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Abstract	MicroRNAs (miRNAs) are 18-24 nucleotide (nt) non-coding RNAs that are deeply involved in development, especially in morphogenesis. However, the detailed biological information about 25-45 nt long small RNAs remains unclear. Here, I aimed to perform a comprehensive analysis of 25-45 nt-long small RNAs in Triops cancriformis (Tadpole shrimp), whose morphology dramatically changes during development. I especially focused on stage-specific mitochondrial and genomic tRNA-derived fragments (tRFs). To detect small RNAs, deep sequencing of small RNA libraries in each six developmental stage (egg, 1st-4tn instar larvae, and adult) of T. cancriformis was performed. In order to analyze tRFs, I obtained mitochondrial DNA sequence from NCBI and genomic DNA sequences by deep sequencing, and predicted mitochondrial and genomic tRNA genes by tRNAscan-SE. By the comparative sequence analysis between tRNA genes and small RNAs, it was found that large amount of small RNAs were derived from mitochondrial and genomic tRNA genes. Interestingly, tRPs were derived from the particular positions of its tRNAs. For example, approximately 25-30 nt-long tRFs were produced from the almost similar positions around the 55 half regions of mitochondrial and genomic tRNALys(CUU) at every six stage. Moreover, expression pattern analysis suggested that the expression of many mitochondrial and genomic tRFs was altered in six developmental stages. Northern blot analysis confirmed that two genomic tRFs were actually expressed in T. cancriformis. In particular, several tRFs in different length were produced from genomic tRNALys(CUU) depending on the developmental stages. These results suggest that tRFs are not random degradation products, but may have important role(s) in T. cancriformis development.
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Analysis of tRNA-derived fragments (tRFs) in *Triops cancriformis* development

広瀬　友香

Abstract

MicroRNAs (miRNAs) are 18-24 nucleotide (nt) non-coding RNAs that are deeply involved in development, especially in morphogenesis. However, the detailed biological information about 25-45 nt long small RNAs remains unclear. Here, I aimed to perform a comprehensive analysis of 25-45 nt-long small RNAs in *Triops cancriformis* (Tadpole shrimp), whose morphology dramatically changes during development. I especially focused on stage-specific mitochondrial and genomic tRNA-derived fragments (tRFs).

To detect small RNAs, deep sequencing of small RNA libraries in each six developmental stage (egg, 1^{st}-4^{th} instar larvae, and adult) of *T. cancriformis* was performed. In order to analyze tRFs, I obtained mitochondrial DNA sequence from NCBI and genomic DNA sequences by deep sequencing, and predicted mitochondrial and genomic tRNA genes by tRNAscan-SE. By the comparative sequence analysis between tRNA genes and small RNAs, it was found that large amount of small RNAs were derived from mitochondrial and genomic tRNA genes. Interestingly, tRFs were derived from the particular positions of its tRNAs. For example, approximately 25-30 nt-long tRFs were produced from the almost similar positions around the 5' half regions of mitochondrial and genomic $tRNA^{Lys}$(CUU) at every six stage. Moreover, expression pattern analysis suggested that the expression of many mitochondrial and genomic tRFs was altered in six developmental stages. Northern blot analysis confirmed that two genomic tRFs were actually expressed in *T. cancriformis*. In particular, several tRFs in different length were produced from genomic $tRNA^{Lys}$(CUU) depending on the developmental stages. These results suggest that tRFs are not random degradation products, but may have important role(s) in *T. cancriformis* development.

Key Words

tRNA-derived fragment, small RNA, development, *Triops cancriformis*, deep sequencing

Introduction

Recently, large amount of small RNAs have been identified in eukaryotes. MicroRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), which play as gene regulators, are one of well-characterized classes of small RNAs (Farazi *et al.*, 2008). In addition to miRNAs and piRNAs, transfer RNA (tRNA)-derived fragments (tRFs) have been found as a novel class of small RNAs (Yang and Lai, 2011). 30-50 nt-long tRFs are often produced under a variety of stress conditions, and this stress response is conserved from yeast to human (Lee and Collins, 2005; Thompson *et al.*, 2008). It should be noted that the 5' half regions of tRNAs-derived fragments cooperate with a translational repressor, YB-1, to inhibit protein translation in the stressed human cells (Ivanov *et al.*, 2011). tRFs are also expressed even if cells or organisms are not under the stress conditions. For examples, 20-30 nt long tRFs that are derived from the 5' and 3' end regions of mature tRNAs (5'- and 3'-tRFs), and the 3' end regions of precursor tRNAs (pre-tRNAs) found in *Tetrahymena*, mouse ES cells, and several human cell lines (Babiarz *et al.*, 2008; Lee *et al.*, 2009; Cole *et al.*, 2009; Couvillion *et al.*, 2010). It has been demonstrated that 3'-tRF binds to PIWI protein, and tRF/PIWI complex promotes rRNA processing in *Tetrahymena* (Couvillion *et al.*, 2010, 2012). These previous findings show that several tRFs have important roles in eukaryotic cellular processes. Thus, we cannot regard all tRFs as random degradation products, although the molecular feature and function of them have not been clearly demonstrated yet.

