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LC-MS/MS-based Proteomic Analysis of Locally Advanced Rectal Tumors to Identify Biomarkers for Predicting Tumor Response to Neoadjuvant Chemoradiotherapy

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Abstract : Neoadjuvant chemoradiotherapy (nCRT) is a standard therapy used for locally advanced rectal cancer prior to surgery, which can more effectively reduce the locoregional recurrence rate and radiation toxicity compared to postoperative chemoradiotherapy. The response of patients to nCRT varies, and thus, robust biomarkers for predicting a pathological complete response are necessary. This study aimed to identify possible biomarkers involved in the complete response/non-response of rectal cancer patients to nCRT. Comparative proteomic analysis was performed on rectal tissue samples before and after nCRT. Proteins were extracted for label-free proteomic analysis. Western blot and real-time PCR were performed using rectal cancer cell line SNU-503 and radiation-resistant rectal cancer cell line SNU-503R80Gy. A total of 135 up- and 93 down-regulated proteins were identified in the complete response group. Six possible biomarkers were selected to evaluate the expression of proteins and mRNA in SNU-503 and SNU-503R80Gy cell lines. Lyso-phosphatidylcholine acyltransferase 2, annexin A13, aldo-ketose reductase family 1 member B1, and cathelicidin antimicrobial peptide appeared to be potential biomarkers for predicting a pathological complete response to nCRT. This study identified differentially expressed proteins and some potential biomarkers in the complete response group, which would be further validated in future studies.

Keywords : LC-MS/MS; proteomics; neoadjuvant chemoradiotherapy; locally advanced rectal cancer; biomarker

Introduction

Colorectal cancer (CRC) is estimated to have the third-highest incidence and second-highest mortality rate among tumors in the United States in 2020.[1] Neoadjuvant chemoradiotherapy (nCRT) prior to surgery is considered the standard therapy for locally advanced rectal cancer. It can significantly reduce the locoregional recurrence rate and radiation toxicity, which cannot be achieved using postoperative CRT.[2,3] Tumor regression, including pathologic complete response after nCRT, is associated with good oncologic outcomes as compared to non-response to nCRT.[4,5] Despite the clinical importance of predicting a response, the response of patients with locally advanced rectal cancer to nCRT varies and has not been predicted.[6,7] It is crucial to predict the efficacy of nCRT in patients with rectal cancer in advance. However, there are currently no robust biomarkers for the prediction of a pathological complete response; thus, it remains an essential issue.[8]

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a powerful tool to identify and quantify a wide range of compounds, including small molecules (metabolites),[9-11] peptides,[12,13] and proteins.[14] In recent years, proteomics has become a powerful approach to discover potential biomarkers involved in various diseases, such as breast cancer (blood biomarkers),[15] lung cancer (exhaled breath condensate biomarkers),[16] strokes (plasma biomarkers),[17] and chronic kidney disease (urinary

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biomarker).[18] Proteomics allows the investigation of a large number (up to several thousand) of proteins existing in a sample.[19,20] Technically, proteomics includes top-down (direct separation and identification of intact proteins) and bottom-up approaches (analysis of peptides resulting from enzymatic proteolysis of proteins).[21,22] Current proteomics studies use LC to separate proteins and peptides prior to MS analysis.[23,24] With the development of LC separation techniques as well as MS speed and accuracy, proteomics can identify changes in the proteome and thereby elucidate mechanisms related to diseases and treatment therapies.[25,26]

In this study, we aimed to use a proteomic strategy to identify differentially expressed proteins (DEPs) involved in the complete response (CR)/non-response (NR) of rectal cancer patients to nCRT. We collected rectal tissue samples from patients with advanced rectal cancer and performed a comparative proteomic analysis. From DEPs, possible biomarkers were selected for evaluation through protein and mRNA expression in rectal cancer cell lines.

Experimental

Materials

Formic acid (FA), ammonium bicarbonate, and iodoacetamide (IAA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tris (2-carboxyethyl)phosphine (TCEP) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). HPLC-grade acetonitrile and water were purchased from J.T. Baker (Phillipsburg, NJ, USA). All chemicals were of analytical grade and were used as received without further purification.

Proteomic study

Sample collection

Patients who would receive nCRT were asked to join the study before the treatment and ten patients with advanced rectal cancer were recruited for this study. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Gil Hospital, South Korea (approval number GCIRB2013-223). Informed consent was obtained from all subjects involved in the study. All patients were treated with long-course nCRT according to the National Comprehensive Cancer Network guidelines. Radiation that delivered 50.4 Gy in 28 fractions over five weeks in the pelvis was given to patients. Dosing schedules for concurrent chemotherapy were capecitabine (825 mg/m^2) orally twice daily, five days per week during pelvic radiotherapy, or 5-fluorouracil (400 mg/m^2) intravenous bolus with leucovorin (20 mg/m^2) intravenous bolus for four days during weeks 1 and 5 of nCRT. Rectal tissue samples were collected twice from each patient: a colonoscopic tumor sample (before nCRT) and a surgical tumor sample (after cancer extraction on the day of surgery). All tissues were immediately frozen after collection and stored in liquid nitrogen until analysis. The pathological response to nCRT in rectal cancer was assessed by the pathologist. The absence of viable cancer cells in the tissue after nCRT to rectal cancer was considered a CR. Otherwise, it was NR. Among ten patients, five were in the CR group, and the other five were in the NR group. Patient characteristics are shown in **Table S1**. There were no differences in gender, age, and preoperative stage (clinical stage) between CR and NR groups. Five tissues from patients in each group were pooled to obtain four final samples prior to the proteomic study: CRpre, CRpost, NRpre, and NRpost.

