Whole genome gene expression changes and hematological effects of rikkunshito in patients with advanced non-small cell lung cancer receiving first line chemotherapy

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Abstract. It has been demonstrated that the traditional Chinese medicine rikkunshito, ameliorates anorexia in several types of human cancer and attenuates lung injury by inhibiting neutrophil infiltration. The current study investigated the clinical and hematological effects of rikkunshito and its underlying mechanisms of action in the treatment of advanced non-small cell lung cancer (NSCLC). The Illumina microarray BeadChip was used to analyze the whole-genome expression profiles of peripheral blood mononuclear cells in 17 patients with advanced NSCLC. These patients were randomized to receive combination chemotherapy (cisplatin and gemcitabine) with (n=9, CTH+R group) or without (n=8, CTH group) rikkunshito. The primary endpoint was the treatment response and the categories of the scales of anorexia, nausea, vomiting and fatigue; secondary endpoints included the hematological effect and whole genome gene expression changes. The results of the current study indicated that there were no significant differences in clinical outcomes, including treatment response and toxicity events, between the two groups. Median one-year overall survival (OS) was 12 months in the CTH group and 11 months in the CTH+R group (P=0.058 by log-rank test), while old age (≥60 years old) was the only independent factor associated with one-year OS (hazard ratio 1.095, 95% confidence interval, 1.09-1.199, P=0.030). Patients in the CTH+R group experienced significantly greater maximum decreases in both white cell count (P=0.034) and absolute neutrophil count (P=0.030) from the baseline. A total of 111 genes associated with neutrophil apoptosis, the cell-killing ability of neutrophils, natural killer cell activation and B cell proliferation were up-regulated following rikkunshito treatment. A total of 48 genes associated with neutrophil migration, coagulation, thrombosis and type I interferon signaling were down-regulated following rikkunshito treatment. Rikkunshito may therefore affect the blood neutrophil count when used with combination chemotherapy in patients with NSCLC, potentially by down-regulating prostaglandin-endoperoxidase synthase 1, MPL, AMICA1 and junctional adhesion molecule 3, while up-regulating elastase, neutrophil expressed, proteinase 3, cathepsin G and cluster of differentiation 24.

Introduction

Lung cancer is a major cause of mortality worldwide, with more than 1.8 million new cases and almost 1.6 million deaths estimated in 2012 (1). The age-standardized incidence rate of lung cancer was 64.8/10^5 for men and 48.6/10^5 for women in the USA from 2011 to 2014, while the age-standardized mortality rate was 57.8/10^5 for men and 37/10^5 for women (1,2). The incidence rate of lung cancer in China was 63.9/10^5 for men and 31.9/10^5 for women in 2011, while the mortality rate was 59.9/10^5 for men and 23.8/10^5 for women (3). Non-small cell lung cancer (NSCLC) accounts for ≥80% of all lung tumors, and ~66% of patients initially present with an inoperable form.
of the disease (4). Treatment with cisplatin (CDDP)-based chemotherapy (CTH) leads to a 10% increase in the 1-year survival rate and increases median survival time by 2 months. It is currently considered the first line treatment for patients with advanced NSCLC who lack a sensitizing mutation of epidermal growth factor receptor (EGFR) (5). The addition of a second drug [either gemcitabine (GEM), vinorelbine, docetaxel or paclitaxel] generates only small improvements in tumor responses and survival rates (6). Recently it was determined that the addition of bevacizumab, a monoclonal antibody for vascular endothelial growth factor, increased the survival and response rates in patients with advanced NSCLC receiving CTH (7). However, bevacizumab is expensive and thus may not be developed for wider use (7). CTH-induced neutropenia is independently associated with increased survival rates of patients with advanced NSCLC, possibly due to a reduction in tumor-related leukocytosis (8-11). Neutropenia occurring in patients with early stage breast cancer is also an independent predictor of increased survival (12). The mechanisms that the body uses to counter CTH-induced neutropenia remain unknown and may be caused by a combination of factors, such as variations in drug metabolism.

Rikkunshito (also known as TJ-43 or Liu-Jun-Zi-Tang) is a common prescription of traditional Chinese and Japanese Kampo medicines consisting of 8 herbs (Atractylodis lanceae rhizoma, Ginseng radix, Pinelliae tuber, Hoelen, Zizyphi fructus, Aurantii nobilis pericarpium, Glycyrrhizae radix and Zingiberis rhizoma) and is commonly used to treat gastrointestinal diseases (13). It has been demonstrated that rikkunshito ameliorates CDDP-induced anorexia, improves patient quality of life (14) and relieves functional dyspepsia via antagonistic action of the 5-Hydroxytryptamine (HT) 2B/2C/3 receptor pathway (15-17). Rikkunshito has been used to ameliorate anorexic symptoms, such as dysmotility-like dyspepsia, postprandial fullness, early satiety and epigastric burning, in cancer cachexia-anorexia syndrome, particularly in patients with colon and breast cancer (18,19). Intervention with herbs used in traditional Chinese medicine herbs may increase efficacy and reduce toxicity when used in combination with EGFR-tyrosine kinase inhibitor to treat advanced NSCLC (20). Thus, it was hypothesized that rikkunshito may be effective at increasing patient responses to treatment and decreasing the side effects of combination CTH in patients with advanced stage NSCLC. In the present study, a randomized double blind clinical trial was conducted to determine whether rikkunshito may be appropriate to use as an adjuvant treatment during chemotherapy for patients with NSCLC. Furthermore, a microarray genomic expression method was used to identify the molecular processes involved in CTH and the whether rikkunshito induced any changes in peripheral blood mononuclear cells (PBMC).