The modulation of gene expression by small RNAs is implicated in various processes such as cell differentiation, antiviral defense, and tumorigenesis (He and Hannon, 2004; Esquela-Kerscher and Slack, 2006; Takane and Kanai, 2011). Small RNAs also play important roles in developmental processes. For examples, miRNA is deeply involved in morphogenesis and histogenesis, and piRNA is involved in germ line development (Stefani and Slack, 2008). According to previous studies on miRNAs and piRNAs, it can clearly be seen that small RNAs play key roles in regulation of eukaryotic development. However, the detailed information of small RNAs, which are especially longer molecular size than miRNAs (18-24 nt), on development remains obscure.

In order to understand the biological importance of 25-45 nt-long small RNAs in development, I aimed to identify stage-specific small RNAs in non-model species, tadpole shrimp *Triops cancriformis* (*T. cancriformis*). *T. cancriforsmis* belongs to Crustacean, and their form almost no changes during approximately 200 million years, so called "living fossil". Interestingly, their morphology dramatically changes in the short term during larval development (Figure 1). Here, the preceding studies showed that some small RNAs are expressed in stage-specific manner, and regulate developmental processes. Therefore, I made a hypothesis that expression of *T. cancriformis* small RNAs dynamically change in developmental stage to regulate their rapid morphological changes. I expected that novel small RNAs, which are associated with development, might be found in this study.

For this purpose, *T. cancriformis* 25-45 nt long small RNAs were analyzed using bioinformatics and experimental techniques. As a result, mitochondrial and genomic tRFs were found in *T. cancriformis* developmental stages. In this master's thesis, the specific features and expression patterns of these tRFs were described. Possible roles of *T. cancriformis* tRFs were also discussed in light of the prior studies of tRFs.

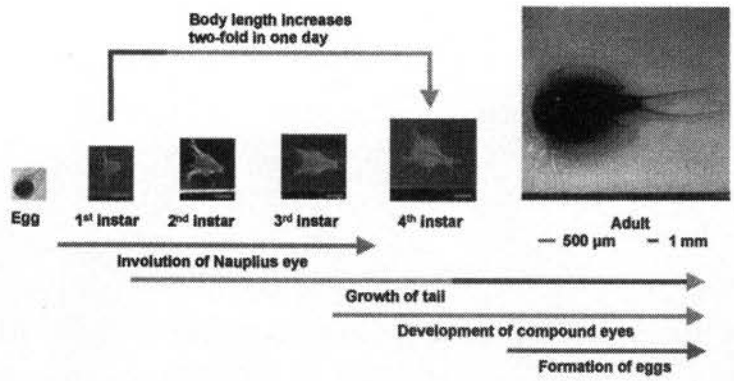

Figure 1: Developmental morphological changes of *T. cancriformis*

Morphologies of six developmental stages (egg , 1st to 4th instar larvae, and adult) of *T. cancriformis* were depicted with their characteristics of dynamic morphological alteration. Grey bars indicate 100 μm and a red bar indicates 1 mm.

Materials and Methods

See Master's Thesis.

Results

For detail, see Master's Thesis.

(The figures are not shown due to confidentiality of unpublished manuscript.)

Prediction of mitochondrial and genomic tRNA genes in *T. cancriformis*.

In order to find tRFs, I predicted mitochondrial and genomic tRNA genes in mitochondrial and genomic DNA of *T. cancriformis* using tRNAscan-SE (Lowe and Eddy, 1997). Total of 22 mitochondrial tRNAs corresponding to 22 anticodons and 254 genomic tRNAs corresponding to 45 anticodons were predicted and used for further analysis.

