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（新任者論文）

# 5-fluorouracil-inducible proteins in a colorectal cancer cell line

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**ABSTRACT** : 5-fluorouracil (5-FU) is the most common chemotherapeutic agent used in the treatment of colorectal cancer. To understand the intracellular changes induced by 5-FU, we attempted to identify the specific proteins affected using a proteomics analysis. A human colorectal carcinoma cell line, WiDr, was treated with 1.0 μM 5-FU. After 24-72 h, the cellular proteins were analyzed by two-dimensional gel-electrophoresis in combination with ESI Q-TOF mass spectrometry. A differential display of the proteins using a computer-aided image analysis revealed several protein spots that varied in quantity after 5-FU exposure. The up-regulated proteins were heat shock protein (hsp) 70 protein 5, hsp60 protein 1 and thyroid hormone-binding protein precursor, while the down-regulated proteins were lactate dehydrogenase (LDH)-A and gamma-synuclein. The distributions of these five protein varied widely, and included the cytoplasm, mitochondria, endoplasmic reticulum and plasma membrane. The results of this study demonstrate that hsp70, hsp60, thyroid hormone-binding protein precursor, LDH-A and gamma-synuclein may represent candidates for diagnostic and prognostic markers. Furthermore, the results demonstrate that the protein expression profile of colon cancer cells can be used to establish the potential of this methodology as a means by which rational decisions regarding the choice of therapy can be approached.

**Keywords** : Proteomics, 5-FU, colorectal cancer, ESI Q-TOF mass spectrometry

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## 1. Introduction

Various types of malignant tumors, including colorectal cancer, have been treated by multiple chemotherapy, such as fluoropyrimidine 5-fluorouracil (5-FU) treatment. 5-FU is the most common chemotherapeutic agent used in the treatment of colorectal cancer. It is anabolized to the respective deoxynucleotide monophosphate, FdUMP, which competes with the normal metabolite, dUTP, as a potent inhibitor of thymidylate synthase (TS) [1,2]. This enzyme governs the de novo synthesis of thymidylate, and its inhibition results in specific depletion of dTTP. This depletion is associated with inhibition of DNA replication and the ribonucleoside triphosphate of 5-FU, FUTP, may be incorporated into RNA, and affect the function of the transcripts [3]. In vitro and in vivo studies have demonstrated that increased TS expression is correlated with increased resistance to 5-FU [3-5]. It is likely that events downstream of TS inhibition, such as activation of DNA damage response pathways, also play key roles in determining the cellular response to 5-FU. Identification of such pathways would greatly facilitate the development of new therapeutic strategies to improve the efficacy of 5-FU-based chemotherapy. Recently, Maxwell et al. identified 5-FU-inducible target mRNAs, such as annexin II, spermine/spermidine acetyl transferase (SSAT), thymosin-beta-10, chaperonin-10 and MAT-8 [7]. However, the changes in the intracellular environment after 5-FU treatment may not only involve the expression of mRNAs but also the amounts of certain proteins. To identify the downstream mediators of the tumor cell response to 5-FU, we used proteomics technology to identify affected proteins in the WiDr colorectal cancer cell line after 5-FU treatment.

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## 2. Methods

### *2.1. Cell Culture*

The WiDr cell line was purchased from the Japan Health Science Foundation (HSRRB, Osaka, Japan), and maintained

in Dulbecco's modified Eagle's medium (D-MEM) supplemented with heat-inactivated fetal bovine serum. After culture for 48 h, the cells were treated with 1.0 μM 5-FU (Kyowa, Tokyo, Japan) for 0, 24, 48 or 72 h. All cells were grown in 5% $CO_2$ at 37°C. Apoptosis was assessed using an in situ cell death detection kit (Roche Molecular Biochemicals Diagnostic, IN, USA) (Fig.1). Milli-Q water (Millipore, MA, USA) was used for all solutions.