Patients and methods

Patients. The study participants were recruited from the pulmonary clinics of Kaohsiung Chung Gung Memorial Hospital (Kaohsiung, Taiwan) between August 2007 and January 2009. A total of 17 patients were enrolled in the current study. Patients were aged ≥20 years with histologically confirmed, newly diagnosed, untreated stage IIIB or IV NSCLC based on the international staging system for lung cancer (21), ≥1 measurable (uni-dimensional) lesion with a diameter ≥10 mm using spiral computerized tomography and an ECOG performance status (22) of 0, 1 or 2. Exclusion criteria included hypersensitivity to any herbal drug, receipt of concurrent immunotherapy or radiotherapy, uncontrolled medical illness and history of other malignancies within the last 5 years. The study protocol followed the guidelines of the Declaration of Helsinki and was approved by the institutional review boards of Kaohsiung Chang Gung Memorial Hospital (certificate no. 96-0363B). All patients provided written informed consent for inclusion in the current study.

Treatment plan. All enrolled patients were randomly assigned into one of two groups: A CTH+R group (n=9) or a CTH group (n=8). All patients received combination CTH with CDDP (Platinex®; Dabur India Limited, New Delhi, India; mean dose 60 mg/m²) on day 1 plus GEM (GEMZAR®; mean dose 1000 mg/m²; Lilly, Indianapolis, IN, USA) on day 1, 8 and 15 of a 28-day cycle, as well as dry powder placebo (corn starch; Chuang Song Zong Pharmaceutical Co., Ltd., Kaohsiung, Taiwan) 30 g administered orally daily (CTH group), or combination chemotherapy plus rikkunshito 30 g administered orally daily (CTH+R group). The powder extract of rikkunshito was a mixture of 5.00 g ginseng radix, 5.00 g atractylodis rhizoma, 5.00 g hoelen, 5.00 g pinelliae tuber, 2.50 g glycyrrhizae radix, 2.50 g auranti nobilis pericarpium, 2.50 g gingersibis rhizoma, 2.50 g zizyphi Fructus and 7.80 g starch for each 100.00 g powder with a crude drug: extract ratio of 30.00:7.80 (LIOW JIUN TZYY TANG extract powder; no. 2088; Chuang Song Zong Pharmaceutical Co., Ltd.). Treatment was planned for a maximum of six cycles unless discontinued due to disease progression, unacceptable toxicity, physician or patient decision, requirement for palliative radiotherapy or patient mortality. All patients received the 5-HT3 receptor antagonist granisetron (Kytril®; 2 mg; TTY Biopharm, Taipei, Taiwan), metoclopramide (Promeran®; 7.68 mg; Standard Chem. & Pharm. Co., Ltd., Tainan, Taiwan) and dexamethasone (Methasone®; 10 mg; Taiwan Veterans Pharm. Co., Ltd., Chung-Li, Taiwan) as anti-emetic agents in each combination CTH regimen. Following baseline evaluation, tumor status was assessed following last course of chemotherapy, according to the Response Evaluation Criteria in Solid Tumors (23). Leukocyte, hemoglobin levels and platelet counts as well as events of subjective toxicity were recorded on day 1, 8 and 15 in each treatment course. Maximum changes in the hematological parameters were defined as the value prior to treatment minus the mean lowest value for each treatment course. Toxicity was graded prior to each treatment cycle using the National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0 (24). Overall survival (OS) was defined as time from enrollment until mortality from any cause within a 1- to 3-year observation period. Progression-free survival (PFS) was defined as the time from date of randomization to the date of first observation of progression [defined as ≥20% increase in the sum of the largest diameter of target lesions (23)] or mortality due to any cause. The primary endpoint was the treatment response and the categories of the scales of anorexia, nausea, and vomiting [based on evaluation of event frequency and clinical impact (24)] during the
treatment course (duration, ~4 months); secondary endpoints included hematological effects (hemoglobin level, white cell count, neutrophil count and platelet count) during the treatment course and whole genome gene expression changes at the end of the treatment course (24).

**RNA isolation and cRNA synthesis.** Approximately 15 ml peripheral whole blood was collected by a phlebotomist using mini-puncture following four courses of chemotherapy treatment with or without concurrent use of rikkunshito. PBMCs were isolated, washed in PBS, transferred and stored in RNAlater® (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at -80˚C until RNA isolation. A RiboPure-Blood kit (Ambion; Thermo Fisher Scientific, Inc.) was used to isolate high quality total RNA. Samples were run using an RNA 6000 Nano Gel kit on an Agilent 2100 Bioanalyzer (both from Agilent Technologies, Inc., Santa Clara, CA, USA) to determine RNA quality and 2 µl RNA was used to determine RNA concentration using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Only samples with A260/A280 ratios of 1.9 to 2.1 were used for further studies. A total of 300 ng RNA was used for *in vitro* transcription of cRNA using the Illumina® Totalprep™ RNA Amplification kit (Ambion; Thermo Fisher Scientific, Inc.).

