Doctoral Dissertation Evaluation Review

Degree requested: Doctor of Life Science Applicant's name Urunanont Puntakarn

Examiner :

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Title of Doctoral Dissertation

Studies on the function of SRY with an A-to-S substitution in the HMG domain in the Okinawa spiny rat
（A-to-S アミノ酸置換が生じたオキナワトゲネズミ SRY タンパク質 HMG ドメインの機能解析）

Results of Evaluation of the Doctoral Dissertation (Report)

The mammalian sex-determining gene SRY is highly conserved on Y chromosome across species. Over time, the progressive degradation of the Y chromosome led to a significant loss of their size and the number of genes they carry. However, despite the widespread degeneration of the Y chromosome in many species, certain exceptional mammals have managed to preserve their sex determination mechanisms by developing alternative systems independent of the Y chromosome. These systems involve the evolution of new sex-determining genes or chromosomal rearrangements that allow sex determination to function without reliance on the Y chromosome. Such adaptations highlight the dynamic nature of sex chromosome evolution and the potential for species to develop novel mechanisms to maintain reproductive viability. Among those exceptions, some native Japanese murine rodent species belonging to the genus *Tokudaia* exhibit unique characteristics in their sex chromosomes and sex determination mechanisms. The Okinawa spiny rat *Tokudaia muenninki* (TMU) acquired neo-sex chromosomes with multiple *Sry* copies by sex chromosome-autosome fusions. All SRY copies in TMU have a substitution from alanine (A) to serine (S) at position 21 in the high-mobility group (HMG) box, a critical DNA-binding domain, suggesting that they are nonfunctional. However, the sex determination system in TMU remains unclear, in part because the species is endangered and it is therefore extremely difficult to obtain experimental samples. Therefore, the author investigated the molecular properties and function of SRY *in silico*. A comparison of SRY sequences from 225 species showed that TMU is the only species with a substitution at the 21st position. This result highlights the rarity and specificity of this substitution, suggesting that the locus is critical for sex determination. Structural predictions, DNA docking simulations, showed that DNA-binding ability was retained, at least *in silico.*

As one of the most known functions of SRY, is its transcriptional activity. In order to up regulate its relative, SOX9 activities, SRY need to precisely bind to its DNA target, Enh13.the author identified the molecular properties of TMU, the muti SRY copies critically endangered species using in silico methods. The results showed that it might possibly bound to the Enh.13 DNA. However, the amount of SRY required to be bound to the DNA (DNA binding affinity) is yet to be confirmed. Therefore, author performed *in vitro* experiments included western blotting, electrophoretic mobility shift assays, and fluorescence anisotropy have been conducted using recombinant protein from *E. coli*. The results showed that although the affinity was slightly lower than that of the mouse homolog, DNA-binding ability was retained. These findings provide insight into the effects of the A-to-S substitution in SRY on sex determination.

From above, the author hypothesized that A to S substitution might not critically effected to the DNA binding activity of SRY. However, to determine the function of SRY as sex determining factor, the other properties of SRY, DNA bending must be investigate. DNA bending could be critical for functional protein. The changing in protein structure due to some amino acid substitution proximal in those specific residues of the DNA-binding domain might not only consequence to the DNA binding ability, but also confrontational reduced the transcriptional activity. Therefore, the author discovered that the HMG domain of TMU's SRY which

employed A-to-S substitution still maintained the DNA binding ability, although with slightly reduced in binding affinity. Therefore, the author performed Permutation Gel Electrophoresis (PGE) with the recombinant HMG protein from mouse and TMU and five labeled DNA to measure the DNA bending capacity. The results showed that both mouse and TMU explicit on par DNA bending angles, 86.3° in Mouse, and 79.3° in TMU, indicating that TMU's SRY should work as sex determination properly if it could be expressed in the animals.

In conclusion, these findings provide a first report, in detail demonstrating that the SRY gene can tolerate an A-to-S substitution in its HMG domain without losing its DNA-binding and bending capabilities, the study challenges previous assumptions about the fragility of sex-determination pathways results in losing SRY dependence properties. It invites further inquiry into the molecular and evolutionary flexibility of these systems and stands as a valuable contribution to our understanding of genetic regulation, development, and adaptation.

Therefore, we acknowledge that the author is qualified to be granted a Doctorate of Life Science from Hokkaido University.