氏　　名	TANZIMA NUHAT SHARNA
学位の種類	博士（　医学　）
学位記番号	博甲第　　８８４６　　号
学位授与年月	平成　３０年　９月　２５日
学位授与の要件	学位規則第４条第１項該当
審査研究科	人間総合科学研究科
学位論文題目	Genetic analysis to evaluate disease specific mutations in non-Hodgkin's lymphoma.（非ホジキンリンパ種における疾患特異的遺伝子変異の解析）
主　　査	筑波大学教授　博士（医学）　関根　郁夫
副　　査	筑波大学教授　医学博士　小島　寛
副　　査	筑波大学准教授　博士（医学）　坂下　信悟
副　　査	筑波大学助教　博士（医学）　川﨑　綾

The report of the Assessment of Ms TANZIMA NUHAT SHARNA's Dissertation Defense

論文の要旨
Abstract of thesis

Research 1: Droplet digital PCR assay for RHOA mutation detection in angioimmunoblastic T-cell lymphoma

Purpose: Angioimmunoblastic T-cell lymphoma (AITL) is a subtype of nodal peripheral T-cell lymphoma (PTCL). In addition, a part of peripheral T-cell lymphoma not otherwise specified (PTCL-NOS), referred to nodal PTCL with T_{FH} phenotype, share similar genetic features of AITL. The hotspot mutation is the G17V RHOA mutations, which are observed in approximately 60% of the cases. Routine detection of the G17V RHOA mutations is troublesome because tumor contents are low in clinical specimen. In addition, quality of genomic DNAs derived from biopsy specimens is relatively poor. Therefore, the author tried to establish a sensitive method to detect G17V RHOA mutations in clinical samples.

Methods: The author analyzed 67 PTCL (40 AITL and 27 PTCL-NOS) patient samples by droplet digital PCR (ddPCR). A 20 µl reaction mixture containing 10 µl of ddPCR supermix specific for the

G17V RHOA mutation and 40 ng of DNA template was mixed with 70 µl of droplet generation oil by using QX200 Droplet Generator. The water-in-oil droplets in a 96-well PCR plate were placed on a thermocycler for PCR amplification, and then were read by Droplet Reader using QuantaSoft software. For PNA-LNA PCR clamp, PCR primers, fluorogenic probes, and a PNA clamp primer and DNA template were added to the Premix Ex taq (Probe qPCR) Master Mix. Total probe (fluorescent dye Cy5) targeted normal sequences and LNA probe (fluorescent dye FAM) targeted the sequences with the G17V mutation. Quantitative polymerase chain reaction was then performed. The results of ddPCR and PNA-LNA clamp were compared with those of next generation sequencing (NGS).

Results: Initially, the lower limits of detection (LOD) and quantification (LOQ) for ddPCR were established with serial dilution samples of the G17V mutation at 13%, 3.25%, 0.81%, 0.20%, 0.05%, 0.01%, and 0.0025%. The LOQ of ddPCR was determined as 0.05%, and that of the PNA-LNA PCR clamp method was 0.01%. Totally, c.50G>T, p.Gly17Val mutation was identified in 31 of 67 (46.3%) PTCL samples (AITL 23/40 [57.5%] and PTCL-NOS 8/27 [29.6%]). Both ddPCR and PNA-LNA clamp assays detected the mutation in all 31 of 67 samples. The sensitivity of ddPCR and PNA-LNA clamp assays for detection of c.50G>T, p.Gly17Val mutation was 100% (31/31) with 100% (36/36) specificity, while NGS had 87% (27/31) sensitivity with 100% (36/36) specificity. Three other RHOA mutations (p.Gly17Val, p.Gly17Leu, and p.Gly17Glu) were detected by NGS, but they could not be detected by either of the ddPCR or PNA-LNA clamp assay. Variant allele frequencies by ddPCR and those by NGS showed high concordance ($p<0.001$).

Conclusion: The author concluded the ddPCR and PNA-LNA clamp assays were more sensitive to detect low frequency G17V RHOA mutations than NGS in AITL tissue samples, although NGS was convenient for detection of all mutational types. A combination of ddPCR or PNA-LNA clamp method and NGS was required for exact diagnosis of AITL.

Research 2: Analysis of control region of mitochondrial DNA in samples of diffuse large B-cell lymphoma.