**Gene expression profiling.** The Illumina HumanRef-8V2 BeadChip (Illumina, Inc., San Diego, CA, USA) was used to generate expression profiles of >22,000 transcripts with 750 ng labeled cRNA. All expression profiles are available at NCBI Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) under the series number GSE18309. The probe sets and genes were grouped into functional categories using the Gene Ontology Biological Processes Classification (25).

**Microarray data analysis.** Statistical analysis of the microarray data was performed using the GeneSpring™ software version 11 (Sigeneics Inc., Chicago, IL, USA). A total of 8,186 probe sets passed the signal filter (which filtered out genes that had low signal close to background level), and were used for further statistical analysis. A non-parametric Wilcoxon signed-rank test for paired comparison of the data prior to and following treatment in the two groups was applied. A change

### Table I. Comparison of the baseline characteristics of CTH and the CTH+R group.

| Characteristic                  | CTH group, n=8 | CTH+R group, n=9 | P-value |
|--------------------------------|----------------|------------------|---------|
| Age, years                     | 56.7±15        | 67.5±8.7         | 0.135   |
| Male, n (%)                    | 6 (75)         | 7 (77.8)         | 0.893   |
| TNM tumor stage, n (%)         |                |                  | 0.086   |
| IIIb                           | 6 (75)         | 3 (33.3)         |         |
| IV                             | 2 (25)         | 6 (66.7)         |         |
| Histology, n (%)               |                |                  | 0.893   |
| Adenocarcinoma                 | 6 (75)         | 7 (77.8)         |         |
| Squamous cell carcinoma        | 2 (25)         | 2 (22.2)         |         |
| ECOG performance status        |                |                  | 0.363   |
| 0                              | 0 (0)          | 2 (22.2)         |         |
| 1                              | 7 (87.5)       | 6 (66.7)         |         |
| 2                              | 1 (12.5)       | 1 (11.1)         |         |
| Co-morbidity, n (%)            |                |                  |         |
| Diabetes mellitus              | 1 (12.5)       | 2 (22.2)         | 0.600   |
| Hypertension                   | 4 (50)         | 4 (44.4)         | 0.819   |
| Chronic bronchitis             | 5 (62.5)       | 3 (33.3)         | 0.229   |
| Chronic hepatitis              | 1 (12.5)       | 1 (11.1)         | 0.929   |
| Chronic kidney disease         | 0 (0)          | 1 (11.1)         | 0.331   |
| Congestive heart failure       | 1 (12.5)       | 0 (0)            | 0.274   |
| Smoking history, n (%)         |                |                  | 0.959   |
| Never                          | 3 (37.5)       | 4 (44.4)         |         |
| Former                         | 4 (50)         | 4 (44.4)         |         |
| Current                        | 1 (12.5)       | 1 (11.1)         |         |
| Mean dosage of chemotherapy agent (mg/m²) |       |                  |         |
| Cisplatin                      | 67.4±6.2       | 69.2±6.9         | 0.590   |
| Gemcitabine                    | 973.1±65.1     | 968.7±82.4       | 0.782   |

All data are presented as the mean ± standard deviation unless otherwise specified. CTH, combination chemotherapy group; CTH+R group, combination chemotherapy plus rikkunshito group.
in expression of >1.5-fold and P<0.05 was used to define the optimal subsets of significantly up- and down-regulated genes.

**Statistical analysis.** Continuous values are presented as mean ± standard deviation. Mann-whitney, Wilcoxon ranked sum, Kruskal-Wallis H and \( \chi^2 \) tests were used to assess the differences between different groups, where appropriate. Survival curves were constructed using the Kaplan-Meier method. Cox regression was used to identify independent survival factors. Stepwise multiple linear regression analysis was used to adjust for age, tumor stage, and other confounding factors and obtain adjusted P-values comparing continual variables between the two study groups. All tests were two tailed and P<0.05 was considered to indicate a significant difference. The SPSS statistical software package version 15.0 (SPSS Inc; Chicago, IL, USA) was used for data analysis.