*2.2. 2-D electrophoresis*

Two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE) analysis was performed using a previously described standard protocol [8]. Briefly, proteins from untreated cells (control) or cells after treatment with 1.0 μM 5-FU for 24, 48 or 72 h were extracted with 8.5 M urea, 2% Triton X-100, 0.1% sodium dodecyl sulfate, 1.2% DeStreak™ reagent (Amersham Biosciences Corp., NJ, USA), 1% phosphatase inhibitor cocktail 1&2 (Sigma, MO, USA), 1/10 tablet/mL protease inhibitor cocktail tablets, complete mini (Roche Molecular Biochemicals Diagnostic) and 2% pharmalyte™ pH 3-10 (Amersham Biosciences). The suspension was sonicated for approximately 30 sec and then centrifuged at 10000 xg for 30 min to sediment any undissolved material. The protein concentration was approximately 50 mg/mL after filtration through an Ultrafree UFV5BGC25 filter (Millipore). Samples containing approximately 0.5 mg were applied to immobilized pH 3-10 linear gel strips (Bio-Rad, Hercules, CA, USA) in a rehydration buffer (6 M urea, 2 M thiourea, 2% Triton X-100, 1.2% DeStreak™ reagent, 1% pharmalyte™ pH 3-10, 6 mM Orange G). The first dimension of isoelectric focusing (IEF) was carried out using a Protean IEF Cell System (Bio-Rad). Electrofocusing was performed at 250 V for 1 h, followed by a linear increase in voltage from 250 V to 10000 V within 6 h, and then at 10000 V for 6 h. The 2-D separation was performed in vertical 7.5%T polyacrylamide gels (Anatech, Tokyo, Japan) in a Tris/Tricine buffer using a CoolPhoreStar Tetra-200 vertical slab gel electrophoresis apparatus (Anatech) under a constant current of 20 mA/gel. After electrophoresis, the gels were stained using a SYPRO Ruby protein gel stain kit (Invitrogen Corp., Carlsbad, CA, USA), and scanned in a FluoroPhoreStar 3000 blue diode scanner (Anatech). Noise reduction, background subtraction, spot detection, quantification, gel-to-gel matching and differential analysis were carried out using PDQuest software ver. 7.1 (Bio-Rad) (Fig. 2).

*2.3. Identification of proteins*

Protein spots on stained 2-D electrophoresis gels were excised and digested with 0.4% (w/v) trypsin overnight. The digested peptides were desalted and cleaned with $ZipTip_{C18}$ pipette tips (Millipore). Briefly, the $ZipTip_{C18}$ pipette tips were equilibrated in 0.1% trifluoroacetic acid (TFA), and the digested peptides were then bound to the tips by pipetting ten times. The bound peptides were washed with 0.1% TFA, and eluted with 0.1% TFA and 50% acetonitrile. The eluted peptides were determined using an electrospray ionization (ESI)-quadrupole-time of flight (Q-TOF) system (Q-TOF Ultima™ API mass spectrometer; Waters, Milford, MA, USA). Database searches were carried out using the Mascot Search engine in Matrix Science

(http://www.matrixscience.com/) (Fig. 3).

## 3. Results

Remarkable apoptosis was not observed at any of the time points (Fig. 1). The protein expression profiles of the treated and untreated populations are shown in Figure 2. We found three proteins that were up-regulated by >2-fold, and two proteins that were down-regulated by >2-fold (Table 1). The up-regulated proteins were heat shock protein (hsp) 70 protein 5 (BiP), hsp60 protein 1 and thyroid hormone-binding protein precursor, while the down-regulated proteins were lactate dehydrogenase (LDH)-A and gamma-synuclein. Thyroid hormone-binding protein precursor and LDH-A were cytoplasmic proteins, hsp60 protein 1 was a mitochondrial protein, hsp70 protein 5 was distributed in the endoplasmic reticulum, and gamma-synuclein was distributed the cytoplasm and plasma membrane, indicating that the distributions of the up- and down-regulated proteins varied

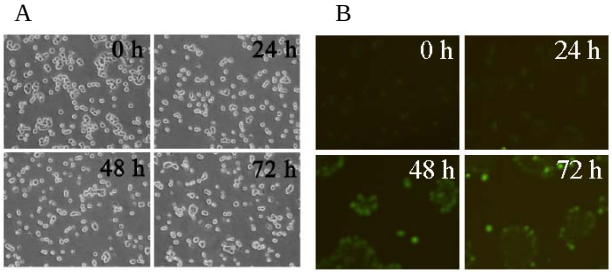



**Fig. 1. Apoptosis in the WiDr cell line.**

A: Untreated WiDr cells (0 h) and WiDr cells after treatment with 1.0 μM 5-FU for 24, 48 and 72 h. No differences are observed among the cells. B: Apoptosis in 5-FU-treated WiDr cells. Remarkable apoptosis is not observed.

widely. The possibility that the changes in the identified proteins could have resulted from artifacts was excluded, since triplicate experiments based on independent cell extractions yielded consistent results.