Purpose: Diffuse large B-cell lymphoma (DLBCL) is the most frequent lymphoid malignancy among adult population. Due to the involvement of multiple oncogenic pathways a wide variety of genetic lesions in the nuclear genome have been identified as causative agents in DLBCL. There are, however, few studies of mitochondrial genome (mtDNA) in the pathogenesis of DLBCL. The human mtDNA is a small circular, double stranded (outer heavy strand, and inner light strand), 16.6kb molecule which contains 37 genes encoding 13 subunits of respiratory chain complexes, 22tRNAs and 2 ribosomal RNAs. Each mitochondrion contains an average of 2 to 10 copies of mtDNA. Tumor derived mutant mtDNA can serve as a predictive biomarker, because, mitochondrial D-loop region is known as a hotspot for mutations and control region (CR) controls mitochondrial transcription and replication process. The purpose of this study was to evaluate the mutation frequency in the CR of mtDNA in DLBCL samples.

Methods: The author collected archived fresh frozen paraffin embedded (FFPE) samples from tumor biopsy of 20 DLBCL patients with matched normal bone marrow (BM) samples from 14 patients. DNAs were extracted from FFPE samples using QIAamp FFPE tissue Kit and from BM samples using and QIAamp DNA Blood Mini Kit. Amplification reactions were carried out with 20 ng of extracted DNA as the template and 300 nM concentration of ten pairs of forword and reverse primers in a 10 µl reaction volume with Kod-Plus-Neo PCR reagent. To prepare DNA libraries for NGS, PCR amplicons were ligated to barcode adapters and P1 adapters, and amplified using an IonPGM fragment library preparation kit. Libraries were then subjected to deep sequencing on the Ion Torrent PGM platform for 300 base-pair single-end reads. Sequencing data were analyzed using Variant Caller 5.0.

Results: Somatic single nucleotide variants were found in two of 14 (14%) tumor samples in the CR of mtDNA. They were m.150C>T (allele frequency [AF] 24.5%), m.152T>C (AF 21.2%), m.189A>G (AF 5.5%), m.194 C>T (AF 8.8%), m.195T>C (AF 15.2%), m.199T>C (AF 10.7%), and m.16129G>A (AF 32.6%) in a patient, and m.235A>G in another patient. These mutations were absent in the paired normal bone marrow samples.

In addition, the author found four heteroplasmic mutations in both tumor and paired BM samples out of total 14 samples. Among them two site-specific heteroplasmic mutations were present at m.16093T>C in a patient (tumor AF 14.1% and BM AF 86%) and another (tumor AF 2.6% and BM AF 11.2%). This site is known to be a common site of tissue-specific heteroplasmy. The other two heteroplasmic mutations were mtDNA sequence variants.

Conclusion: The author showed that disease-specific somatic mutations were noted in mtDNA of DLBCL patients. For studying the molecular mechanism of DLBCL, mtDNA can be considered as a potential target to detect mutations other than genomic DNA.

審査の要旨

Abstract of assessment result

【批評　Review】

This dissertation is comprised of two studies. In the first study, “Droplet digital PCR assay for RHOA mutation detection in AITL”, the applicant clearly showed ddPCR was more sensitive than NGS to detect the G17V RHOA mutations in patients with AITL. Because tumor cell contents in biopsy specimens from these patients are quite low, the results of this study are important to make a diagnosis of AITL in the clinical setting. The second study, “Analysis of control region of mitochondrial DNA in samples of DLBCL, is challenging and attractive, because thus far there have been few studies on mutations of mtDNA in DLBCL using whole genome sequencing. This study raised new questions to be addressed in future studies. For example, a key feature of the mtDNA is its high copy number in cells (10–10,000 copies), and mutated mtDNA is in the state of heteroplasmy, coexistence with wild type mtDNA. Thus, dysfunction of mitochondria and cellular consequence depend on the percentage of mutated allele as well as the site of mutation. The threshold of the

percentage of heteroplasmy and function of the mutated mitochondrial gene are good subjects for future experiments.

【最終試験の結果　Assessment】

The final examination committee conducted a meeting as a final examination on 18 June, 2018. 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】

The final examination committee approved that the applicant is qualified to be awarded Doctor of Philosophy in Medical Sciences.