**Results**

**Clinical effects of combination chemotherapy with or without rikkunshito in patients with advanced stage NSCLC.** Between August 2007 and January 2009, 26 patients with newly diagnosed stage IIIb or IV NSCLC were screened. A total of 9 patients were excluded; 6 refused to participate in the study, 2 had other concomitant malignancies and one was receiving concurrent radiotherapy. A total of 17 patients were thus enrolled in the current study. Patients were randomized to the CTH arm (n=8) or CTH+R arm (n=9) and all patients completed follow-up. One-year survival follow-up was completed in January 2010. Patients in the CTH arm received a median of 4 (range 3-5) cycles of combination chemotherapy as well as a daily dose of rikkunshito (cycle numbers of combination chemotherapy, CTH vs. CTH+R arm, P=0.705). The demographic
and clinicopathological characteristics of the 17 patients are presented in Table I. Both groups were matched in age, sex, smoking history, tumor stage, ECOG performance status, histopathology subtype, tumor size, co-morbidity and doses of chemotherapy agents administered. Blood cell counts at baseline were similar, apart from significantly lower hemoglobin levels (P=0.014) observed in the CTH+R group compared with the CTH group. Following a median of four courses of chemotherapy, the patients in the CTH+R group experienced greater maximum changes in white cell count (P=0.034) and absolute neutrophil count (P=0.030), compared with patients in the CTH alone group, following adjustment for age, tumor stage and other confounding factors by stepwise multiple linear regression analysis (Table II). This means that patients with NSCLC in the CTH+R group exhibited more severe CTH-induced neutropenia compared with patients in the CTH group. There were no significant differences in other outcomes between the two groups in terms of PFS, OS, treatment response, change in tumor size, subjective adverse reactions and maximum changes in hemoglobin level or platelet count from baseline (Table II). Based on Kaplan-Meier estimates (Fig. 1A), median PFS was 6 months in both the CTH and CTH+R groups (P=0.390, Log-Rank test). There was 1 case of patient mortality in the CTH group and 5 cases in the CTH+R group. Median one-year OS was 12 months in the CTH group and 11 months in the CTH+R group (P=0.058, Log-Rank test; Fig. 1B). The one-year survival rate was 87.5% for the CTH arm and 44.4% for the CTH+R arm. Multivariate Cox regression analysis determined that old age [aged >60 years; hazard ratio 1.095, 95% confidence interval (CI) 1.09-1.189; P=0.030] was the only independent factor associated with one-year mortality when adjusting for blood laboratory data, tumor stage, ECOG performance status, histology subtype and treatment group. Kaplan-Meier estimates determined that patients aged ≤60 years had a better one-year survival rate than those aged >60 years (P=0.015, Log-Rank test; Fig. 1C). The three-year PFS (median 6 vs. 7 months, P=0.591; Fig. 2A) and OS (median 27 vs. 15 months, P=0.261; Fig. 2B) were similar in both groups. Clinical benefit, defined as a complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD), as well as the frequency of subjective toxicity events, was similar between all patients in the two arms (Table II). There were 2 (25%) PR cases, 3 (37.5%) SD cases and 3 (37.5%) PD cases in the CTH arm, compared with 4 (44.4%) PR cases, 4 (44.4%) SD cases and 1 (11.1%) PD case in the CTH+R arm (P=0.415).
Table III. Selected microarray gene sets significantly altered before and after chemotherapy in the CTH+R group and regulated in the opposite direction before and after chemotherapy in the CTH group by Gene Ontology analysis.

| Gene symbol | Fold change | Ensembl ID | Description |
|-------------|-------------|------------|-------------|
| GNLY        | 1.9396      | ENSG00000115523 | Granulysin |
| LTF         | 1.8497      | ENSG00000012223 | Lactotransferrin |
| CAMP        | 1.9233      | ENSG00000164047 | Cathelicidin antimicrobial peptide |
| DEFA3       | 3.5452      | ENSG00000239839 | Defensin, α3, neutrophil-specific |
| DEFA4       | 3.1757      | ENSG00000164821 | Defensin, α4, cortico statin |
| PRF1        | 1.6845      | ENSG00000180644 | Perforin 1 (pore forming protein) |
| DDIT4       | 2.0889      | ENSG00000168209 | DNA-damage-inducible transcript 4 |
| RNASE3      | 2.2979      | ENSG00000169397 | Ribonuclease, RNase A family, 3 |
| CTSG        | 3.1563      | ENSG00000100448 | Cathepsin G |
| MPO         | 2.0649      | ENSG0000005381 | Myeloperoxidase |
| AZU1        | 2.1764      | ENSG00000172232 | Azurocidin 1 |
| PGLYRP1     | 1.6233      | ENSG0000008438 | Peptidoglycan recognition protein 1 |
| BPI         | 2.1614      | ENSG00000101425 | Bactericidal/permeability-increasing protein |
| MMP8        | 1.5489      | ENSG00000118113 | Matrix metallopeptidase 8 (neutrophil collagenase) |
| GZMB        | 1.5678      | ENSG00000100453 | Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) |
| PRTN3       | 1.6904      | ENSG00000196415 | Proteinase 3 |
| ELANE       | 2.8886      | ENSG00000197561 | Elastase, neutrophil expressed |
| LTF         | 1.84967     | ENSG0000012223 | Lactotransferrin |
| CTSG        | 3.15631     | ENSG00000100448 | Cathepsin G |
| AZU1        | 2.17641     | ENSG00000172232 | Azurocidin 1 |
| MPL         | -1.7269     | ENSG0000017400 | myeloproliferative leukemia virus oncogene protein S (alpha) |
| PROS1       | -1.6982     | ENSG00000184500 | glycoprotein IX (platelet) |
| GP IX       | -2.1117     | ENSG00000169704 | platelet factor 4 variant 1 |
| PF4V1       | -1.8459     | ENSG00000109272 | pro-platelet basic protein (chemokine (C-X-C motif) ligand 7) |
| PPBP        | -1.5451     | ENSG00000163736 | coagulation factor XIII, A1 polypeptide |
Distinct gene expression signature changes associated with Rikkunshito and chemotherapy relative to chemotherapy alone. Gene expression data prior to and following treatment was compared between the CTH+R and CTH groups, respectively and fold change was obtained by dividing the log ratios of gene expression intensity following treatment by the data collected prior to treatment. A total of 159 differentially expressed genes (DEG) were identified to be significantly up or down regulated in the CTH+R group following treatment, which were regulated in the opposite direction or remained unchanged in the CTH group. Among these, 111 genes were up-regulated and 48 genes were down-regulated following rikkunshito treatment. Gene ontology analysis demonstrated that gene sets mapping to defensive responses to other organisms, immune cell killing and serine-type endopeptidase activity pathways were up-regulated following rikkunshito treatment, whereas gene sets mapping to blood coagulation, thrombosis, leukocyte migration and integral component of plasma membrane pathways were down-regulated (Table III). Genes up-regulated following with rikkunshito treatment (Table IV) were associated with neutrophil apoptosis [elastase (ELANE), proteinase 3 (PRTN3), cathepsin G (CTSG), cluster of differentiation (CD)24], cell-killing or bactericidal ability of neutrophil [defensin α 1-3 (DEF A1, A2, A3), bactericidal/permeability-increasing protein (BPI)], natural killer (NK) cell activation [killer cell immunoglobulin-like receptor (KIR2DL1-3), killer cell lectin-like receptor (KLR), KIR2DS5, NK group 7 (NKG7)] and B cell proliferation [CD19, membrane-spanning 4-domains (MS4A3), CD79A, CD79B and tumor necrosis factor receptor superfamily (TNFRSF13B/17)]. Genes that were down-regulated following rikkunshito treatment (Table IV) were associated with neutrophil migration [prostaglandin endoperoxide synthase 1 (PTGS1; cyclooxygenase 1, COX1), monophosphoryl lipid A (MPL), junctional adhesion molecule 3 (JAM3), adhesion molecule interacts with CXADR antigen 1 (AMICA1; JAML), coagulation [coagulation factor XIII (F13A1)], thrombosis [glycoprotein IX (GPIX), platelet factor 4 variant 1 (PF4V1), pro-platelet basic protein (PPBP), integrin α 2b (ITGA2B;
Table IV. Selected microarray of DEGs significantly altered before and after chemotherapy in the CTH+R group and regulated in the opposite direction before to and after chemotherapy in the CTH group.

