

Clinical Study Protocol

Study about the Efficacy of Metformin to Immune Function in Cancer Patients

Mototsugu Watanabe^a, Hiromasa Yamamoto^a, Shingo Eikawa^b, Kazuhiko Shien^a,
Tadahiko Shien^a, Junichi Soh^a, Katsuyuki Hotta^c, Jun Wada^d,
Shiro Hinotsu^c, Toshiyoshi Fujiwara^e, Katsuyuki Kiura^f, Hiroyoshi Doihara^a,
Shinichiro Miyoshi^a, Heiichiro Udono^b, and Shinichi Toyooka^{a,g*}

Departments of ^aThoracic, Breast and Endocrinological Surgery, ^bImmunology, ^dNephrology, Rheumatology, Endocrinology and Metabolism,
^cGastroenterological Surgery, ^fHematology, Oncology and Respiratory Medicine and ^eClinical Genomic Medicine,
Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, ^cCenter for Innovative Clinical Medicine,
Okayama University Hospital, Okayama 700-8558, Japan

A study to evaluate the effect of metformin on the immune system was commenced in July 2014. Metformin is one of the most commonly prescribed drugs for type 2 diabetes, and previous studies have reported that metformin has an anti-tumor effect. The aim of this study is to evaluate the efficacy of metformin on the immune system in human cancer patients *in vivo*. The primary outcome parameter will be the rate change in the population of CD8⁺ T cells, which produce multiple cytokines.

Key words: metformin, CD8⁺ T cells, cancer immunology

Malignant tumors are intractable diseases and one of the leading causes of death worldwide. Surgery, chemotherapy and radiotherapy are standard therapeutic modalities for various cancers at present. However, the prognosis of cancer patients, especially those with advanced stages, is not satisfactory. Immunotherapy is a means of treating diseases by activating or suppressing an immune response, and it has been applied to the treatment of cancers [1, 2]. However, previous immunotherapy regimens did not show effects comparable to standard treatment modalities for various cancers, although many researchers have focused on the relatively low risk of adverse events associated with immunotherapy. “Cancer vaccination” is one example that was expected to have a large anti-tumor effect. Although this vac-

cine certainly led to an increase in the number of T cells, which were able to attack cancer cells, neither the tumor volume nor the 5-year survival rate were affected [3]. Recently, however, immune checkpoint therapy, such as PD-1/PD-L1 blockade, has been developed and has shown a drastic effect on several malignancies [4]. Thus, cancer immunotherapy has currently come under the spotlight.

Recently, the reason why previous immunotherapy regimens did not show satisfactory anti-tumor effects has become evident [5–7]. In the tumor microenvironment, tumor-infiltrating CD8⁺ T cells are repeatedly exposed to tumor antigens. As a result, CD8⁺ T cells become exhausted and lose their anti-tumor activities, such as their ability to kill tumor cells, their proliferation potency, and the production of several cytokines [6]. Thus, to achieve effective cancer immuno-

Received June 22, 2016; accepted July 19, 2016.

*Corresponding author. Phone: +81-86-235-7436; Fax: +81-86-235-7437
E-mail: toyooka@md.okayama-u.ac.jp (S. Toyooka)

Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.

therapy, it is necessary to prevent immune exhaustion, and this is one of the most important issues to be resolved.

Metformin is one of the most commonly prescribed drugs for type 2 diabetes and has been reported to have an anti-tumor effect or to decrease the frequency of specific cancers [8–10]. Two independent meta-analyses of epidemiological studies concluded that metformin was associated with a 30–40% reduction in the incidence of various cancers among patients with type 2 diabetes, compared with other treatments, indicating that an investigation of the anti-tumor mechanisms of metformin and long-term randomized controlled trials are warranted [11, 12]. In addition, a previous study reported a direct effect of metformin against cancer stem cells [13]. The authors have also shown an anti-tumor effect of metformin that is dependent on CD8⁺ T cells in a mouse model [14].

We have already started *in vitro* studies to evaluate the effects of metformin on CD8⁺ T cells (approval number 1941, Institutional Review Board of Okayama University Hospital: Examination of anti-tumor effects of metformin in malignant tumors) and have detected that metformin induced the production of multiple cytokines in peripheral blood CD8⁺ T cells in cancer patients *in vitro* (preliminary data). We also performed an examination to evaluate the effects of metformin on CD8⁺ T cells in healthy volunteers (approval number m12004, Institutional Review Board of Okayama University Hospital: Study about the efficacy of metformin to immune function in healthy volunteers) and confirmed that there were no severe adverse events when metformin was administered to people without diabetes (preliminary data).

Based on these findings, we have commenced an interventional study, the purpose of which is to evaluate the efficacy of metformin on the immune system in human cancer patients. This study protocol was approved by the Institutional Review Board of Okayama University Hospital in July 2014 (approval number m12005), and patient enrollment started soon after approval. This trial has been registered with the UMIN Clinical Trials Registry (UMIN-CTR) as UMIN000014689.

