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Clinical Study on Nicotinamide Phosphoribosyl Transferase and *SIRT1* Level in Patients With Neutropenia After Chemotherapy for Lung Cancer

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The NAD^+–sirtuin 1 (*SIRT1*) pathway is essential for neutrophil differentiation induced by the granulocyte-colony stimulating factor, and thus may contribute to neutropenia observed after cancer chemotherapies. This study aimed to investigate the relationship between neutropenia and indices for the activity of the NAD-*SIRT1* pathway, i.e., nicotinamide phosphoribosyltransferase (NAMPT) activity and *SIRT1* expression in patients receiving chemotherapy for lung cancer. Blood samples were collected from sixteen patients at the start of chemotherapy, at the nadir, and before the start of the second chemotherapy course. Neutrophil counts, NAMPT activity and *SIRT1* expression level were measured. There were no differences observed either in NAMPT activity or in *SIRT1* expression level between the cases of grade 3–4 neutropenia and those of grade 0–2 neutropenia though the *SIRT1* expression level tended to decrease at the nadir. Further studies are required to elucidate this relationship between post-chemotherapy neutropenia and *SIRT1* expression.

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INTRODUCTION

Chemotherapy-associated neutropenia is the greatest obstacle to anticancer drug treatment [1]. Severe and prolonged neutropenia can hamper planned treatments, which is a significant disadvantage for patients. Cases with a fever over 38℃ during neutropenia are classified as febrile neutropenia (FN) [2], an oncologic emergency that can be life-threatening if appropriate interventions are not provided. Therefore, physicians administering cancer chemotherapy should always pay attention to neutropenia.

According to the National Comprehensive Cancer Network (NCCN) guidelines, risk factors for neutropenia include a history of chemotherapy or radiation therapy, bone marrow infiltration of tumor, recent surgery, liver and renal dysfunction, and age ≥65 years [3]. However, it is currently difficult to predict development of post-chemotherapy neutropenia only with known pre-chemotherapy risk factors listed above.

The sirtuin (*SIRT*) gene, also known as the "longevity gene," was reported to regulate gluconeogenesis in the liver in type 2 diabetes and to be involved in cholesterol metabolism [4, 5]. In addi-

tion, *SIRT* was reported as well to regulate circadian rhythms and to maintain systemic homeostasis [6-8]. The best-known pathway activating *SIRT* is the nicotinamide adenine dinucleotide (NAD^+)–*SIRT1* pathway, in which the nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in NAD^+ biosynthesis, plays an important role [9]. Skokowa *et al*. reported that NAMPT activity was essential for neutrophil differentiation induced by granulocyte-colony stimulating factor (G-CSF) and that the neutrophil count increased when a large amount of nicotinamide (vitamin B3), the substrate of NAMPT, was administered to healthy subjects [10]. Based on the observations above, it was expected that the NAD^+–*SIRT1* pathway might be suppressed either directly by chemotherapies or by deficiency in vitamin B3 in patients with post-chemotherapy neutropenia. We therefore hypothesized that NAMPT level and *SIRT1* expression level at the start of chemotherapy might be useful to predict post-chemotherapy neutropenia.

In this study, we evaluated NAMPT concentration in plasma and *SIRT1* expression in peripheral blood mononuclear cells (PBMCs) before and after cancer chemotherapy in patients of non-small cell lung cancer (NSCLC) and examined their relationship with post-chemotherapy neutropenia.

MATERIALS AND METHODS

Patient Selection and Endpoints

The main eligibility criteria were as follows; (1) histological or cytological diagnosis of NSCLC, (2) chemotherapy-naïve status, (3) sufficient bone marrow function (neutrophil count ≥2,000/μL, platelet count ≥100,000/μL and hemoglobin, ≥8.5 g/dL) and retained renal and liver function, (4) the score of Eastern Cooperative Oncology Group performance status (PS) was 0–3, and (5) age ≥20 years.

The main exclusion criteria were as follows; (1) pathologically proven or clinically suspected bone marrow infiltration of malignant disease, (2) blood disorders that were active or required treatments, (3) concurrent irradiation, (4) concurrent immunotherapy or therapy using epidermal growth factor receptor-tyrosine kinase inhibitor or anaplastic lymphoma kinase–tyrosine kinase inhibitor, and (5) history of systemic steroid administration for non-emetic purposes within 2 weeks prior to the enrollment.