Sample preparation

The samples frozen in liquid nitrogen were pulverized using TT1 tissue TUBE^{TM} and the CryoPrep$^{®}$ system (Covaris, Woburn, MA, USA). Lysis buffer (8 M urea and 0.1 M Tris-HCl, pH 8.5) was used to suspend the samples prior to cell lysis and protein extraction with focus-sonication (12 min, 18^{o}C, Covaris).[27] Proteins were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein digestion was performed on a Microcon-30 kDa centrifugal filter unit with Ultracel-30 membrane (Millipore, Darmstadt, Germany) using a filter-aided sample preparation (FASP) protocol.[28] In brief, proteins (100 μg) were reduced with 1 μL of 500 mM TCEP (37^{o}C, 30 min), alkylated with 3 μL of 500 mM IAA (25^{o}C, 30 min, in the dark), and digested with trypsin at an enzyme:protein ratio of 1:50 (*w/w*) (37^{o}C, 18 h). FA was used to stop digestion. Sample desalting was conducted using C18 Ultra-Micro SpinColumns (Harvard Apparatus, Holliston, MA, USA). The resultant peptides were dried in a ScanSpeed 40 centrifugal evaporator (1,800 rpm, 3 h). Samples were dissolved in 50 μL of solvent A (0.1% FA in water) and quantified using a NanoDrop^{TM} 2000/2000c Spectrophotometer (Thermo Fisher Scientific) prior to LC-MS/MS analysis.

LC-MS/MS analysis

A peptide amount of 3 μg was injected into the LC-MS/MS system for analysis. The LC-MS/MS system consisted of a Dionex Ultimate 3000 HPLC coupled with a Q Exactive^{TM} Hybrid Quadrupole-Orbitrap MS (Thermo Fisher Scientific). The peptide mixtures were loaded onto an Acclaim^{TM} PepMap^{TM} 100 C18 nano-trap column (75 μm × 2 cm, 3 μm particles, 100 Å pores, Thermo Fisher Scientific) using solvent A at a flow rate of 2.5 μL/min for 5 min. Subsequently, the peptides were separated on an Acclaim^{TM} PepMap^{TM} C18 100A RSLC nano-column (75 μm × 50 cm, 2 μm particles, 100 Å pores, Thermo Fisher Scientific) at a flow rate of 300 nL/min. The mobile phase solvent consisted of solvents A and B (0.1% FA in acetonitrile). The gradient was set up as

follows for solvent B: equilibration at 4% for 10 min, 4–40% for 110 min, 40–96% for 0.1 min, holding at 96% for 9.9 min, 96–4% for 0.1 min, and holding at 4% for 19.9 min for re-equilibration of the column. The following parameters were set: spray voltage, 2.2 kV; capillary temperature, 320 °C; isolation width, ± 2 *m/z*; scan range, 400–2000 *m/z*; resolution in full-MS scans, 70,000; and resolution in MS/MS scans at 200 *m/z*, 17,500. The MS was operated using a data-dependent acquisition method. The top ten precursor ions with the highest intensity were isolated in the quadrupole and fragmented by higher-energy collisional dissociation with 27% normalized collisional energy. Dynamic exclusion was set at 20 s to minimize repeated analyses of the same abundant precursor ions.

Data analysis

The mass spectrometry proteomics data were deposited into the ProteomeXchange Consortium via the PRIDE[29] partner repository with the dataset identifier PXD027445. Proteome Discoverer (version 1.4, Thermo Fisher Scientific) was used to search the MS/MS spectra against a human database obtained from Uniprot. The following parameters were set for the search: maximum of two missed cleavages with trypsin; semitryptic cleavage; 10 ppm and 0.02 Da tolerances of precursor ion masses and fragment ion mass, respectively; static carbamidomethylation of cysteine; and variable modifications including methionine oxidation (+15.995 Da) and carbamylation of protein in N-term (+43.0006 Da). Peptides and proteins were filtered at a false discovery rate (FDR) of ≤0.01. Proteins were quantified using the label-free quantification (LFQ) method. Statistical analysis was performed using Mass Profiler Professional (MPP) version 12.6.1 (Agilent, Santa Clara, CA, USA). DEPs were filtered with a corrected p-value of <0.05, and fold-change (FC) of >1.5 or <-1.5 (Log_2FC of >0.58 or <-0.58). Data were processed and visualized using Microsoft Excel 2016. Gene ontology (GO)[30] was categorized using Panther.[31] Protein interactions and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway[32] were analyzed using the STRING database.[33] GO terms and KEGG pathways were filtered using an FDR of ≤0.05. A heatmap was generated using MPP, whereas volcano plots were constructed and Principal component analysis (PCA) was conducted using R version 3.6.1.

In vitro evaluation of DEPs on cells

Cell culture

Rectal cancer cell lines (SNU-503 and SNU-503R80Gy) were purchased from the Korean Cell Line Bank (Seoul, South Korea). The catalog numbers of the cell lines were 00503 and 00503/R80Gy. A total of 80 Gy of fractionated ionizing radiation was irradiated to the rectal cancer cell line, SNU-503, over 40 times using a Cesium-137 irradiator. The two cell lines were grown in RPMI-1640 medium (WelGENE, Daegu, South Korea) supplemented with 10% FBS and 1% penicillin/streptomycin (Thermo Fisher Scientific, CA, USA) in 5% CO_2 at 37°C.

