 statistical software (San Diego, CA). Statistical significance was set at $P < 0.05$.

Results

Measurement of Expression of rTSβ by Western Blotting Method

Titre of antibodies was measured by enzyme-linked immunosorbent assay (ELISA). Specificity of antibodies was determined by Western immunoblotting analysis. The antibody recognised a 47-kDa band in the cell lysate of the human colon cancer cell line (Fig. 1) as well as a much weaker protein band at 43-kDa that corresponded to the rTSα protein.

Of the 172 colon cancer samples, rTSβ expression was found in 115 samples (66.9%), including 102 slides and 13 pairs of tissue. We selected 5 pairs with clear staining from these 13 samples. Results of other 8 samples might be disturbed by the enzymes in the colon tissue. All 5 samples revealed that the expression of rTSβ in the tumour was higher than in the surrounding tissue (Fig. 2).

We found that from the 151 patients studied from slides, those with expression of rTSβ had a survival rate of 5 years less than patients without expression of rTSβ (35% vs. 70%) ($P = 0.0023$) (Fig. 3).

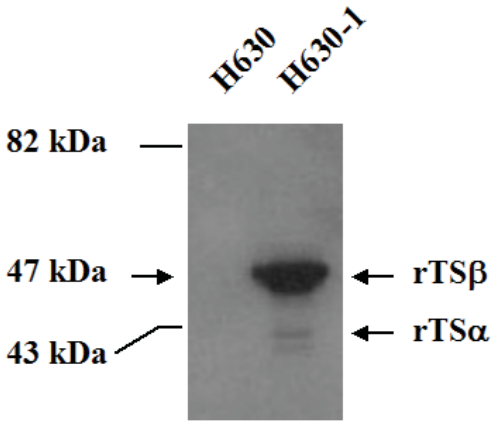

Fig. 1. Immunological characterisation of monoclonal antibodies to rTS. Colon cancer cell line, H630-1, extract was used as a positive control. The antibodies interacted with a 43-kDa protein (rTSα) and a 47-kDa protein (rTSβ). However, the signals of rTSα proteins are much weaker than that of rTSβ.

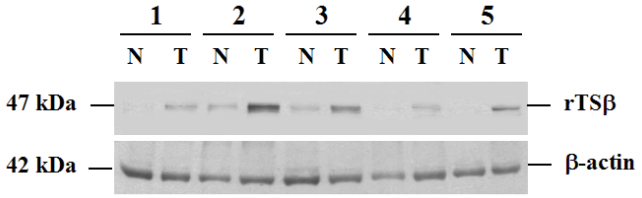

Fig. 2. Expression of rTSβ in the tumour was higher than in the surrounding tissue. From 21 pairs of colon cancer samples, 13 pairs expressed rTSβ. 5 pairs of samples were selected randomly from these 13 pairs and all revealed higher expression of rTSβ in tumour cells than non-tumour cells. (N: non-tumour, T: tumour)

Immunocytochemistry

Cells without expression of rTSβ did not reveal cytoplasmic colour after staining by ACE and haematoxylin (Fig. 4A). On the other hand, the cytoplasm in cells with expression of rTSβ was dense in colour following the same staining procedure (Fig. 4B). Figure 4B also revealed cells without expression of rTSβ.

Measurement of Cytotoxicity of 5-FU and 5-FU with Leucovorin in Colon Cancer Cells

Two human colon cell lines, H630 (without expression of rTSβ) and H630-1 (with expression of rTSβ), were cultured with 2M 5-FU for 72 hours. The results revealed that 5-FU had higher sensitivity to H630 than H630-1 (Fig. 5).

According to the method described above, H630 and H630-1 were cultured with 5-FU and 200 μM leucovorin in order to measure the cytotoxicity. Results revealed that leucovorin decreased the IC_{50} of H630 and H630-1 in the presence or absence of rTSβ expression (Fig. 5).

Discussion

Drug resistance is a serious problem for cancer patients undergoing chemotherapy. Inconsistencies in chemotherapy response exist even among patients in similar stages of the disease. Thus the availability of a drug resistance gene may help decrease unnecessary drug exposure prior to patients receiving chemotherapy.

In our study, rTSβ was found in 66.9% of colon cancer samples and tumour expression was higher than in corresponding normal tissue. Results also demonstrated that the expression of rTSβ correlated with 5-FU resistance and that leucovorin decreased IC_{50} of 5-FU. In addition, we found that the survival rate of patients with expression of rTSβ was significantly lower than those without rTSβ expression ($P = 0.0023$). Because the patient demographics

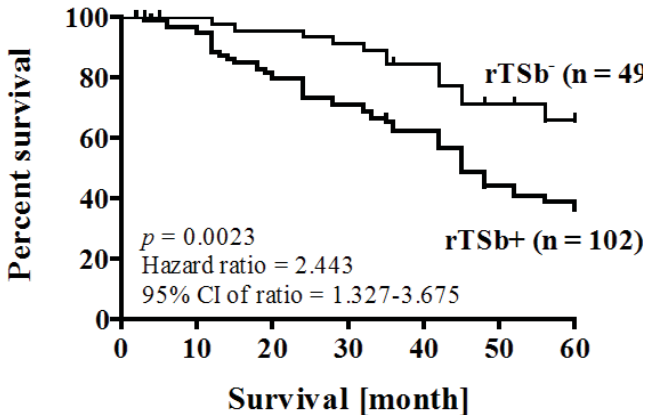

Fig. 3. Expression of rTSβ and patients' survival. Survival difference between patient groups that were divided by the presence or absence of rTSβ expression ($P = 0.0023$).