The primary endpoint of this study was to evaluate the association of plasma NAMPT level and *SIRT1* expression in PBMC with the incidence of FN and severe neutropenia at the common terminology criteria for adverse events (CTCAE) grade 4 during chemotherapy. The secondary endpoints were changes in plasma NAMPT level and *SIRT1* expression in PBMC before and after chemotherapy.

Study Schedule and Measurement Procedures

Blood samples were collected from enrolled patients three times; before the start of the first course of chemotherapy, at the nadir during the chemotherapy, and before the start of the second course of chemotherapy. As the time considered to be a nadir varied depending on the chemotherapy regimen, the timing of blood collection at the nadir was chosen by the attending physicians according to clinical status of patients. Two blood samples (2 mL each) were collected; one in a hemogram test tube (containing ethylenediaminetetraacetic acid/2 K) for a hematological test and the other in a PBMC preparation tube (BD Vacutainer® CPT™, BD, Tokyo, Japan) for measurement of plasma NAMPT concentration and *SIRT1* expression in PBMC. Blood samples collected in PBMC preparation tubes were gently inverted and mixed, and centrifuged at 20–25°C at 1800 × g for 15 min. Plasma and PBMC fractions were aspirated separately, transferred into microtubes, and immediately stored at -80°C.

Plasma NAMPT concentration was measured using an enzyme-linked immunosorbent assay (CircuLex Human NAMPT/PBEF ELISA Kit, MBL, MA, USA). *SIRT1* expression in the PBMC fraction was measured using digital PCR (QuantStudio 3D Digital PCR system, Thermo Fisher Scientific, MA, USA). Probe for *SIRT1* (TaqMan Gene Expression Assay, Assay ID: Hs01009006_m1) was purchased from Thermo Fisher Scientific. The PCR chips were prepared according to manufacturer's protocol using a QuantStudio™ 3D Digital PCR Chip Loader followed by PCR using a GeneAmp® PCR system 9700 (Thermo Fisher Scientific) with PCR parameters of 96°C for 10 min; 40 cycles of 2 min at 58°C and 30s at 98°C; followed by 60°C for 2 min. After

PCR amplification, to acquire these data, the chip was loaded into the QuantStudio™ 3D Digital PCR System. The data were analyzed using QuantStudio™ 3D AnalysisSuiteTM software (Thermo Fisher Scientific, version 3.0).

Statistical Analysis

Since no previous data were available to perform a power calculation, it was difficult to estimate a number of cases necessary for the analysis. Therefore, we enrolled as many cases as possible within the study period (May 2017 to March 2019). Statistical analyses were performed using Statistical Analysis System version 9.4 (SAS Institute Inc., NC, USA). $P < 0.05$ was statistically significant.

RESULTS

***Patient Characteristics* (*Table 1*)**

There were 16 registered cases. The median age was 68.5 years, which seemed younger when considering the lung cancer statistics in Japan [11]. Male patients and patients with a good PC (i.e., 0-1) were prevalent (81.3% and 93.8%, respectively). Among the chemotherapy regimens used, carboplatin (CBDCA)/S-1, cisplatin (CDDP), pemetrexed (PEM), and bevacizumab (BEV) were the most used (three patients each).