| Gene symbol | Fold change | Description |
|-------------|-------------|-------------|
| CD19        | 1.9084      | CD19 molecule |
| MS4A1 (CD20)| 1.5698      | membrane-spanning 4-domains, subfamily A, member 1 |
| CD24        | 1.5597      | CD24 molecule |
| CD79A       | 2.0026      | CD79a molecule, immunoglobulin-associated α |
| CD79B       | 1.6759      | CD79b molecule, immunoglobulin-associated β |
| TNFRSF13B (CD267)| 1.5603 | tumor necrosis factor receptor superfamily, member 13B |
| TNFRSF17 (CD268)| 1.5128 | tumor necrosis factor receptor superfamily, member 17 |
| PRL         | 1.52987     | Prolactin |
| KIR2DL1 (CD158A)| 1.5137 | Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 1 |
| KIR2DL2 (CD158B1)| 1.5478 | Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 2 |
| KIR2DL3 (CD158B2)| 1.5771 | Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 3 |
| KIR2DL4 (CD158D)| 1.6675 | Killer cell immunoglobulin-like receptor, two domains, long cytoplasmic tail, 4 |
| KLRK1 (CD314)| 1.5105      | Killer cell lectin-like receptor subfamily K, member 1 |
| KLRD1 (CD94)| 1.5437      | Killer cell lectin-like receptor subfamily D, member 1 |
| KIR2DS5 (CD158G)| 1.5378 | Killer cell immunoglobulin-like receptor, two domains, short cytoplasmic tail, 5 |
| PTGS1       | -1.8308     | Prostaglandin-endoperoxide synthase 1 |
| PGRMC1      | -1.7732     | Progesterone receptor membrane component 1 |
| MPL         | -1.72699    | Proto-oncogene, thrombopoietin receptor |
| AMICA1      | -1.55819    | Adhesion molecule, interacts with CXADR antigen 1 |
| CYBB        | -1.50661    |            |
| PPBP        | -1.54508    |            |
**Table IV. Continued.**

| Gene symbol | Positive regulation by activated neutrophils | Fold change | Ensembl ID       | Description                                         |
|-------------|----------------------------------------------|-------------|------------------|-----------------------------------------------------|
| IFIT1       | After/ before CTH+R | -1.6140      | 1.2178           | Interferon-induced protein with tetratricopeptide repeats 1 |
| PF4V1       | After/ before CTH | -1.3522      | -1.8459          | Platelet factor 4 variant 1                          |
| ITGA2B      | After/ before CTH | -1.83201     | -1.59619         |                                                     |

CTH, combination chemotherapy group; CTH+R group, combination chemotherapy plus rikkunshito group; DEG, differentially expressed genes; CD, cluster of differentiation.