Cell viability assay

The two cultured cell lines (7×10^3 cells/96-well plates) were irradiated with 0, 2, 4, and 6 Gy radiation. Cells were irradiated into 96-well plates and incubated for the indicated times at 6, 24, 48, and 72 h. Cell viability was confirmed using the EZ-Cytox reagent (DoGenBio, Seoul, South Korea). After applying 4 Gy irradiation in a time-dependent manner, 10 µl of reagent was added to each well and incubated in an incubator for 30 min. The mixture was shaken gently for 1 min before measuring the absorbance. The absorbance was measured at 450 nm using a plate reader. The rectal cancer cell line and radiation-resistant rectal cancer cell lines were tested.

Western blot analysis

SNU-503 and SNU-503R80Gy cells were seeded at a density of 1×10^6 cells/100 mm dishes. The cells were irradiated with 4 Gy using a CLINAC ix-SN5895. The cells were extracted using RIPA cell lysis buffer (GenDEPOT, TX, USA) containing protease inhibitors. The lysate was separated by 6–15% SDS-PAGE depending on the molecular weight and transferred to a polyvinylidene difluoride (PVDF) membrane using the standard technique. The membrane was incubated with primary antibodies, including aldo-ketose reductase family1, member B1 (AKR1B1, Abcam, Cambridge, UK), annexin A13 (ANXA13, Abcam), carbamoyl-phosphate synthetase 2 (CAD, also known as aspartate transcarbamylase or dihydroorotase, Cell Signaling, MA, USA), cathelicidin antimicrobial peptide (CAMP, Abcam), lysophosphatidylcholine acyltransferase 2 (LPCAT2, Santa Cruz, TX, USA), signal transducer and activator transcription (STAT3, Cell Signaling), and β-actin (Santa Cruz) overnight at 4°C. The proteins were visualized using an enhanced chemiluminescence detection system (Amersham ECL Plus, PA, USA).

Real-time RT-PCR

Total cellular RNA was extracted from SNU-503 and SNU-503R80Gy cells plated in 60 mm dishes using an RNA Extraction Kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer's instructions. Aliquots of total RNA (1 µg) were reverse transcribed into cDNA using the Prime Script RT Reagent Kit (Takara Bio). Real-time RT-PCR was performed using the CFX384 real-time DNA amplification system (BioRad, Hercules, CA, USA), and the fluorescent dye SYBR green was used to monitor cDNA synthesis (Takara Bio). PCR conditions included a denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Table 1 lists the forward and reverse primer sequences for the various genes of interest. The expression levels of each

Table 1. Forward and reverse primer sequences for real-time RT-PCR

Gene	Forward primer	Reverse primer
AKR1B1	5′-GAGGAACTTGGTGGTGATCC-3′	5′-AGGTGGTCATATCCTGGCTG-3′
Annexin13	5′-TCGCAATGAAGGAGATGACG-3′	5′-TGAAAGGTGGCTCGTAACTG-3′
CAD	5′-CAGAGATCGGAGAGCATGTG-3′	5′-TGTTAGAGGCAAAGCCAGAG-3′
CAMP	5′-GCAGTCACCAGAGGATTGTG-3′	5′-AACTGATGTCAAAGGAGCCC-3′
LPCAT2	5′-TCCAGGTGGCATTTAAGCTG-3′	5′-GGAAGCCTGTAGAATGGTGG-3′
STAT3	5′-TCCTGGGAGAGATTGACCAG-3′	5′-TGGCTTCTCAAGATACCTGC-3′

gene of interest were normalized to that of GAPDH mRNA. All experiments were independently performed three times.

Results and Discussion

Protein quantification and differentially expressed proteins

Currently, nCRT is considered the standard therapy for locally advanced rectal cancer prior to surgery, with numerous oncologic benefits.[2-5] However, the response to nCRT varies among patients and is difficult to predict. A complete pathological response has been reported in 12% to 38% of patients.[6,7] Thus, the prediction of nCRT efficacy for rectal cancer patients is essential to determine whether nCRT or some other therapy should be implemented. There are no current robust biomarkers for the prediction of pathological complete response.[8] This study investigated two groups of patients who showed complete response and non-response. We performed proteomic analysis to identify DEPs that might be involved in the response of rectal tumors to nCRT. These proteins could be used as a prognosis before nCRT for predicting the complete response in patients with locally advanced rectal cancer.

A total of 1266 proteins were quantified using the LFQ method and are listed in Table S2. The distribution of these