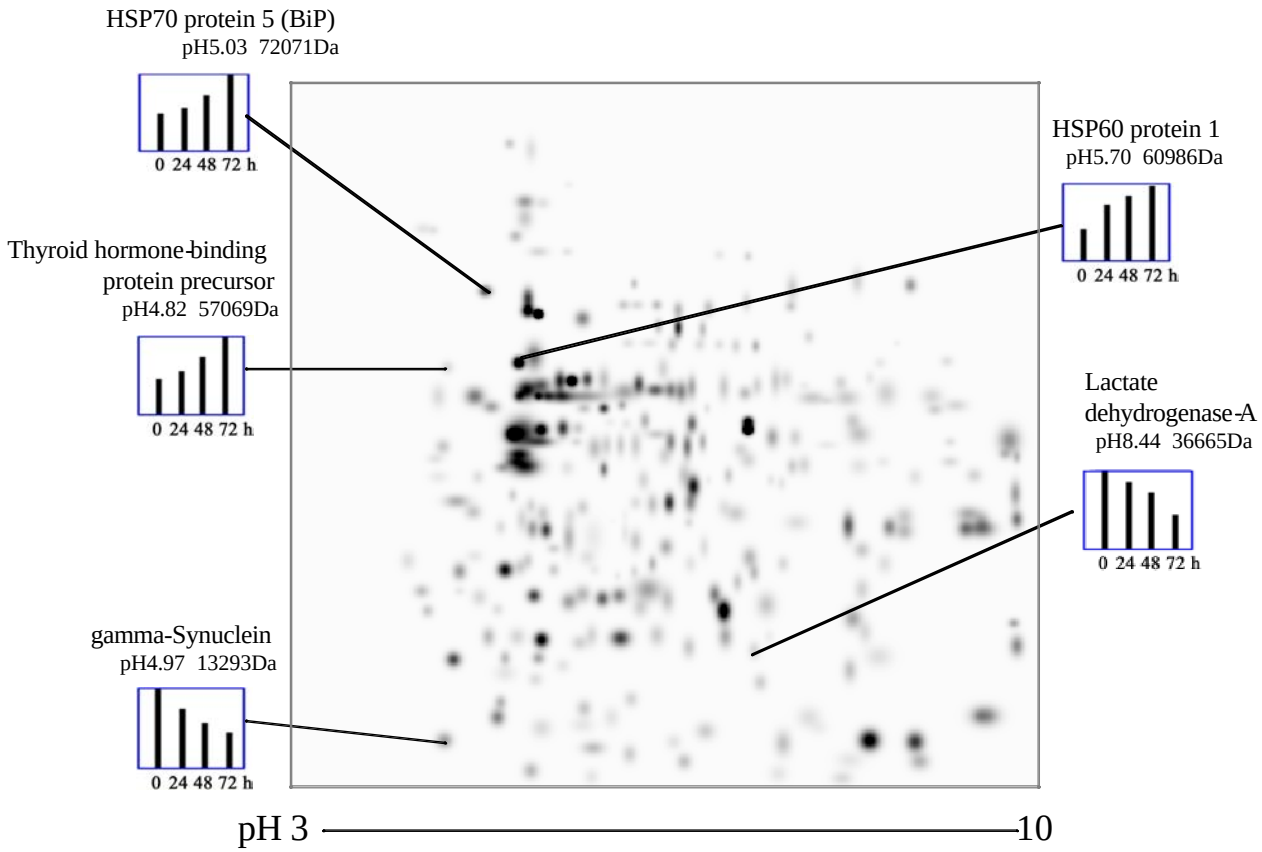


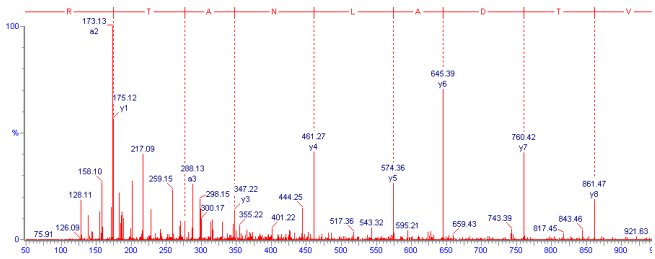

**Fig. 2. Protein expression map of the WiDr cell line.**

Spot detection, quantification and gel-to-gel matching were carried out using the PDQuest software. The newly identified spots were excised, digested with trypsin and subjected to ESI Q-TOF mass spectrometry.