Endpoints

The objectives of this study are to evaluate the

efficacy of metformin on immune function and the safety of its use in cancer patients, so as to determine whether further investigation is warranted. The primary outcome parameter will be the rate change in the population of CD8⁺ T cells that produce multiple cytokines (IFN γ , TNF α , and IL-2). The secondary outcome measures will be toxicity and treatment compliance.

Eligibility Criteria

All the patients who meet the main inclusion and exclusion criteria will be invited for screening. The main inclusion and exclusion criteria of the current study are listed in Table 1.

Treatment Methods

The current study has been designed as a single-

Table 1 Eligibility criteria for this study

Inclusion criteria

1. Age of 20 years or older
2. Eastern Cooperative Oncology Group (ECOG) performance status of 0–1
3. Pathologically proven malignancies
4. Written informed consent
5. Subjects who can complain of abnormal and/or subjective symptoms if they suffer from them during the study

Exclusion criteria

1. Previous medical history of insulin therapy for diabetes mellitus
2. Previous medical history of lactic acidosis
3. Hemodialysis
4. Serious hepatic dysfunction
5. Previous medical history of shock status, heart failure, cardiac infarction, pulmonary infarction, or hypoxia
6. Drinking habit
7. Dehydration, Diarrhea or Vomiting with gastrointestinal disturbance
8. Previous medical history of severe ketosis, diabetic coma, or type 1 diabetes
9. Severe infection and serious trauma
10. Malnutrition, starvation, adrenal insufficiency, or pituitary gland dysfunction
11. Pregnancy
12. Allergy to medicines
13. Subjects who have been enrolled in another clinical trial within the last 4 months before the administration of metformin and who have been treated with any new investigational drugs.
14. Other subjects who are judged to be ineligible for this trial by the investigator

arm, prospective, non-randomized, open label interventional trial. An investigator must obtain the patient's written informed consent before any screening or inclusion procedures are performed. This study will be conducted in compliance with the principles of the Declaration of Helsinki, and the protocol has been approved by the Institutional Review Board of Okayama University Hospital (approval number m12005).

Patients will take a daily dose of metformin orally for 7 to 21 days before receiving the treatment for malignancy such as surgery, chemotherapy and radiotherapy. If the patients still keep good condition after the treatment for malignancy, they resume to take metformin. Total duration of administration will be less than 2 months (8 weeks). The initial dose will be 500mg/day (250mg twice daily), and the dose will be increased up to 1,500mg/day (500mg thrice daily) over a few days in the absence of adverse effects. Because three out of five patients felt nausea when they took metformin 1,500mg/day orally, we amended protocol to regarding maximum dose of metformin from 1,500mg/day (500mg thrice daily) to 1,000mg/day (500mg twice daily) on 22th March 2016 (approved by the Institutional Review Board of Okayama University Hospital, Table 2). The dose of metformin will be reduced from 500mg to 250mg as a single dose, from

250mg thrice daily to 250mg twice daily, or terminated if the dose of metformin is 250mg twice daily if patients exhibit adverse effects such as diarrhea or nausea or if they have any difficulty continuing to take metformin. The protocol treatment will be terminated if patients exhibit Grade-4 non-hematologic toxicities. If the patients require examinations using iodinated contrast material or invasive therapies such as surgery, they will stop taking metformin 24h before the procedure and will restart taking metformin 48h thereafter. Blood samples and biopsies of tumor specimens will be obtained before treatment with metformin, and blood samples will also be obtained after the initiation of metformin treatment. In principle, blood samples will be obtained 3 times after the treatment for malignancy, that is, they will be obtained 1 and 3 weeks after the treatment and at the point of completion of metformin treatment. All adverse events will be defined according to the Common Terminology Criteria for Adverse Events, version 4.0.

About 20ml blood will be collected from cancer patients. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using a Ficoll-Paque™ (GE Healthcare Bio-Sciences, Uppsala, Sweden). $1.0\text{--}2.0 \times 10^6$ PBMCs will be suspended in 500μl of

Table 2 Study design

		Duration of administration					4 weeks after the completion or stop
	Enrollment	1-3 weeks	Treatment for malignancy (Surgery, Chemotherapy, Radiation)	2 weeks (1 week after the treatment)	2 weeks (3 weeks after the treatment)	2 weeks (completion) or stop	
		① ② ③		③			
Metformin		←→		←→			
Physical examination ^a	○	●		●	●	●	●
Blood sampling ^b	○	●		● ^c	● ^d	● ^e	

Administration of metformin will be less than 2 months (8 weeks).

a: Physical examination for checking adverse events will be performed in outpatient clinic and at the time of hospital admission.

b: Blood sample will be obtained after taking metformin for more than 1 week.

c: Blood samples will be obtained 1 week after the treatment for malignancy.

d: Blood samples will be obtained 3 weeks after the treatment for malignancy.

e: Blood samples will be obtained at the end of intervention.