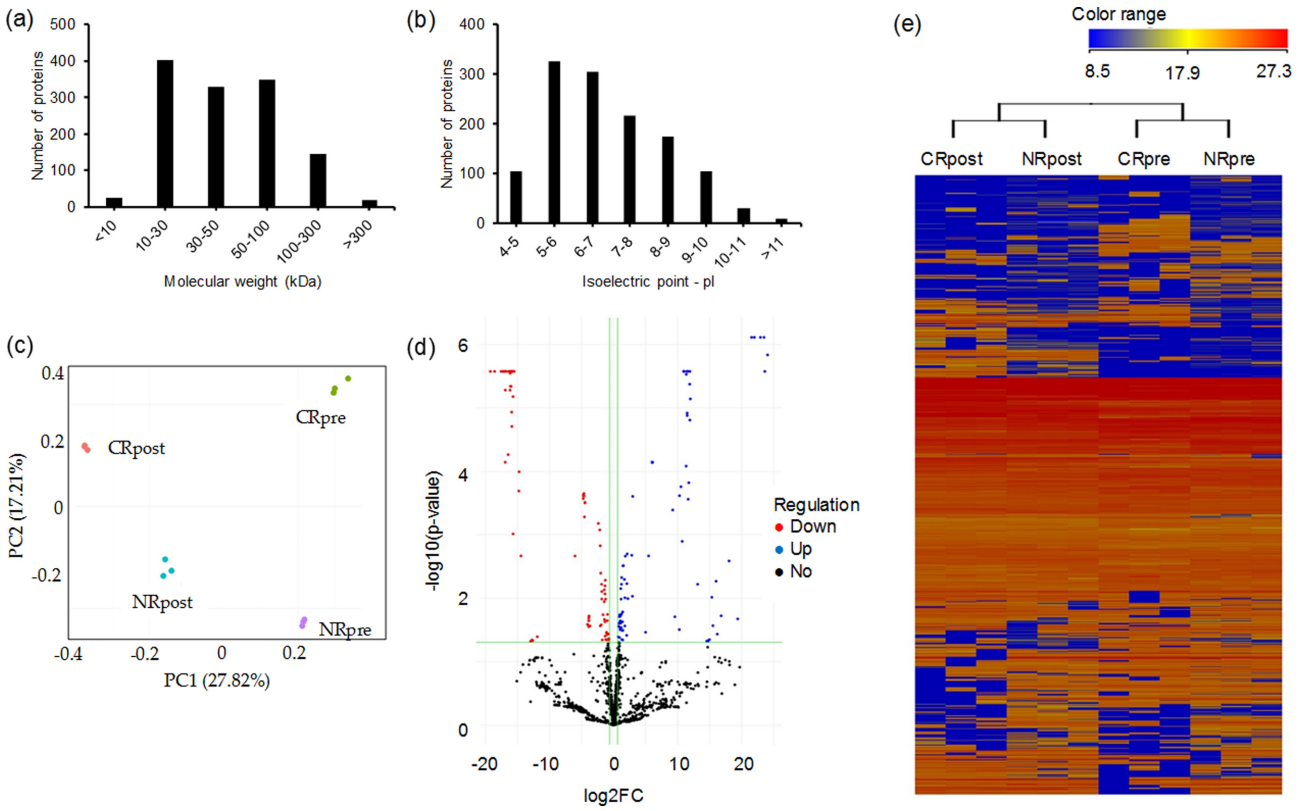

Figure 1. Analysis of quantified proteins. Distribution of quantified proteins by (a) molecular weight and (b) isoelectric point. (c) Principal component analysis. (d) Volcano plot (CRpre versus NRpre). (e) Hierarchical clustering by samples and quantified proteins.

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proteins by molecular weight (MW) and isoelectric point (pI) is shown in Figures 1a and b. Most of the quantified proteins had an MW in the range of 10–100 kDa and pI in the range of 5–9. PCA clearly indicated the differences among CRpre, CRpost, NRpre, and NRpost (Figure 1c). To identify possible biomarkers, we focused on the CRpre and NRpre groups. DEPs of CRpre versus NRpre are indicators of successful nCRT before the therapy is applied. A total of 228 DEPs were defined for CRpre versus NRpre, including 135 up-regulated and 93 down-regulated proteins (as listed in Table S3 and visualized by a volcano plot in Figure 1d). Hierarchical clustering (Figure 1e) illustrates an overview of protein abundance among the four groups.

Bioinformatic analysis

The DEPs (for CRpre versus NRpre) were subjected to GO analysis. Various biological processes, cellular components, and molecular functions were defined as up- and down-regulated proteins (Tables S4 and S5). The GO terms with the highest coverage% are shown in Figure 2. Protein-protein interactions (from curated databases and experimentally determined) are illustrated in Figure S1. Approximately 50 up-regulated proteins interact closely, whereas other proteins are alone or in a small group. Most down-regulated proteins interact with each other. KEGG pathways were defined and are listed in Tables 2 and 3.