Small RNAs were derived from both mitochondrial and genomic tRNAs in *T. cancriformis*

To reveal overall features of *T. cancriformis* small RNAs, deep sequencing analysis of small RNA libraries in each six developmental stage were performed, and total of 151,340,419 reads were obtained. After discarding of low-quality reads (<20), reads containing sequence errors (N), and sequence reads with low count (<5), 1,162,917 non-redundant (unique) reads were extracted in all. Subsequently, total of 900,174 small RNA reads ranging in length from 25 to 45 nt were extracted. To characterize 25-45 nt-long small RNAs, approximately nine hundred thousand reads were compared with mitochondrial DNA and genomic DNA contigs. I extracted 1,182 unique reads and 743,650 unique reads that were mapped to mitochondrial DNA and genomic DNA contigs, respectively. Further analysis has revealed that 181 unique reads and 1,620 unique reads were derived from mitochondrial and genomic tRNAs, respectively. These results suggest that 25-45 nt-long small RNAs are produced from both mitochondrial and genomic tRNAs in *T. cancriformis*. For further analyses, I focused on mitochondrial and genomic tRFs to understand the biological significance of tRFs on *T. cancriformis* development.

Mitochondrial tRFs were derived from the particular positions of its tRNAs.

To reveal *T. cancriformis* mitochondrial tRF species, mitochondrial tRFs were categorized by their corresponding anticodons. Analysis of mitochondrial tRFs locus reveled that they were derived from 16 of

22 mitochondrial tRNA genes, and tRF^{Ser}(GCU) was the most abundantly detected tRF. Interestingly, a significant number of tRF^{Ser}(GCU) were aligned to the 5' half region of its tRNAs, while a small number of tRFs were aligned to the 3' half region of them. Analyses of the other 15 tRFs revealed that patterns of tRF regions aligned to its tRNAs were differed depending on anticodons. These results suggest that mitochondrial tRFs were not random degradation products, but they may be regulated to be produced from the particular position of its tRNAs.

Altered expression of mitochondrial tRFs was observed in developmental stages.

To discuss the characteristics of mitochondrial tRFs in development of *T. cancriformis*, expression pattern analysis was carried out. As a result, it has revealed that expression of mitochondrial tRFs was slightly altered during development. Based on their expression patterns, 16 tRFs were mainly divided into three groups: 1) tRFs that were strongly detected in late larval stages, 2) tRFs that were strongly detected in egg, and 3) intermittently expressed tRFs. Considering these data together, it is suspected that expression of mitochondrial tRFs might be regulated intricately depending on the developmental stages.

The highly abundant tRFs were preferentially produced from the 5' end regions of its tRNAs.

My deep sequencing data shows that tRFs were derived from genomic tRNAs as well as mitochondrial tRNAs. In order to reveal the distribution of genomic tRF types, I classified genomic tRFs by their corresponding anticodons. It can clearly seen that approximately 70% of tRFs were derived from $tRNA^{Gly}$(GCC). Additionally, tRF^{Gly}(CCC), tRF^{Glu}(CUC), tRF^{Lys}(CUU), and tRF^{Asp}(GUC) reads were also comparatively higher than the other tRFs. To examine whether genomic tRFs were derived from the particular position of its tRNAs, nucleotide sequences of the highly abundant genomic tRFs (i.e., tRF^{Gly}(GCC), tRF^{Gly}(CCC), tRF^{Glu}(CUC), tRF^{Lys}(CUU), and tRF^{Asp}(GUC)) were compared with that of the corresponding tRNA genes. The comparative sequence analysis showed that large amounts of these tRFs were mapped to the 5' half region of its tRNAs. These results suggest that the location of highly abundant tRFs were biased towards the 5' end of tRNAs.

Expression of genomic tRFs was also altered in six developmental stages.

To examine whether expression of genomic tRFs were altered in the development stages, expression patterns of tRFs were analyzed based on sequencing reads. In order to estimate excise expression of genomic tRFs, I analyzed 14 tRNAs, because all these tRFs were mapped to only one tRNA gene, and no reads were mapped to non-tRNA regions. Analysis of the accumulation of the all tRF reads that mapped to mature genomic tRNA sequences showed that expression of genomic tRFs was also altered during development, and their expression patterns were able to be categorized into four groups: 1) increasingly expressed tRFs, 2) decreasingly expressed tRFs, 3) tRFs that were strongly detected in egg, and 4) other tRFs. These data suggest that expression of not only mitochondrial tRFs, but also genomic tRFs were altered in *T. cancriformis*.