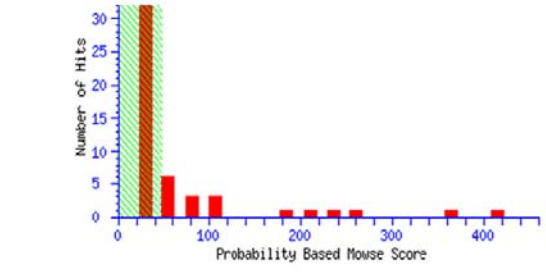
## 4. Discussion

Proteomics analysis usually employs the separation of a protein mixture by 2-D electrophoresis and the identification of the separated proteins by mass spectrometry. The major advantage of 2-D electrophoresis is that it enables the simultaneous separation and visualization of thousands of unknown proteins in different modification states. No other method can achieve this at the present time. The assessment of protein expression profiles by 2-D electrophoresis after treatment with chemotherapeutic agents has the potential to identify novel signaling pathways involved in mediating the downstream responses to these therapies, and could greatly facilitate the discovery of novel potential therapeutic targets and/or markers of chemoresistance. Studies examining the post-transcriptional expression profiles of cancer cell lines and tumors have begun to identify genes that may be associated with a response or resistance to these anticancer agents [9,10]. In the present study, we used such an approach to identify proteins up- or down-regulated in WiDr colorectal cancer cells after 5-FU treatment. We found three proteins that were up-regulated by>2-fold, and two proteins that were down-regulated by>2-fold after 5-FU treatment. Hsp represents a complex family of proteins exerting chaperone-like activities that are classified according to their molecular weight [11-13]. The up-regulated hsp60 protein 1 is a member of the hsp family and has been attributed numerous roles, including regulation of DNA synthesis, cell proliferation and apoptosis [13,14]. Hsp60 protein 1 binds hsp10 (chaperonin-10) to regulate the folding of mitochondrial proteins [15]. Hsp10 mRNA was

**A**




**B**




| Query | Observed | Mr(expt) | Mr(calc) | Delta | Miss | Score | Expect | Rank | Peptide |
|---|---|---|---|---|---|---|---|---|---|
| 11 | 417.21 | 832.48 | 832.38 | 0.82 | 0 | 31 | 0.22 | 1 | APGFGDNR |
| 13 | 422.77 | 843.53 | 843.51 | 0.03 | 0 | 23 | 1.4 | 1 | VGEVIVTK |
| 15 | 438.76 | 859.50 | 859.48 | 0.02 | 0 | 11 | 24 | 1 | IPAMTIAK + Oxidation (M) |
| 24 | 456.78 | 911.55 | 911.50 | -0.03 | 0 | 51 | 0.0017 | 1 | VGLQVVAVK |
| 28 | 480.75 | 959.49 | 959.50 | -0.01 | 0 | 51 | 0.002 | 1 | VTDALNATR |
| 37 | 506.30 | 1010.59 | 1010.56 | 0.03 | 1 | 22 | 1.3 | 1 | GAMPVEIRR |
| 62 | 401.59 | 1201.74 | 1201.71 | 0.03 | 1 | 30 | 0.10 | 1 | TLKIPAMTIAK + Oxidation (M) |
| 70 | 608.33 | 1214.64 | 1214.65 | -0.01 | 0 | 59 | 0.00023 | 1 | NAGVEGSLIVEK |
| 73 | 617.31 | 1232.61 | 1232.59 | 0.02 | 0 | 52 | 0.0016 | 1 | VGGTSDVEVNEK |
| 82 | 454.58 | 1368.73 | 1368.68 | 0.05 | 1 | 44 | 0.0095 | 1 | VGGTSDVEVNEKK |
| 99 | 549.60 | 1646.81 | 1645.90 | 0.11 | 1 | 40 | 0.011 | 1 | VGCVIVTKDDAMLLK + Oxidation (M) |
| 184 | 806.44 | 1770.87 | 1770.85 | 0.02 | 0 | 14 | 0.6 | 1 | CIPALDSLIPAMLDQK + Carbamidomethyl (C) |