① Metformin: 500 mg/day (250 mg twice daily) for 3 days

② Metformin: 750 mg/day (250 mg thrice daily) from day 4 to day 7

③ Metformin: 1,000 mg/day (500 mg twice daily) from day 8

BAMBANKER™ (LYMPHOTEC, Tokyo, Japan) media, and the cells are placed in cryotubes for freezing and preservation. Then, the cells are frozen and preserved at -150 degrees centigrade without preliminary freezing. After more than 5 samples will be collected, these samples are thawed and cultured with or without stimulation by 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO, USA)/2 μ M ionomycin (Sigma-Aldrich) in a presence of BD Golgi Stop (BD Biosciences, San Jose, CA, USA) for 6 h. Then, PBMCs are harvested from culture plate, resuspended in staining buffer, and stained with fluorochrome-conjugated mAb binding for CD8, PD-1, Tim-3, IFN γ , TNF α , and IL-2. These samples are analyzed by BD FACSCantoII™ Flow Cytometer (BECTON DICKSON, Franklin Lakes, NJ, USA), equipped with three lasers, having the ability to detect eight colors at department of immunology in Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences [15].

To determine efficacy of metformin, the rate change in the population of CD8⁺ T cells capable of producing multiple cytokines (IFN γ , TNF α , and IL-2) will be evaluated. To evaluate tumor-infiltrating lymphocytes (TILs) and cancer cells, we also plan to perform immunohistochemistry and to check the number and characteristics of TIL by staining for CD3, CD4, CD8, CD45RA, Foxp3, TUNEL and ALDH1.

Statistical Considerations

The planned sample size is 30 patients. All the statistical analyses in this study will be performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) [16]. Specifically, the software is a modified version of R commander designed to add statistical functions frequently used in biostatistics. A value of $p < 0.05$ will be considered statistically significant.

Acknowledgments. This work was supported by JSPS KAKENHI Grant Number JP16K15637.

References

1. Rosenberg SA: A New Era for Cancer Immunotherapy Review Based on the Genes that Encode Cancer Antigens. *Immunity* (1999) 10: 281–287.
2. Rosenberg SA: Progress in human tumour immunology and immunotherapy. *Nature* (2001) 411: 380–384.
3. Meiliana A, Dewi NM and Wijaya A: Cancer Immunotherapy: A Review. *Indones Biomed J* (2016) 8: 1–20.
4. Sharma P and Allison JP: The future of immune checkpoint therapy. *Science* (2015) 348: 56–61.
5. Schreiber RD, Old LJ and Smyth MJ: Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* (2011) 331: 1565–1570.
6. Wherry EJ: T cell exhaustion. *Nat Immunol* (2011) 131: 492–499.
7. Speiser DE, Utzschneider DT, Oberle SG, Münz C, Romero P and Zehn D: T cell differentiation in chronic infection and cancer: functional adaptation or exhaustion? *Nat Rev Immunol* (2014) 14: 768–774.
8. McFarland MS and Cripps R: Diabetes Mellitus and Increased Risk of Cancer: Focus on Metformin and the Insulin Analogs. *Pharmacotherapy* (2010) 30: 1159–1178.
9. Bodmer M, Meier C, Krahenbuhl S, Jick SS and Meier CR: Long-term metformin use is associated with decreased risk of breast cancer. *Diabetes Care* (2010) 33: 1304–1308.
10. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD and Evans JM: New users of metformin are at low risk of incident cancer: a cohort study among people with type 2 diabetes. *Diabetes Care* (2009) 32: 1620–1625.
11. Decensi A, Puntoni M, Goodwin P, Cazzaniga M, Gennari A, Bonanni B and Gandini S: Metformin and cancer risk in diabetic patients: a systematic review and meta-analysis. *Cancer Prev Res (Phila)* (2010) 3: 1451–1461.
12. Noto H, Goto A, Tsujimoto T and Noda M: Cancer risk in diabetic patients treated with metformin: a systematic review and meta-analysis. *PLoS One* (2012) 7: e33411.
13. Hirsch HA, Iliopoulos D, Tschlis PN and Struhl K: Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. *Cancer Res* (2009) 69: 7507–7511.
14. Eikawa S, Nishida M, Mizukami S, Yamazaki C, Nakayama E and Uono H: Immune-mediated antitumor effect by type 2 diabetes drug, metformin. *Proc Natl Acad Sci U S A* (2015).
15. Eikawa S, Mizukami S and Uono H: Monitoring Multifunctionality of Immune-Exhausted CD8 T Cells in Cancer Patients. *Methods Mol Biol* (2014) 1142: 11–17.
16. Kanda Y: Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* (2013) 48: 452–458.