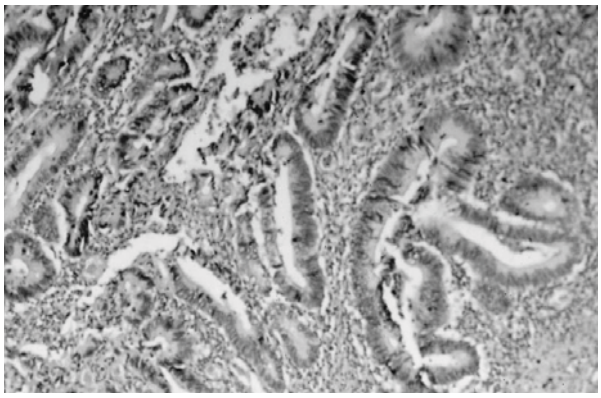

Fig. 4A.

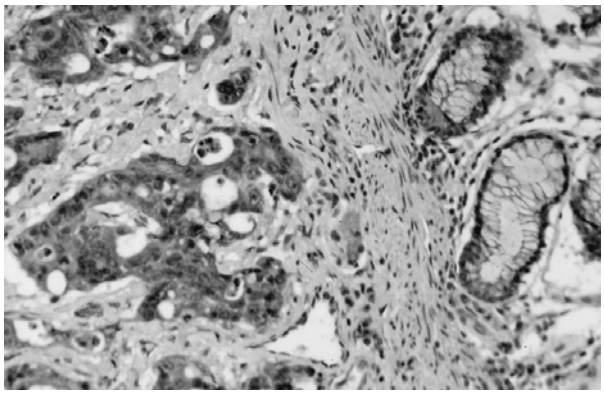

Fig. 4B.

Fig. 4. Expression of rTSβ by immunohistochemical staining. (A) No specific staining was found in the cytoplasm without expression of rTSβ (original magnification x200). (B) Left: dense staining was found in the cytoplasm with expression of rTSβ. Right: no specific staining was found in the cytoplasm without expression of rTSβ.

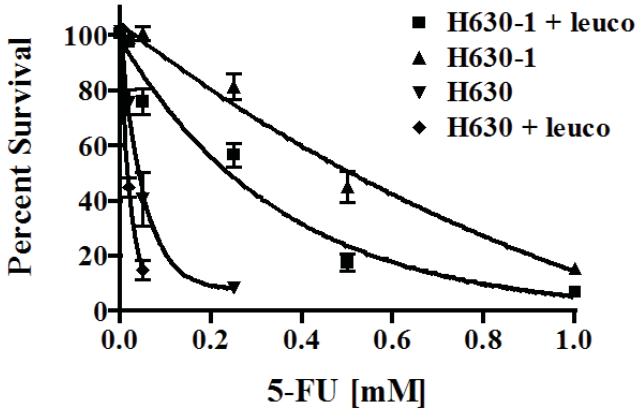

Fig. 5. Sensitivity of colon cancer cell line H630 and H630-1, to 5-fluorouracil and 5-fluorouracil plus leucovorin. Percent survival is plotted as a function of 5-FU concentration. Survival curves are the mean of triplicate experiments. ■: H630-1 + leucovorin, ▲: H630-1, ▼: H630, □: H630 + leucovorin.

were absent, we did not support the expression of rTSβ might be a prognostic factor of patients with colon cancer but it is an important resistance marker for patients receiving 5-FU. As previously suggested, a multivariate analysis is needed.

Previous studies have revealed a correlation between 5-FU resistance and TS.[23] Nonetheless, recent studies show an overexpression of rTSβ but not of TS in cancer patients with 5-FU resistance.[9,16,24] Studies conducted by Dolnick indicate that TSβ could be a negative control of TS.[16] A recent study further demonstrates that the phosphorylation state of rTSβ may be a marker for 5-FU resistance.[12]

Although 5-FU is the most common drug for chemotherapy, it is usually combined with leucovorin to elevate therapeutic efficiency.[25] Similarly, our study showed 5-FU plus leucovorin to be more efficient than 5-FU alone. Previous studies also indicate that this combination is better in patients with higher 5-FU sensitivity.[25]

Analysis of the amino acid sequence of rTSβ reveals that it is similar to L-alanine-DL-glutamate epimerase and related enzymes of the anolase superfamily. Considering that this superfamily is related to cell membrane biogenesis,[26,27] and the results of this study, it seems likely that rTSβ may induce drug resistance via the effect of cell cycle, especially in the G2/M phase.[15,16,28,29]

While studying colon cancer cell lines H630 and H630-1, Dolnick found that H630-1 (overexpression of rTSβ) secretes a novel molecule that resembles Acyl Homoserine Lactone (AHL) and that the production of this molecule was elevated with increasing rTSβ expression.[18] Although the structure of this molecule was unknown, Dolnick revealed that it could control expression of TS by a mechanism resembling the quorum sensing pathway of gram-negative bacteria.

Conclusions

We conclude that the expression of rTSβ is a novel 5-FU resistance marker of human colorectal cancer. However, further studies are necessary to determine the exact mechanism through which rTSβ operates.

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