**Fig. 3. Protein identification results from the mass spectrum obtained in a Q-TOF Ultima™ API mass spectrometer and a database search on the Mascot Server.**

A: Mass spectrum obtained after in-gel digestion and ESI Q-TOF analysis of one of the identified proteins, hsp60 protein 1. B: Probability-based Mowse Score on the Mascot Server. The identified peptides and their molecular masses are reported using a root mean square (RMS) error graph. The amino acid sequence "VTDALNATR" corresponds to amino acids 421–429 of hsp60 protein 1. There are three oxidation peptides and a carbamidomethyl peptide among the identified peptides.

consistently up-regulated in the MCF7 breast cancer cell line after treatment with 10 μM 5-FU [7]. In addition, it is known that hsp70 can control the decomposition of LDH [16,17], and both the up-regulation of hsp70 protein 5 and down-regulation of LDH-A we identified will be related to this phenomenon. Galetto *et al.* also reported that hsp70 was up-regulated in granulocyte macrophages after gamma-radiation and 5-FU treatment [18]. Gamma-synuclein was previously identified as a breast cancer-specific gene [19], and Jiang *et al.* demonstrated that it had a chaperone activity in the hsp-based multiprotein chaperone complex for stimulation of estrogen receptor (ER)-alpha signaling [20,21]. The gamma-synuclein-mediated stimulation of ER-alpha transcriptional activity is consistent with its stimulation of mammary tumorigenesis in response to estrogen. The anti-proliferative effects of tamoxifen plus 5-FU on KATOIII (poorly differentiated gastric adenocarcinoma) cells were not dependent on ER-alpha expression [21]. Gamma-synuclein is thought to have many functions as an hsp besides its participation with ER-alpha. No previous reports regarding any relationship between thyroid hormone-binding protein and 5-FU or hsp were found, and we therefore have no explanation for this at the present time.

Using a cDNA microarray analysis, Maxwell *et al.* identified 5-FU-inducible target mRNAs, including SSAT, annexin II, thymosin-beta-10, chaperonin-10 and MAT-8 [8]. They used 10 μM 5-FU for their transcriptional study, which was 10-fold the concentration used in the current study. Therefore, we are unable to validate the 5-FU-inducible genes they identified. However, hsp10, which was identified as an up-regulated gene by Maxwell *et al.* in their cDNA microarray analysis, binds hsp60 protein 1, which was identified as an up-regulated protein in this study. It is considered that only a few protein changes were observed in the present study because the sensitivity of WiDr cells to 5-FU is comparatively low.

The results in the current study demonstrate the potential of 2-D electrophoresis to identify novel genes involved in mediating the response of tumor cells to chemotherapy. Since there are five candidate proteins that appear to be involved in

**Table 1. Functional grouping of the proteins identified by 2-D electrophoresis as being up- or down-regulated in the WiDr cell line after 5-FU treatment.**

| Regulation | Fold induction | Protein name | Functional classifications | Structial classifications |
|---|---|---|---|---|
| Up | 2.53 | HSP 70 protein 5 (BiP) | Heat shock protein | Endoplasmic reticulum |
| Up | 2.22 | HSP60 protein 1 | Heat shock protein | Mitochondrial protein |
| Up | 2.00 | Thyroid hormone-binding protein precursor | Hormone | Cytoplasmic protein |
| Down | 2.12 | LDH-A | Simple carbohydrate metabolism | Cytoplasmic protein |
| Down | 2.06 | Gamma-synuclein | Heat shock protein | Plasma membrane, cytoplasmic protein |

tumor proliferation and drug resistance, it is crucial to correlate the severity of the disease with the different expressions in colorectal cancer samples to generate diagnostic and prognostic markers. However, it is not feasible to try and confirm the involvement of only five candidates in the resistance/response to therapy. The probability that these five candidates alone will serve as markers is low, and many more candidates are likely to be identified. Before application to clinical studies, many validation studies for these markers are required. Further improvement of this method and the development of other methods are crucially important for the identification of new diagnostic and prognostic markers for cancer therapies.

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