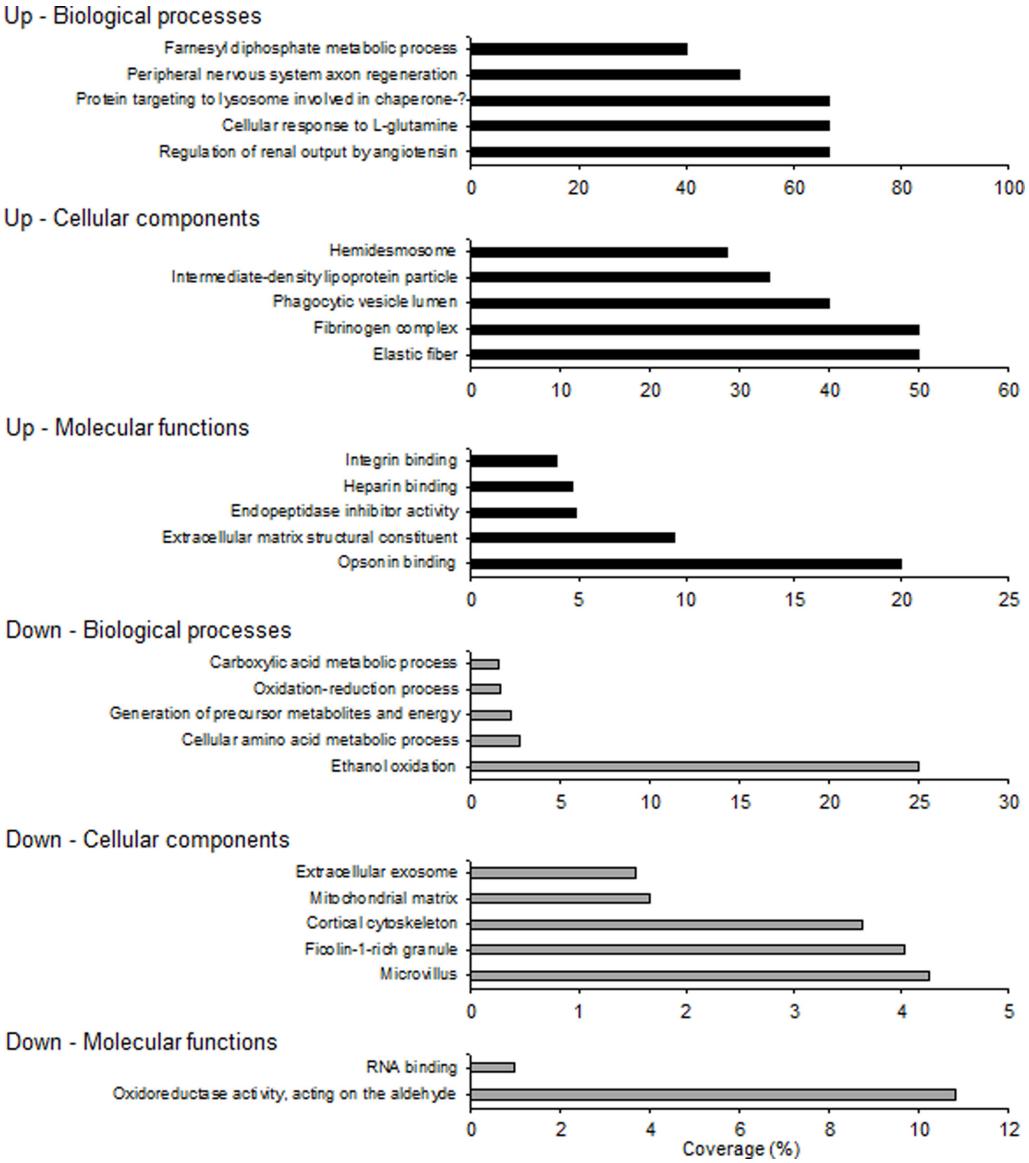

Figure 2. Top five gene ontology terms with highest coverage%. The proteins used for analysis were up- and down-regulated proteins (for CRpre versus NRpre).

Table 2. KEGG pathways defined from up-regulated proteins (for CRpre versus NRpre)

ID	Description	Input /Total proteins	FDR
hsa04610	Complement and coagulation cascades	15/78	4.00×10^{-15}
hsa04145	Phagosome	11/145	3.70×10^{-07}
hsa05133	Pertussis	6/74	6.10×10^{-04}
hsa04512	ECM-receptor interaction	6/81	7.40×10^{-04}
hsa05150	Staphylococcus aureus infection	5/51	8.60×10^{-04}
hsa05322	Systemic lupus erythematosus	5/94	1.10×10^{-02}
hsa03320	PPAR signaling pathway	4/72	2.80×10^{-02}
hsa04611	Platelet activation	5/123	2.80×10^{-02}
hsa05140	Leishmaniasis	4/70	2.80×10^{-02}
hsa04510	Focal adhesion	6/197	2.90×10^{-02}
hsa03050	Proteasome	3/43	4.30×10^{-02}

Table 3. KEGG pathways defined from down-regulated proteins (for CRpre versus NRpre)

ID	Description	Input/ Total proteins	FDR
hsa01100	Metabolic pathways	25/1250	4.30×10^{-08}
hsa00071	Fatty acid degradation	6/44	7.30×10^{-06}
hsa00010	Glycolysis / Gluconeogenesis	6/68	5.20×10^{-05}
hsa00280	Valine, leucine and isoleucine degradation	5/48	1.50×10^{-04}
hsa00410	beta-Alanine metabolism	4/31	5.30×10^{-04}
hsa01200	Carbon metabolism	6/116	5.30×10-04
hsa01230	Biosynthesis of amino acids	5/72	5.40×10^{-04}
hsa00020	Citrate cycle (TCA cycle)	3/30	7.90×10-03
hsa00051	Fructose and mannose metabolism	3/33	9.10×10^{-03}
hsa00350	Tyrosine metabolism	3/36	1.00×10^{-02}
hsa00620	Pyruvate metabolism	3/39	1.20×10^{-02}
hsa00970	Aminoacyl-tRNA biosynthesis	3/44	1.50×10^{-02}
hsa00330	Arginine and proline metabolism	3/48	1.80×10^{-02}
hsa00561	Glycerolipid metabolism	3/59	2.90×10^{-02}
hsa00190	Oxidative phosphorylation	4/131	3.20×10^{-02}
hsa00980	Metabolism of xenobiotics by cytochrome P450	3/70	4.00×10^{-02}
hsa00340	Histidine metabolism	2/23	4.60×10^{-02}
hsa05204	Chemical carcinogenesis	3/76	4.60×10^{-02}
hsa04146	Peroxisome	3/81	4.90×10^{-02}

The complement and coagulation cascade pathways were the most important for up-regulated proteins, whereas metabolic pathways, fatty acid degradation, glycolysis/ gluconeogenesis, carbon metabolism, and biosynthesis of amino acids were the five featured pathways for down-regulated proteins. Among the quantified proteins, six proteins were selected, which showed quantitative changes between the two groups before treatment (CRpre versus NRpre). They have been reported to be involved in CRC. They included carbamoyl-phosphate synthetase 2 (CAD, also known as aspartate transcarbamylase or dihydroorotase), lysophosphatidylcholine acyltransferase 2 (LPCAT2), annexin A13 (ANXA13), aldo-ketose reductase family1, member B1 (AKR1B1), cathelicidin antimicrobial peptide (CAMP), and signal transducer and activator transcription (STAT3). These proteins have different functions, including ligase (CAD), DNA-binding transcription factor (STAT3), antimicrobial response protein (CAMP), calcium ion binding (ANXA13), transferase (LPCAT2), and oxidoreductase (AKR1B1). The LFQ intensities are shown in Figure 3.

