氏　　名	TRAN THI PHUONG DUNG
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	（職名）	（学位）	（氏名）
主　　査	筑波大学教授 （グローバル教育院）	医学博士	佐藤　孝明
副　　査	筑波大学准教授	博士（薬学）	鈴木 裕之
副　　査	筑波大学教授	博士（人間・環境学）	森川　一也
副　　査	筑波大学教授 （グローバル教育院）	Ph.D.	Peter ten Dijke

論文の要旨
Abstract of thesis

Squamous epithelial cells exist in the tissues of the external surfaces of the body, such as the upper aero digestive tract and the lower female genital tract. Squamous tissues are usually exposed to oxygen and need a cytoprotective mechanism for oxidative stress. Squamous tissue has a stratified structure with a stemcells and progenitorcells containing basal layer. Accumulated mutations and alterations of genomic DNAs in stem or progenitor cells in basal layer lead to squamous cell carcinoma (SCC) development and/or progression. Although the information of genomic DNA alterations in SCC has been accumulated, 5-year survival is still low due to theineffective treatments.

NF-E2 related factor 2 (Nrf2) is a cap ‘n’ collar basic leucine zipper (CNC-bzip) transcription factor. Nrf2 binds to the antioxidant response elements (ARE) and upregulates several antioxidant, drug metabolism enzyme, and drug transporter genes. Therefore, increased expression of Nrf2 leads to detoxification and elimination of numerous exogenous and endogenous stimuli. Under the normal condition, Nrf2 is kept transcriptionally inactive by binding to its negative regulator Kelch-like ECH associated protein 1 (Keap1). Keap1 acts as an adaptor to recruit cullin3

(CUL3) to the complex and catalyzes polyubiquitination of Nrf2 protein. In response to electrophiles and ROS, which have the ability to interact with a sulfhydryl group, Keap1 cysteines are covalently modified and Keap1-mediated ubiquitination of Nrf2 is diminished. Thereby, Nrf2 accumulates and translocates into the nucleus, where it activates ARE-genes. However, persistent Nrf2 activation in cancer cells has detrimental effects by enhancing cancer cell drug resistance, metabolic activity and aggressive tumorigenic activity.

Transforming growth factor beta-1 stimulated clone 22 - isoform 4 (Tsc22D4/THG-1) is overexpressed in more than 80% of SCC specimens including esophagus SCC, lung SCC and cervical SCC while strictly resided in mitotically active basal layer of normal squamous epithelial tissue. Knockout of THG-1 caused reduction of growth, invasion and tumorigenesis in cancer cells. Experimentally, protein-protein interactions (PPIs) were suggested as THG-1 mechanism to promote cancer progression. Well-known functional interactions of cellular regulators, Keap1, PHD2, TBLR1 and NRBP1, are disrupted in the present of THG-1. In this thesis project, THG-1 specific macrocyclic peptide ligands were identified and their application for research involving THG-1 as well as their potential use for disrupting THG-1 PPIs were experimentally and scientificallyevaluated.

The applicant employed Random non-standard Peptide Integrated Discovery (RaPID) system for screening of macrocyclic peptides against purified recombinant THG-1 protein. In this system, D-stereochemistry, unusual side chains and N-methylation containing macrocyclic peptides were generated and screened against THG-1. Identified macrocyclic peptides were used for fluorescein staining, pull-down and ELISA-like assay. Moreover, PPI inhibition activity of identified macrocyclic peptide was also evaluated both *in vitro* and in cells.

By using RaPID system, the applicant successfully identified macrocyclic peptides that specifically bind to both recombinant THG-1 and endogenous THG-1. R4-1 macrocyclic peptide successfully pulled-down both recombinant THG-1 as well as endogenous THG-1 protein in esophagus squamous cell carcinomas cell lines. In addition, fluorescein-tagged R4-1 stained THG-1 overexpressed HaCaT cells with low non-specific staining in the negative control HaCaT-mock cells. Especially, R4-1 showed antagonist activity to THG-1 and Keap1 interaction by NanoBiT and co-IP assays. In addition, transfected R4-1 using Xfect reagent downregulated Nrf2 target genes expression and caused reduced cells proliferation. Furthermore, to improve the delivery of macrocyclic peptide into cells, the applicant modified the R4-1 with cyclic heptapeptidecyclo FΦRRRRRRQ (cFΦR4). cFΦR4 were reported to have the cell-penetrating ability through endocytosis. Synthesized fluorescein tagged-R4-1-cFΦR4 (R4-1CPP-F) conjugate was significantly incorporated into cells and showed the promising biological activities including the inhibition of THG-1 and Keap1 interaction, the downregulation of Nrf2 target genes and suppression of cell proliferation.

The applicant discussed the future application of identified macrocyclic peptide against THG-1 in staining, ELISA-like assay and pull-down in replacement for antibody. Furthermore, the binding characteristic including binding affinity and binding location between R4-1 and THG-1 should be confirmed. Co-crystallization of R4-1 and THG-1 can be used to understand binding between THG-1 and R4-1. From the results of co-crystallization, modification of R4-1 to improve its serum stability, permeability, specificity, and affinity will be easier to carry out and with more confidence.Importantly, identified macrocyclic peptide R4-1 is THG-1 PPI antagonist. While THG-1 and Keap1 interaction implies important role in cancer progression, R4-1 inhibitory activity may serve as therapeutic for treatment of squamous cell carcinomas.

審査の要旨
Abstract of assessment result

【批評 Review】

The applicantsuccessfully identified macrocyclic peptides against Transforming growth factor beta-1 stimulated clone 22 - isoform 4(THG-1)proteinby Random non-standard Peptide Integrated Discovery (RaPID) system and well characterized the property *in vitro* and in cultured cell system.The applicant demonstrated the important effectof R4-1 and its membrane permeable macrocyclic peptide that can inhibits the THG-1-Keap1 protein-protein interaction and suppresses cell proliferation.These results suggest the therapeutic potential for squamous cell carcinomas.This study provided a steady step towards the final purposeby creating a promising macrocyclic peptide whose physical propertyincluding binding affinity and membrane penetration need to be tested and further improved in future research.

【最終試験の結果 Result】

The final examination committee conducted a meeting as a final examination on 1stAugust, 2019. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

【結論 Conclusion】

Therefore, the final examination committee approved that the applicant is qualified to be awarded a Doctor of Philosophy in Human Biology.