My bioinformatics analyses showed that numerous tRFs were derived from genomic tRNAs. However, it was unclear whether they are expressed in *T. cancriformis*. To confirm the expression of genomic tRFs, I performed northern blot analysis of the total RNA isolated from each six developmental stage, using probes specific to genomic tRFs. Especially, the expression of tRF^{Gly}(CCC) and tRF^{Lys}(CUU) was analyzed, because these two tRFs were also detected in another deep sequencing data of *T. cancriformis* small RNAs obtained before (46,610,484 reads in all). By northern blot analysis, the expression of both tRFs

was detected in *T. cancriformis*. Despite the uniform expression of 5.8S rRNAs and mature tRNAs, approximately 30 nt-long tRF^{Gly}(CCC) and tRF^{Lys}(CUU) were particularly increased at 3rd instar and the late larval stages, respectively. These data demonstrate that expression of tRFs was actually altered in development of *T. cancriformis*. Furthermore, approximately 60 nt-long tRF^{Lys}(CUU) were strongly expressed in egg, and apploximately 45 ng-long tRF^{Lys}(CUU) were expressed only in adult stage, suggesting that several tRFs in different length were produced from $tRNA^{Lys}$(CUU) depending on the developmental stages. Meanwhile, it should be noted that the expression patterns revealed by northern blot analysis slightly differed from those by deep sequencing analysis. This appears to be the cause of design of the oligonucleotide probes and/or individual differences of *T. cancriformis*. Thus, in order to identify tRF expression, additional northern blot analyses will be performed in future. I conclude that genomic tRFs were actually expressed in *T. cancriformis*, and their expression may be regulated depending on the developmental stages.

Discussion

In this study, I report the overall features and expression patterns of mitochondrial and genomic tRFs in the development of *T. cancriformis*. For a long time, tRFs have been widely assumed to random by-products of the degradation of tRNAs. Recently, several groups have been identified tRFs as a new class of small RNAs (Lee *et al.*, 2009; Cole *et al.*, 2009). This study also demonstrates that tRFs are not random degradation products, and newly suggests that they may have important role(s) in eukaryotic development.

Until now, the molecular feature of mitochondrial tRFs has not been investigated well. In this research, total of 181 mitochondrial tRFs corresponding to 16 of 22 anticodons were discovered. Although further experimental analysis will be required to examine whether 16 mitochondrial tRFs were actually expressed in *T. cancriformis*, this is the first report of a comprehensive analysis of mitochondrial tRFs. Thus, my data will be a valuable source for understanding the biological significance of mitochondrial tRFs. Deep sequencing analysis also allowed me to detect 1,620 genomic tRFs in *T. cancriformis*. Intriguingly, approximately 70% of tRFs were derived from $tRNA^{Gly}$(GCC), and they were preferentially aligned to the 5' half regions of its tRNA. It is noted that the 5' end region of $tRNA^{Gly}$(GCC)-derived fragment has been also found in other eukaryotic organisms (Peng *et al.*, 2012; Zheng *et al.*, 2013). Considering these results together, highly abundant tRFs, tRF^{Gly}(GCC), may have important role(s) in eukaryotes.

The visualization of the aligned tRF sequences showed that many mitochondrial and genomic tRFs were derived from the particular regions of its tRNAs such as 5' half region, 5' end region, 3' half region, 3' end region, and AC-stem loop region, suggesting that these tRFs are not random degradation products. Why only the particular regions of tRNA-derived fragments were accumulated still remains unclear. Thus, the underlying mechanism of tRNA cleavage should be analyzed in future.

I also observed that expression of mitochondrial and genomic tRFs were altered in *T. cancriformis* developmental stages. To my surprise, in the case of tRF^{Lys}(CUU), different length of tRFs were produced in different developmental stages, indicating that the mechanism of tRFs production may differed depending on the developmental stages. An interesting issue is whether temporal tRFs regulate developmental process such as the rapid morphological alteration of *T. cancriformis*. Recently, it has been demonstrated that tRF corresponding to the 5' half region of tRNA cooperates with a translational repressor to regulate protein translation in the stress-induced human cells (Ivanov *et al.*, 2011). Similar to previously reported tRFs, tRFs are likely to function as gene regulatory molecules in developmental stages. Additionally, one should

consider the possibility that developmentally regulated-tRNA cleavage occurs to reduce tRNA level. To exclude this possibility, I will precisely quantify the expression levels of mature tRNAs in each six stage of *T. cancriformis*, although my results showed the mature genomic $tRNA^{Gly}$(CCC) and $tRNA^{Lys}$(CUU) were almost uniformly detected in the developmental stages.

To understand the biogenesis, mechanism, and biological function of mitochondrial and genomic tRFs, much more works will be needed. In future research, additional northern blot analysis for mitochondrial and genomic tRFs will be performed. I will also try to construct a gene transfection system adapted to *T. cancriformis* for functional analysis of tRFs. Taken together with previous and additional results, I believe that this study will provide a new and valuable insight into tRF function.

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