Expression of selected DEPs in cells

The results in Figures 4a and b show the cell viability of the rectal cancer cell line SNU-503 and the radiation-resistant rectal cancer cell line SNU-503R80Gy. It was confirmed that there was a difference in the cell viability of

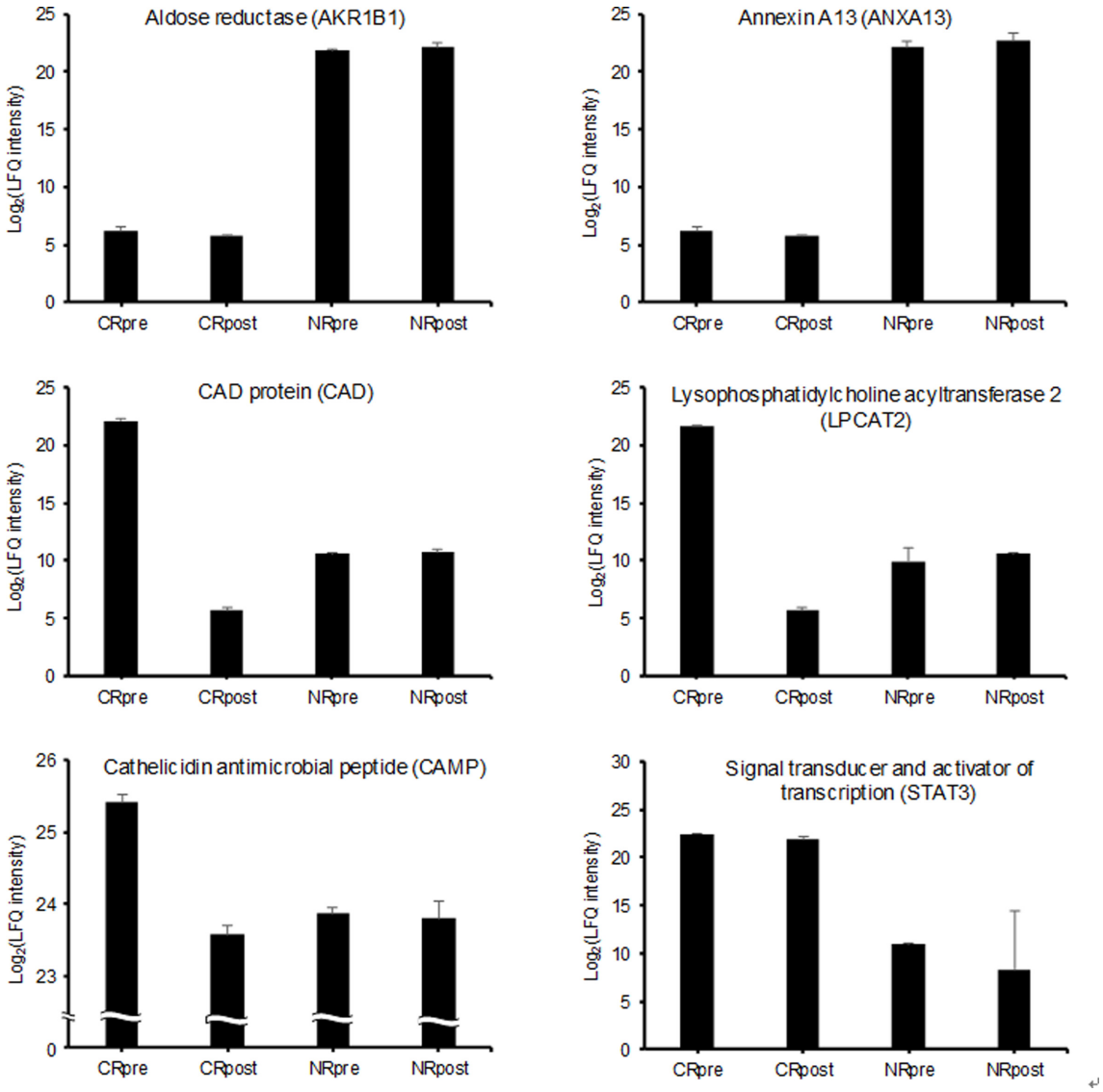

Figure 3. Relative comparison of intensities of six proteins among 4 groups.

the two cell lines according to the radiation dose and time. Briefly, 48 h after 4 Gy irradiation, the viability of the SNU-503 and SNU-503R80Gy cells was 46.9% and 86.0%, respectively, suggesting that they are suitable cell lines for studying differences between CR and NR patients. The expression of AKR1B1, ANXA13, LPCAT2, and CAMP in cell studies was similar to the proteomic results (Figures 4c and d). However, CAD and STAT3 show different results between proteomic analysis of tumors and cell studies. Proteomic data revealed a high intensity of CAD in CRpre compared to other groups, whereas the expression of CAD was low in SNU-503 cells compared to that in SNU-503R80Gy cells. STAT3 was highly expressed in CRpre and CRpost in proteomic data, while its expression was similar in the two cell lines. These differences between proteomic analysis of tumors and cell studies (western blot and mRNA expression) could be due to the differences between tumors and cell lines. Similar differences were observed in the proteomes of colon adenocarcinoma tissues and tissue-derived primary cell lines in a previous study.[34] Furthermore, the cell lines were treated with radiation, while the rectal cancer patients were subjected to both radiation and chemotherapy.