Changes in Neutrophils and CTCAE Grading

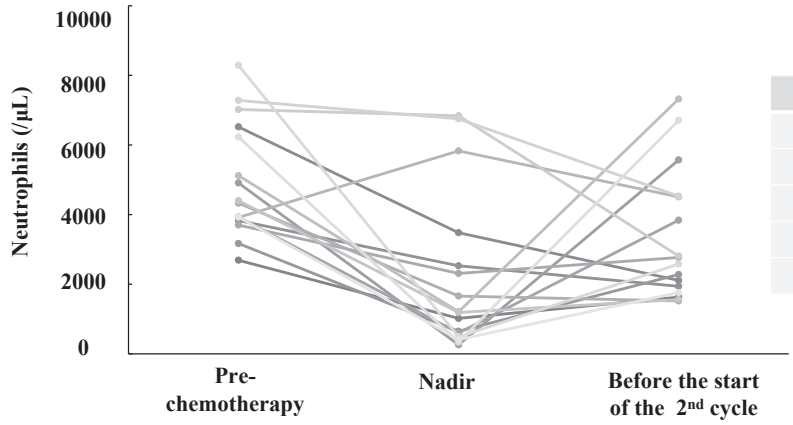
The neutrophil count at the start of chemotherapy varied widely (2690–8290/μL). Even if the neutrophil count was high enough before chemotherapy, some cases showed a rapid decrease of neutrophils at the nadir (Fig. 1). Neutropenia at CTCAE grade 3–4 was observed in six patients (37.5%), while no FN cases were found in the present study.

Although treatment with G-CSF was done in three of four patients with neutropenia at CTCAE grade 4, neutropenia persisted in one patient, in whom the

Table 1. Patient background variables and chemotherapy regimens

Patient number		**N = 16**
Median age (range)		68.5 (50-75)
Sex, male/female (%)		13(81.3) / 3(18.8)
ECOG PS (%)	0	5 (31.3)
	1	10 (62.5)
	2	1 (6.3)
Histology (%)	Adenocarcinoma	10 (62.5)
	Squamous cell carcinoma	5 (31.1)
	Adenosquamous carcinoma	1 (6.3)
Stage (%)	IV	2 (12.5)
	Postoperative setting	14 (87.5)
Chemotherapy regimen (n, %)		
CBDCA/S-1, CDDP/PEM/BEV (3, 18.8)		
CBDCA/PEM/BEV, CDDP/PEM, CDDP/GEM, CDDP/VNR (2, 12.5)		
CBDCA/PTX/BEV, CBDCA/nab-PTX (1, 6.3)		

BEV, bevacizumab; CBDCA, carboplatin; CDDP, cisplatin; ECOG PS, Eastern



CTCAE grade	N (%)
0	6 (37.5)
1	0
2	4 (25.0)
3	2 (12.5)
4	4 (25.0)

Fig. 1. Changes in neutrophil count and CTCAE grade
(Left panel) Changes in neutrophil count in individual patients were indicated. (Right panel) CTCAE grade of participants were shown. CTCAE; Common Terminology Criteria for Adverse Events

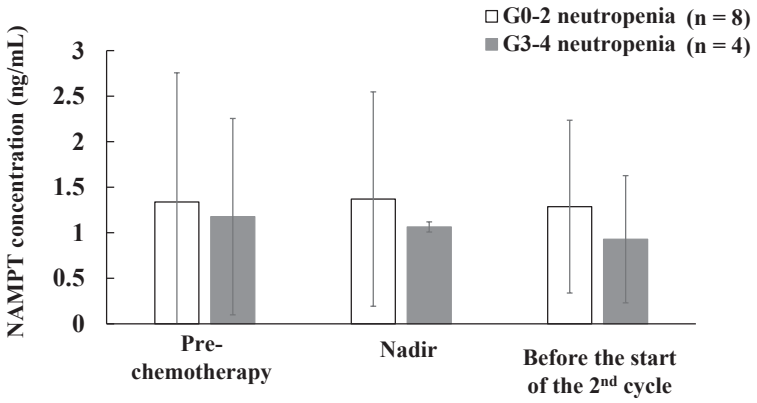


Fig. 2. Changes in plasma NAMPT concentration before and after chemotherapy
The NAMPT concentrations in patients of CTCAE grades 0–2 and of grade 3–4 were not statistically different from each other at three time points examined (Student's t test). Error bars indicate standard errors.
CTCAE; Common Terminology Criteria for Adverse Events, NAMPT; nicotinamide phosphoribosyltransferase

second cycle of chemotherapy was delayed. In three of six patients with neutropenia at CTCAE grade 2–3, the start of the second cycle was delayed due to persistent neutropenia as well.

