Kwansei Gakuin University
Report of Research Outcome

2023/02/06

To President

Department : Science and Technology
Position : Postdoctoral fellow
Name : Ferbian Milas Siswanto

I report the outcome of the research as follows.

Name of the Fund/Program	□Sabbatical leave with grant □Sabbatical leave with no grant □KGU Joint Research □Individual Special Research ☒Postdoctoral fellow ※Please report by designated form as for "International Research Collaboration".
Research Theme	New biological functions and regulation of Parkin under hypoxia and oxidative stress
Research Site/Venue	Imaoka Lab., Graduate School of Science and Technology, Kwansei Gakuin University
Research period	2022/04/01 ～ 2023/03/31 （ 12 months）

◆ **Summary of the research outcome** **（approx. 2,500 words）**

Please write down the outcomes in detail regarding the research theme above.

All metazoans require a sufficient supply of oxygen (O_2), an essential substrate in various metabolic processes, bioenergetics, and cellular signaling. O_2 deprivation or hypoxia (0.1–1% O_2) is a fundamental feature of physiological and pathophysiological conditions, such as respiratory and circulatory disorders, as well as cancer. In solid tumors, rapidly proliferating cancer cells cause tumor mass outgrowth and a shortage of blood supply, resulting in the formation of a hypoxic region. Hypoxia plays a significant role in tumor development, contributes to therapeutic resistance, and is a critical microenvironmental factor promoting metastatic progression, thereby serving as a prognostic factor as well as a compelling therapeutic target. Central to the molecular and cellular responses to hypoxia, hypoxia-inducible factors (HIFs) sense and orchestrate various metabolic adaptation pathways and the transcriptional cascades of stress factors regulating cell death or survival. The transcriptional activity of HIF is regulated by various factors, including reduction/oxidation (redox) factor 1 (Ref-1). Due to its central role in hypoxia responses, HIF and its target genes have emerged as promising therapeutic targets for solid tumor therapy. However, HIF inhibitors have not

yet been approved for the treatment of cancer patients due to limited selectivity, specificity, and therapeutic efficacy or safety.

Oxidative stress refers to excessive levels of reactive oxygen species (ROS) compared to antioxidant defense. ROS represents highly reactive oxygen-containing chemical intermediates, such as superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). ROS are generated endogenously as byproducts of normal metabolism and, at low concentrations, are required for cellular signaling. Upon exceeding a threshold level, ROS leads to cell death in normal cells. However, in pathological conditions, oxidative stress leads to the development of a diseased state, including carcinogenesis and neurodegenerative diseases (i.e., Parkinson's). The elevated metabolism driven by aberrant cell growth in cancer causes excess production of ROS. To adapt to constant high ROS levels, tumor cells optimize the activity of antioxidant transcription factors. This particular feature has been widely exploited in efforts to search for new anticancer treatments. In Parkinson's disease, the clearance ability of oxidative stress-induced damaged and unwanted proteins is impaired. Parkin impairment is strongly associated with oxidative stress-induced Parkinson's, but its role in the oxidative stress pathway in cancer is currently unknown.

Parkin is an E3 ubiquitin ligase belonging to the RING-between-RING family that catalyzes the covalent attachment of ubiquitin onto substrate proteins and subsequently leads to its degradation via the ubiquitin-proteasome system, a major intracellular protein degradation machinery. Parkin is involved in numerous cellular processes, particularly pathways which are tightly connected to mitochondrial quality control. In the present study, we found that Parkin protein levels were elevated and depleted under hypoxia and oxidative stress, respectively. These changes were found to be critical for cancer cell survival under hypoxic and oxidative stress conditions.

Under hypoxia, we found that Parkin mRNA and protein levels were elevated in a HIF-1α-dependent manner. Stabilized HIF-1α under hypoxia translocates to the nucleus, where it binds to the hypoxia response element (HRE) located in the -107/-103 bp of the *Parkin* proximal promoter, increasing transactivation of *Parkin* and its protein levels. The elevation of Parkin under hypoxia is critical for cancer survival under hypoxia, as Parkin knockdown reduces the viability of hypoxia-treated Hep3B cells. We determined that upon activation, Parkin is responsible for ubiquitination and proteasomal degradation of sequestosome 1 (SQSTM1/p62). p62 sequesters and negatively regulates extracellular signal-regulated kinase (ERK), reduces O_2 consumption, decreases CO2 release, and lowers energy expenditure; therefore, the suppression of p62 by Parkin is a critical step in response to hypoxia. These findings show the potential of targeting Parkin/p62 as a novel strategy to eradicate hypoxic solid tumors.

Under oxidative stress, we found that Parkin mRNA and protein levels were depleted. Mechanistically, we found that H_2O_2 activates NF-κB. The RelA/p65 component of the NF-κB pathway inhibits the expression of *Parkin*, likely by interfering with ATF4 activity. Next, we found that Parkin is responsible for the ubiquitination and proteasomal degradation of Ref-1; thus, H_2O_2-suppressed Parkin expression is responsible for the elevated Ref-1. Liver cancer patients with high Ref-1, low Parkin, and high RelA expression had a lower probability of survival. Additionally, the expression levels of Parkin and RelA were negatively and positively correlated with Ref-1 levels, respectively, in the TCGA liver cancer cohort. We concluded that an increase in Ref-1 via inhibition of Parkin is critical for cancer cell survival under oxidative stress. These results show the potential of the Parkin/Ref-1 axis as a prognostic factor and therapeutic strategy to eradicate liver cancer.

CONFERENCE

1. **Siswanto FM,** Imaoka S. Yeast β-glucan possesses a resistance-modifying property by modifying the levels of drug-metabolizing enzymes through the NF-kB and Nrf2 pathways. *The 37th Annual Meeting of the Japanese Society for the Study of Xenobiotics.* Yokohama, Japan. 7-10 Nov 2022.

PUBLICATIONS

2. **Siswanto FM,** Mitsuoka Y, Nakamura M, Oguro A, Imaoka S. Nrf2 and Parkin-Hsc70 regulate the expression and protein stability of p62/SQSTM1 under hypoxia. *Sci Rep*. 2022 Dec 8;12(1):21265. doi: 10.1038/s41598-022-25784-0. PMID: 36481701; PMCID: PMC9731985.
3. **Siswanto FM,** Oguro A, Imaoka S. Nrf2 regulates the expression of CYP2D6 by inhibiting the activity of Krüppel-Like Factor 9 (KLF9). *Genes to Cells*. Under revision.
4. **Siswanto FM,** Okugawa K, Tamura A, Imaoka S. Hydrogen peroxide activates Ref-1/APE1 via NF-κB and Parkin: role in liver cancer resistance to oxidative stress. Manuscript In preparation.

Deadline : Within two months after finishing the research period.
Sabbatical leave with grant: Submit this report to President with confirmation by the dean of school you belong to.
※Postdoctoral fellow is required to submit this report with confirmation by the dean of graduate school before the end of employment period.
Where to submit : Organization for Research and Development and Outreach (NUC)
◆We put this report on the web of KGU. If there is any problem about it because of difficulties on your research, please let us know.