**Discussion**

Rikkunshito is a Chinese and Japanese herbal medicine that is widely used to treat upper gastrointestinal symptoms, including functional dyspepsia, gastroesophageal reflux disease, dyspeptic symptoms in patients following post gastrointestinal surgery, and chemotherapy-induced dyspepsia in patients with cancer (18,19). The anti-emetic effect of rikkunshito occurs via the stimulation of endogenous ghrelin secretion by blocking the serotonin (5-HT) 2b/2c/3 receptor pathway and inhibiting the expression of substance P/calcitonin gene-related peptide/phosphorylated extracellular signal-regulated kinase 1/2 (26). In the current randomized controlled trial, no significant differences in the frequency of anorexia, nausea or vomiting were observed between the CTH+R and CTH groups. Notably, it was determined that patients with NSCLC in the CTH+R group exhibited more severe CTH-induced neutropenia than patients in the CTH group, however both groups had similar PFS and OS. Previous studies have demonstrated that CTH-induced neutropenia is an independent factor of better survival in patients with lung cancer (8-11), therefore it has been suggested that rikkunshito may exert potential effects on disease control when used in combination with the CDDP+GEM CTH regimen. Abnormal expression of transport protein, enhancement of intracellular detoxification, an increase in DNA repair capacity and the blocking of apoptosis are the primary mechanisms underlying cancer resistance to CDDP (27). Furthermore, ginseng radix, one of the primary ingredients of rikkunshito, has been found to exert antitumor, antioxidant, and immunomodulation activities through its pro-apoptotic and anti-cell cycle effects (28-30). The netopenic effect may only occur when rikkunshito is used in combination with anti-cancer drugs, since such an effect is not universally recognized under its general usage.

Microarray gene expression comparison between pre- and post-treatment data for the CTH+R and CTH groups identified a number of signaling pathways and molecules that were significantly altered following the addition of rikkunshito. Enhanced PTGSI expression is responsible for persistent neutrophil presence as it impairs its apoptosis (31). It has been demonstrated that JAM3 regulates the polarized transendothelial migration of neutrophils (32), whereas the exogenous expression of AMICA1 (JAML) in leukocytes resulted in enhanced cell adhesion to endothelial cells (33). In the present study, PTGSI, JAM3 and AMICA1 were all down-regulated following rikkunshito treatment, indicating the underlying mechanisms by which more severe neutropenia was able to develop in the CTH+R group compared with the CTH group. In accordance with the results of the current study, it has been demonstrated that rikkunshito is able to inhibit the infiltration of neutrophils and macrophages by inhibiting the 5-HT3 receptor and ghrelin release in an animal model (34). Moreover, rikkunshito can ameliorate bleomycin-induced acute lung injury by inhibiting neutrophil alveolar infiltration, pulmonary vascular permeability, the release of pro-inflammatory cytokines, nuclear factor-κB pathway activation and the apoptosis of alveolar epithelial cells in a ghrelin-independent manner (35). The three serine proteases ELANE, PRTN3 and CTSG are major components of the neutrophil primary granules (36-38). Previous studies have demonstrated that neutrophil ELANE provides a negative feedback to granulopoiesis by direct antagonism of granulocyte colony-stimulating factor, whereas PRTN3-mediated caspase-3 activation and the CTSG-dependent pathway contribute to the spontaneous death of neutrophils (36-38). Furthermore, CD24 ligation induces neutrophil death by depolarizing the mitochondrial membrane in a manner dependent on caspase-3 and caspase-9 and reactive oxygen species (39). In the present study, the three neutrophil granules and CD24 genes were all up-regulated following rikkunshito treatment, indicating that it may enhance neutrophil apoptosis. Four human α-defensins from the granules of neutrophils (DEF A1-A4) can attract other immune cells, inducing the release of pro-inflammatory cytokines (40). BPI, stored in the primary azurophilic granula of...
neutrophil granulocytes, is able to efficiently kill Gram-negative bacteria (41). In the present study DEF A1, A2, A3, and BPI were all up-regulated following rikkunshito treatment, indicating a positive effect of rikkunshito on the individual cell-killing function of neutrophils but a negative effect on the recruitment and survival of neutrophils.

The principal inhibitory receptors expressed on NK cells are KIR (KIR2DL1-3, KIR3DL1), while activating
receptors include FcγRIIa, activating forms of KIR (KIR2DS, KIR3DS), KIR2DS1 and NKGD2D (42). The mounting of effective anti-tumor immune responses by cytotoxic NK cells via mediation of cancer stem cell lysis is important to facilitate tumor clearance (43). In the present study, a number of inhibitory receptors (KIR2DL1-3, KLR) and activating receptors (KIR2DS5, NKG7) were all up-regulated following rikkunshito treatment, indicating that rikkunshito may exert its potential anti-neoplasm effect by modulating the function of NK cells. CD19 and MS4A3 both serve important roles in B cell receptor (BCR) activation and signaling (44,45), while the extracellular antigen recognizing domain is complexed with CD79A and CD79B that form the cytoplasmic tail of the BCR (46). TNFRSF13B affects T-cell-independent antibody responses (47) and PRL may promote the increased proliferation of lymphocytes (48). In the present study, CD19, MS4A3, CD79A, CD79B, TNFRSF13B, TNFRSF17 and PRL were all up-regulated following rikkunshito treatment, indicating that it may enhance the activation and proliferation of B cells.

F13A1 and MPL serve important roles in coagulation and thrombosis, respectively (49,50), whereas IFITs are involved in drug resistance and metastasis in breast cancer (51). In response to vascular damage, engagement of the platelet receptors GP Ib-IX-V, GPVI and/or FcγRIa leads to platelet adhesion and activation (52). A complex coagulopathy characterized by activation of clotting mechanisms develops in parallel with malignancy (53). In the present study, F13A1, MPL, GP Ib, GPIX, GP Ibα, IFIT1 and IFIT2 were all down-regulated following rikkunshito treatment, indicating that rikkunshito may exert an anti-neoplasm effect, resulting in the down-regulation of these coagulation/thrombosis pathways.