Relationship between Neutrophil Count and NAMPT Activity/SIRT1 Expression

NAMPT in plasma and *SIRT1* expression in PBMC were detectable in 12 and 15 cases, respectively. When cases with neutropenia at CTCAE grade 0–2 and with grade 3–4 were compared, no differences were observed either in the plasma NAMPT level (Fig. 2) or in the *SIRT1* expression (Fig. 3 (a)) at the three time points examined. Relative decrease in the *SIRT1* expression tended to be greater in cases with severe neutropenia at the grade 3–4 than in those with the grade 0–2 though they did not reach a significant level (Fig. 3 (b)). When *SIRT1* expression was standardized by the neutrophil count, it tended to increase at the nadir in the cases with grade 3–4, which returned to a low level after the first treatment was completed. Such an increase was not observed in those with grade 0–2 (Fig. 3 (c)).

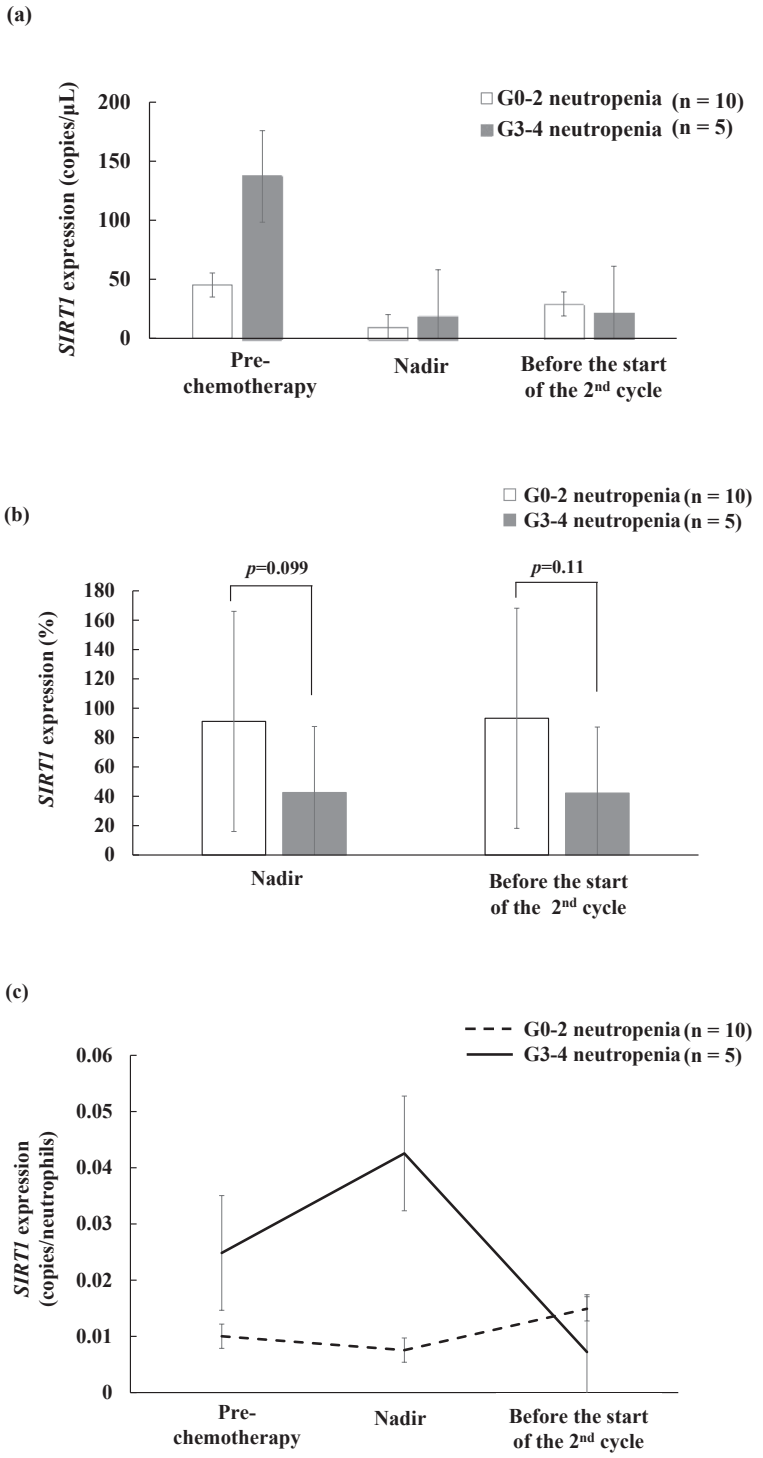


Fig. 3. Changes in *SIRT1* expression before and after chemotherapy

(a) The *SIRT1* expressions in patients of CTCAE grades 0–2 and of grade 3–4 were not statistically different from each other at three time points examined (Student's t test). Error bars indicate standard errors.