ANXA13 is a member of the annexin A subfamily and consists of 12 proteins. In humans, annexin A members are involved in various cellular processes, such as cell signaling, cell division, membrane scaffolding, ion transportation, and apoptosis.[35] ANXA13 promotes lung adenocarcinoma cell proliferation, invasion, and migration, which may be modulated by epithelial-mesenchymal transition.[36] Notably,

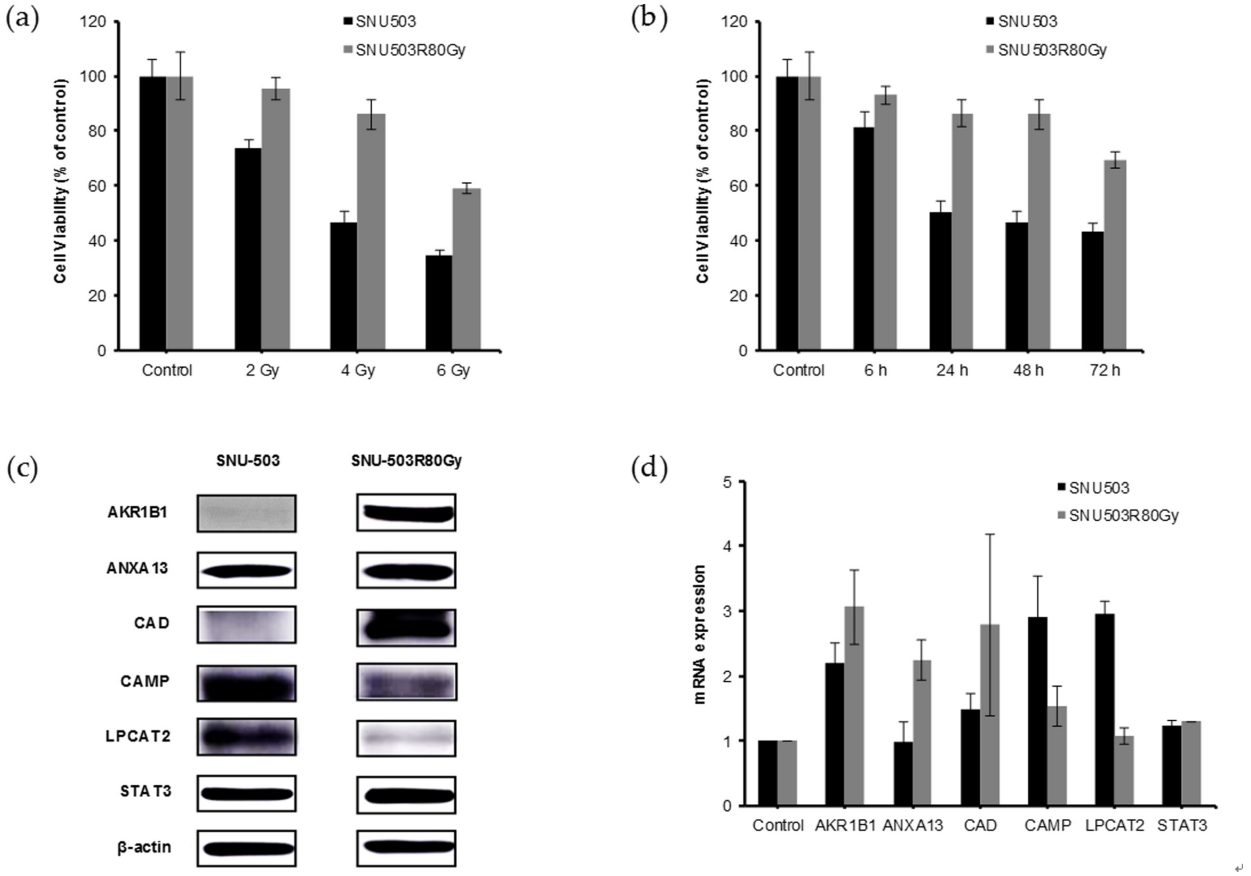

Figure 4. Results of the cell experiment. Radiation sensitivity of SNU-503 and SNU-503R80Gy cell lines de-pending on the radiation dose and time: (a) Cell viability was assessed at 48 h after irradiation with 0, 2, 4, and 6 Gy, and (b) cell viability analysis was performed at 6, 24, 48, and 72 h after ir-radiation with 4 Gy. Expression of biomarker candidate proteins was determined using (c) western blot analysis and (d) expression of mRNA.

ANXA13 is aberrantly up-regulated in cultured CRC cells (SW620, SW480, Rko, HCT116, and HT29), and the overexpression of ANXA13 promotes CRC cell invasion *in vitro*. In human patients, ANXA13 upregulation is associated with lymph node metastasis and poor survival. ANXA13 may be clinically relevant in CRC and can be used as a biomarker for its diagnostic and prognostic value.[37,38] Our findings showed the overexpression of ANXA13 in the NR group, both before and after nCRT; however, in CRpre and CRpost, ANXA13 levels were relatively lower. Thus, this result was in agreement with previous reports, suggesting the potential of ANXA13 as a diagnostic and prognostic biomarker.