It has been demonstrated that compromised immune surveillance and inflammatory microenvironments affect aspects of malignancy including proliferation, survival, angiogenesis and tumor metastasis. Neutrophils are recruited to tumor sites through transendothelial migration. This creates an inflammatory environment, which increases the risk of cancer development. By contrast, T cells and NK cells are able to recognize and destroy cancer cells (54). Antibodies that inhibit the checkpoint molecules of T cells, including cytotoxic T-lymphocyte-associated antigen 4 and programmed death legend 1, improve the survival and response to treatment in patients with NSCLC (55). Based on the rikkunshito-related genetic signatures, rikkunshito may have multiple pharmaceutical action sites to exert its anti-cancer effects by enhancing neutrophil apoptosis, inhibiting neutrophil migration, promoting NK cell activation, inducing B cell proliferation and ameliorating thrombosis/coagulation pathways. However, further studies are required to determine the exact pharmacological sites of rikkunshito with regard to its antimtumor effects.

The limitations and biases of the current study should be addressed. First, only 17 patients with NSCLC were included in the current study and follow-up was completed over short-course study period. The sample size seems to be too small to accurately identify whether there were any significant differences in the major outcomes between the CTH and CTH+R groups. However, despite the small sample size, a greater neutropenic effect following the addition of rikkunshito treatment was observed in the current study. The lack of anti-emetic effect following rikkunshito treatment in the current randomized controlled trial may be due to strong anti-emetic agents, including the 5-HT3 receptor antagonists, metoclopramide and dexamethasone administered, to all 17 participants. Secondly, hemoglobin levels were lower and age was slightly greater in the CTH+R group at baseline. However, the maximum changes following treatment revealed that rikkunshito primarily affected white blood cell and neutrophil count, rather than hemoglobin levels. Moreover, Cox regression model analysis was performed to adjust the effect of the confounding factors on clinical outcomes. Finally, the changes in gene expression identified by the microarray analysis were not validated by quantitative real-time reverse transcript polymerase chain reaction due to the inadequate number of RNA samples. Despite these limitations, to the best of our knowledge, the present study is the first to assess the potential molecular mechanisms and genes associated with the clinical effects of rikkunshito on a genome-wide scale.

In conclusion, the current study identified the potential effect of rikkunshito treatment on reducing blood total leukocyte and absolute neutrophil counts in patients with advanced NSCLC receiving first line CTH with CDDP and GEM. Rikkunshito did not exert a prominent anti-emetic effect or affect other outcomes, including response rates and survival. Microarray gene expression analyses identified a number of signaling pathways and gene sets that may be involved in the underlying mechanisms by which rikkunshito functions to enhance the cell-killing ability of neutrophils, activate NK cells and B cell proliferation and inhibit coagulation/thrombosis. Gene Ontology analyses suggest that PTGSI, MPL, AMICA1 and JAM3 may be involved in the development of more severe CT-induced neutropenia by inhibiting neutrophil apoptosis whereas ELANE, PRTN3, CTSG and CD24 may act by enhancing the transendothelial migration and adhesion of neutrophils following the addition of rikkunshito.