(b) Relative decrease of the *SIRT1* expression level from the baseline (i.e., at the start of chemotherapy) was compared between pateients with grade 0–2 and with grade 3–4. No significant differences between the two groups of patients were observed at the two time points examined (Student's t test). Error bars indicate standard errors.

CTCAE; Common Terminology Criteria for Adverse Events, *SIRT1*; sirtuin 1

(c) Changes in *SIRT1* expression per neutrophil count was shown for patients with CTCAE grade 0–2 and those with grade 3–4. No significant differences were observed between the two groups at three time points examined.

SIRT1; sirtuin 1

DISCUSSION

This study aimed to investigate the relationship between severity of post-chemotherapy neutropenia and NAMPT level in plasma/*SIRT1* expression in PBMC in patients receiving chemotherapy against lung cancer. In progenitor cells of neutrophils, G-CSF stimulation increases the expression of NAMPT, which leads to *SIRT1* expression and promotes neutrophil differentiation [10]. As neutropenia increased a risk of infection, NAMPT activity and intracellular *SIRT1* expression were reported to be associated with prognosis *per se* as well as resistance against chemotherapy in some cancers including NSCLC [12-14]. Therefore, elucidation of roles of the NAD^+–*SIRT1* pathway in neutrophil differentiation may enable to identify causes and predictors of neutropenia, which may eventually promote development of preventive and therapeutic strategies against neutropenia in future.

In a previous study, we evaluated plasma NAMPT activity, vitamin B3 concentration and *SIRT1* expression in PBMC in healthy volunteers and in patients of lung cancer before and after the first-line chemotherapy [15]. We showed that plasma NAMPT activity at the start of treatment was significantly lower in patients with the grade 4 neutropenia than in those without neutropenia. The present study aimed to increase the number of cases and verify the relationship between plasma NAMPT level/*SIRT1* expression in PBMC and neutropenia. However, we failed to confirm the previous results; neither NAMPT nor *SIRT1* level differed between cases with modest neutropenia and those with severe neutropenia. As we had only four patients with the grade 4 neutropenia, such a limited number of cases with severe neutropenia might be responsible for the inconsistency.

Based on the results of randomized phase III clinical trials [16, 17], the Japanese Lung Cancer Society's Lung Cancer Practice Guidelines published in 2018 recommended the use of immune checkpoint inhibitors as the first-line treatment regardless of the programmed death-ligand 1 status [18]. Accordingly, many patients of NSCLC in our hospital started to receive immunotherapy in 2018, which made it extremely difficult to enroll an enough number of cases without immunotherapy in this study. However, the first-line chemotherapy for advanced NSCLC has evolved mainly in the immune checkpoint inhibitor combination regimen, and it is difficult to collect and examine more patients using the same protocol as in this study. These points are limitations of this study. As immunotherapy may give a large impact on neutrophil count as well as on the NAD-*SIRT1* pathway [19], it will be necessary to consider a new strategy to verify the relationship between neutropenia and the NAD^+–*SIRT1* pathway in patients under cancer therapy including immunotherapy. In this study, we focused on the plasma NAPMT and *SIRT1* expression level. Future research also needed to examine the levels of vitamin B3, which is a substrate for NAMPT, and the expression of C/EBP-βin the nucleus, which leads to the production of G-CSF.

In conclusion, this study failed to replicate the association between post-chemotherapy neutropenia and the NAD^+–*SIRT1* pathway. Nevertheless, neutropenia associated with cancer chemotherapy is an important issue for both physicians and patients, and it is essential to continue clinical research to develop strategies to prevent and/or treat this adverse reaction in future.

Ethics

This study was conducted in accordance with the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines. The study protocol was approved by the Shimane University Certified Review Board (March 8, 2017; no. 2574). All the participants provided written informed consent.

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