AKR1B1 is a member of the aldo-keto reductase (AKR) superfamily, which catalyzes the NADPH-dependent reduction of various carbonyl-containing compounds to their corresponding alcohols. The AKR superfamily consists of 16 different families, and AKR1B1 belongs to AKR1 subfamily B. AKR1B1 is broadly over-expressed in human cancers, which relates to shortened patient survival in acute myelogenous leukemia and multiple myelomas.[39] It plays a role in the cell cycle, epithelial to mesenchymal transition, and miR-21 mediating mechanisms such as inflammatory responses, cell survival, and apoptosis. Notably, the inhibition of AKR1B1 has shown anti-cancer effects.[40] Our data showed a relatively high abundance of AKR1B1 in both NRpre and NRpost and lower levels of AKR1B1 in both CRpre and CRpost. This suggests that AKR1B1 could also be considered a potential cancer diagnostic biomarker for rectal cancer. Several studies have also suggested that AKR1B1 inhibition can be used as an adjuvant therapy to render tumor cells more sensitive to anti-cancer therapy or alleviate the adverse effects of therapy.[41]

CAD, LPCAT2, and CAMP exhibited similar patterns in proteomic analysis of tumors. Their intensities in NRpre and NRpost were relatively low compared to those of CRpre. Interestingly, after successful nCRT, the levels of CAD, LPCAT2, and CAMP were significantly reduced. CAD is involved in *de novo* pyrimidine synthesis, negatively regulates WNT/β-catenin signaling, and inhibits CRC cell migration;[42] thus, its high abundance could be an

indicator of a successful nCRT. LPCAT2 could also be used as a potential prognostic factor for early stages of CRC and as a potential predictive factor for the response of patients to conventional neoadjuvant therapies or the more recently described immunotherapies in advanced stages. Furthermore, LPCAT2 positively correlates with lipid droplet accumulation, which drives cell death resistance to chemotherapy (5-fluorouracil and oxaliplatin treatments).[43] In humans, CAMP is constitutively expressed by macrophages, neutrophils, skin epithelial cells, and endothelial cells located in the gastrointestinal, urinary, and respiratory tracts (specialized host defense cells).[44] It plays an essential role in the regulation of different immune functions, including cell proliferation, apoptosis, inflammatory reactions, angiogenesis, cell cycle arrest, and cytokine release.[45] CAMP has been reported to induce tumorigenic effects in various cancers, such as lung, breast, ovary, prostate, and pancreatic cancer. In contrast, it has anti-cancer effects in gastric cancer, oral squamous cell carcinoma, and hematologic malignancy.[46] Accumulating evidence suggests that human CAMP may suppress colon cancer development,[47] whereas contrasting results have also been reported.[48] STAT3 is one of the six members of a family of transcription factors. Its activation can mediate inflammation, cellular invasion, angiogenesis, and metastasis of cancer, as well as transform cells, suppress apoptosis, and cause cellular proliferation.[49] In the proteomics analysis, high levels of STAT3 were found in the CR group, whereas its abundance was significantly lower in the NR group.

Radiation can damage the cell membrane, DNA, and organelles. It regulates tumor cell apoptosis, proliferation, differentiation, migration, and biological functions by changing the immunogenicity and microenvironment of tumor cells.[50] Our results show that CAMP, ANXA13, LPCAT2, and AKR1B1 are highly anticipated to be potential biomarkers that could be clinically useful in predicting the response to nCRT in rectal cancer patients before surgery. This prediction is believed to be of great help in determining the treatment method. Patients classified as CR would be subjected to nCRT, whereas other treatments should be applied to NR patients. A previous study using two-dimensional polyacrylamide gel electrophoresis suggested some possible protein biomarkers, such as tropomodulin, heat shock protein 42, beta-tubulin, annexin V, calsenilin, keratin type II, notch 2 protein homolog, and DNA repair protein RAD51L3.[51] These proteins were not found in the DEP list of our study, except for keratin type II. The differences in DEPs were probably due to the differences in the patients' race. In this study, we observed significant changes in the expression of CAD, CAMP, and LPCAT2 in the CR group after nCRT treatment. The comprehensive elucidation of nCRT effects on tumors needs to be investigated in further studies. This study, however, has a limitation of the small number of patients. Possible protein biomarkers are required to be validated with a large number of patients in future studies using parallel reaction monitoring MS or orthogonal analyses, including western blotting, enzyme-linked immunosorbent assays (ELISA), radioimmunoassay, and immunohistochemistry.[52]

Conclusions

In this study, proteomic analysis was conducted on biopsy tissues of patients with rectal cancer. We identified 228 DEPs between CRpre and NRpre as well as relevant GO terms and KEGG pathways. Six proteins were selected for evaluation on a rectal cancer cell line and a radiation-resistant rectal cancer cell line. Four proteins (LPCAT2, CAMP, ANXA13, and AKR1B1) could be potential to predict the response of rectal cancer patients to nCRT. Further studies will be conducted to validate candidate biomarkers for clinical applications.

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Supplementary Information

Supplementary files (Tables S1, S2, S3, S4, S5, and Figure S1) are available at Mendeley Data https://data.mendeley.com/datasets/prr2nzswdt/draft?a=17642b78-81dc-4898-8fe2-85627d86c698.

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