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References

1. Dubey AK, Gupta U and Jain S: Epidemiology of lung cancer and approaches for its prediction: A systematic review and analysis. Chin J Cancer 35: 71, 2016.
2. Didkowska J, Wojciechowska U, Mańczuk M and Łobaszewski J: Lung cancer epidemiology: Contemporary and future challenges worldwide. Ann Transl Med 4: 150, 2016.
3. Hong QY, Wu GM, Qian GS, Hu CP, Zhou JY, Chen LA, Li WM, Li SY, Wang K, Wang Q, et al: Prevention and management of lung cancer in China. Cancer 121 (Suppl 17): S3080-S3088, 2015.
inhibitor for advanced non-small cell lung cancer: A systematic combined with epidermal growth factor receptor tyrosine kinase
Chen JX and Shen XH:
Fujitsuka N and Uezono Y: Rikkunshito, a ghrelin potentiator,
Mogami S and Hattori T: Beneficial effects of rikkunshito, a
antagonism
Nagai K and Asaka M:
Takeda H, Sadakane C, Hattori T, and 5-HT2C receptor antagonist improve cisplatin-induced
pathway in rats
Yamagami H, Tanigawa T, Watanabe K, Watanabe T, Tominaga K, Kido T, Ochi M,
Ogata K, Mochiki E, Asao T and Kuwano H:
Ohno T, Yanai S, Miura A, Kikuchi K, Saito T, et al: Rikkunshito ameliorates reflux esophagitis. Neurogastroenterol Motil 26: 913-921, 2014.
Liu L and Bian K: Advance in studies on molecular mechanisms of cisplatin resistance and intervention with traditional Chinese medicines. Zhongguo Zhong Yao Za Zhi 39: 3216-3220, 2014 (In Chinese).
Kolaczkowska E, Płytycz B, Arnold B, Piccard H and Opedenaker G: Increased cyclooxygenase activity impairs apoptosis of inflammatory neutrophils in mice lacking gelatinase B/matrix metalloproteinase-9. Immunology 128 (Suppl 1): e262-e274, 2009.
Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapoulis FM, Nash GB, Chavakis T, Albelda SM, Rainiger GE, et al: The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat Immunol 12: 761-769, 2011.
Moog-Lutz C, Cavé-Riant F, Guibal F, Breau MA, Di Gioia Y, Courraud PO, Gayre VE, Bououdoulou S and Lutz PG: JAML, a novel protein with characteristics of a junctional adhesion molecule, is induced during differentiation of myeloid leukemia cells. Blood 102: 3371-3378, 2003.
Endo M, Hori M, Ozaki H, Okawa T and Hanawa T: Rikkunshito, a Kampo medicine, ameliorates post-operative ileus by anti-inflammatory action. J Pharmacol Sci 124: 374-385, 2015.
Tsubouchi H, Yanagi S, Miura A, Iizuka S, Mogami S, Yamada C, Hattori T and Nakazato M: Rikkunshito ameliorates bleomycin-induced acute lung injury in a ghrelin-independent manner. Am J Physiol Lung Cell Mol Physiol 306: L233-L245, 2014.
El Ouraighi F, Fujiwara H, Melenhorst JJ, Sconocchia G, Hensel N and Barrett AJ: Neutrophil elastase enzymatically activates the in vitro action of G-CSF: Implications for the regulation of granulopoiesis. Blood 110: 1752-1758, 2007.
Leutenegger F, Zhu H, Karan S, Zhou A, Liu P, Ye K, Zhou J, Cao S, Gong, H., Jenne DE, et al: Proteinase 3-dependent caspase-3 cleavage modulates neutrophil death and inflammation. J Clin Invest 124: 4445-4458, 2014.
Baumann M, Pham CT and Benarafa C: SerpinB1 is critical for neutrophil survival through cell-autonomous inhibition of cathepsin G. Blood 121: 3900-3907, S1-S6, 2013.
Parlato M, Souza-Fonseca-Guimaraes F, Philippart F, Misset B; Captain Study Group. Adju-Convy M and Cavaillon JM: CD24-triggered caspase-dependent apoptosis via mitochondrial membrane depolarization and reactive oxygen species production of human neutrophils is impaired in sepsis. J Immunol 192: 2449-2459, 2014.
Wilmes M and Sahl HG: Defensin-based anti-infective strategies. Int J Med Microbiol 304: 93-99, 2014.
Bolweg A, Schmale M and Gessler A: The bacterial/cellular permeability-increasing protein (BPI) in the innate defence of the lower airways. Biochem Soc Trans 39: 1045-1050, 2011.
42. Campbell KS and Hasegawa J: Natural killer cell biology: An update and future directions. J Allergy Clin Immunol 132: 536-544, 2013.
43. Jewett A, Tseng HC, Arasteh A, Saadat S, Christensen RE and Cacalano NA: Natural killer cells preferentially target cancer stem cells; role of monocytes in protection against NK cell mediated lysis of cancer stem cells. Curr Drug Deliv 9: 5-16, 2012.
44. Weiland J, Elder A, Forster V, Heidenreich O, Koschmieder S and Vormoor J: CD19: A multifunctional immunological target molecule and its implications for Blineage acute lymphoblastic leukemia. Pediatr Blood Cancer 62: 1144-1148, 2015.
45. Eon Kuek L, Leffler M, Mackay GA and Hulett MD: The MS4A family: Counting past 1, 2 and 3. Immunol Cell Biol 94: 11‑23, 2016.
46. Niemann CU and Wiestner A: B-cell receptor signaling as a driver of lymphoma development and evolution. Semin Cancer Biol 23: 410-421, 2013.
47. Salzer U, Chapel HM, Webster AD, Pan-Hammarström Q, Schmitt-Graef A, Schlesier M, Peter HH, Rockstroh JK, Schneider P, Schäffer AA, et al: Mutations in TNFRSF13B encoding TACI are associated with common variable immuno-deficiency in humans. Nat Genet 37: 820-828, 2005.
48. Auchtung TL and Dahl GE: Prolactin mediates photoperiodic immune enhancement: Effects of administration of exogenous prolactin on circulating concentrations, receptor expression, and immune function in steers. Biol Reprod 71: 1913-1918, 2004.
49. Chou FS and Mulloy JC: The thrombopoietin/MPL pathway in hematopoiesis and leukemogenesis. J Cell Biochem 112: 1491-1498, 2011.
50. Biswas A, Ivaskevicius V, Thomas A and Oldenburg J: Coagulation factor XIII deficiency. Diagnosis, prevalence and management of inherited and acquired forms. Hamostaseologie 34: 160-166, 2014.
51. Motaghed M, Al-Hassan FM and Hamid SS: Thymoquinone regulates gene expression levels in the estrogen metabolic and interferon pathways in MCF7 breast cancer cells. Int J Mol Med 33: 8-16, 2014.
52. Gardiner EE and Andrews RK: Platelet receptor expression and shedding: Glycoprotein Ib-IX-V and glycoprotein VI. Transfus Med Rev 28: 56-60, 2014.
53. Falanga A, Marchetti M and Russo L: The mechanisms of cancer-associated thrombosis. Thromb Res 135 (Suppl 1): S8-S11, 2015.
54. Orozco-Morales M, Souc-Chafre G, Barrios-Bernal P, Hernández-Pedro N and Arrieta O: Interplay between cellular and molecular inflammatory mediators in lung cancer. Mediators Inflamm 2016: 3494608, 2016.
55. Buchbinder EI and Desai A: CTLA-4 and PD-1 pathways: Similarities, differences, and implications of their inhibition. Am J Clin Oncol 39: 98-106, 2016.