Original Article

Cloning and Analysis of piRNAs from Testes of Three Species

Guobin Chang* Rong Chen Ying Zhang Aiqin Dai Teng Ma Guohong Chen

Abstract

piRNAs are a testis-specific class of small RNAs in mammal. Sixteen sequences of piRNAs were cloned from three different species by the method of construction of cDNA library including two sequences from mouse, eight sequences from quail and six sequences from chicken. Herein, the two piRNAs sequences from mouse had already been identified, while the others were cloned for the first time. The sequence analysis indicated that the avian piRNAs had a similar length and a preference of uridine for 5′-end with varying degrees to the mammalian piRNAs. Beside the function in the process of sperm development and maturation, avian piRNAs may also have a role in regulating the development of nerve and tumor cells.

Keywords: chicken, cloning, piRNAs, quail, testis

College of Animal Science and Technology, Yangzhou University, Yangzhou, 225009, China
***Corresponding author E-mail:** passioncgb@163.com*

บทคัดย่อ

การโคลนนิ่งและการวิเคราะห์ piRNAs จากลูกอัณฑะของ สัตว์ 3 สปีชีส์

Guobin Chang Rong Chen Ying Zhang Aiqin Dai Teng Ma Guohong Chen

piRNAs คือ ส่วนของสาย อาร์เอ็นเอขนาดเล็กในกลุ่มความจำเพาะต่อลูกอัณฑะของสัตว์เลี้ยงลูกด้วยน้ำนม ทำการศึกษาลำดับเบส ของ piRNAs ในสัตว์จำนวน 3 สปีชีส์ โดยวิธีการสร้าง cDNA library ประกอบด้วย หนูไมซ์จำนวน 2 ตัวอย่าง นกกระทาจำนวน 8 ตัวอย่าง และไก่จำนวน 6 ตัวอย่าง ซึ่งเป็นการศึกษาครั้งแรกใน นกกระทาและไก่ ผลการวิเคราะห์ลำดับเบสพบว่า piRNAs ของสัตว์ปีกมีความยาว และตำแหน่งของเบส Uridine ท่ตำแหน่งปลาย 5 แตกต่างจาก piRNAs ของสัตว์เลี้ยงลูกด้วยน้ำนม อาจกล่าวว่า piRNAs ของสัตว์ปีกมี บทบาทในการควบคุมการพัฒนาเซลล์ประสาทและเซลล์เนื้องอก นอกเหนือไปจากหน้าที่ในการกระบวนการพัฒนาของการสร้างสเปอร์ม

คำสำคัญ: ไก่ การโคลนนิ่ง piRNAs นกกระทา ลูกอัณฑะ

College of Animal Science and Technology, Yangzhou University, Yangzhou, 225009, China

***ผู้รับผิดชอบบทความ E-mail:** passioncgb@163.com*

Introduction

Small RNAs are a large class of non-coding RNAs regulating all kinds of functions in eukaryotic cells and contributing to a variety of behaviors such as gene expression, cell cycle and individual development (He et al., 2005). According to pathogenesis, non-coding RNAs participating in RNAi are divided into three kinds at present. They are microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). As we all know, siRNAs are cut from double-stranded RNA precursors, while miRNAs are made of double-stranded regions in hairpin RNA precursors. miRNAs can induce the suppression of translation or direct the cleavage of target mRNA by binding complementary or near-perfect complementary sites, to which siRNAs is also Herein, the process of suppression and cleavage is closely related to the members of AGO protein family, which are highly conserved in eukaryotes (Kim, 2006). AGO protein family mainly segregates into two subfamilies: AGO and PIWI (Yan et al., 2003; Bartel, 2004). The AGO subfamily takes part in the inducement of siRNAs RNAi and production of miRNAs, while the PIWI subfamily is involved in the development and maintenance of germ stem cells (Song et al., 2003; Yan et al., 2003).

Although PIWI protein exists in many different species, its expression is almost limited in the period of gametogenesis and development. So far, we only know that PIWI is related to the formation and production of stem and germ cells, whose mechanism of biochemistry is unknown. In 2006, piRNAs were firstly found in the testis of mouse by four research teams at the same time. They are Tuschl, Hannon, Grivna and Kingston who found piRNAs in the study of PIWI proteins how to affect the development of sperm cells from mammal (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006). In mice, PIWI subfamily constitutes three members, namely MIWI, MILI and MIWI2. Tuschl made an experiment with the homologous protein of PIWI protein, MILI protein, while Hannon and Grivna made it with MIWI protein. Simultaneously they all found that a novel class of small RNAs could bind with MILI and MIWI protein in mouse sperm cells, which they had termed PIWI-interacting RNAs (piRNAs). Moreover, they had named the complex containing piRNAs and PIWI protein homologue piRNAs complex (piRC).

In the future, the hot issue of RNAs, especially small RNAs, is the study of RNAs binding protein. Investigating genetic characters of small RNAs can further promote in-depth studies and practical applications of small RNAs and binding proteins. Since piRNAs have led to primary transcriptional gene silencing and played a role in the process of transcription, they are expected to address series of questions such as greater than 30bp of dsRNA leading to apoptosis and the RNAi mechanism of higher organisms and the application of RNAi technology. Moreover, the access of avian gametes, embryos and tissues has unparalleled superiority to other organisms. To date, there has been no research on piRNAs and its binding protein in avain and studies of avain RNAs binding protein mostly concentrate on avian influenza virus nucleoprotein (Chen et al., 2004). Thus, in this study we selected quails and chickens for our research objects, extracting small RNAs from their gonads and cloning avian piRNAs from them by the method of construction of cDNA libraries, which happen for the first time will provide basic foundation for the study

of genetic characters and molecular mechanisms of small RNAs in avain and transgenic chicken.

Materials and Methods

Small RNAs preparation and cloning of piRNAs: Fresh testes were collected from sex-maturity mice (*Mus musculus*), quails (*Coturnix coturnix*) and chickens (*Gallus gallus*) and ground into power in liquid nitrogen. Total RNAs were isolated by using Trizol reagent (Invitrogen, California, USA), then dissolved in RNase-free water (Takara, Shiga, Japan) and stored at -80°C.

Total RNAs were electrophorsed on 15% denaturing polyacrylamide gel [0.5XTBE, 7M urea (Sangon, Shanghai, China), 15% acrylamide (19:1 acryl:bis-acryl) (Sangon, Shanghai, China)]. Small RNAs were size-fractionated ranging from 26 to 30 nucleotides and purified by Takara small RNA Gel Extraction (Takara, Shiga, Japan).

Purified small RNAs were ligated sequentially to 3′ and 5′ DNA/RNA chimeric oligonucleotide adapters according to the manufacturer's instructions from Small RNA Cloning Kit (Takara, Shiga, Japan) (3′ adapters/ RT-Primer/PCR-R: 5′-GTCTCTAGCCTGCAGGATCGA TG-3′; 5′ adapters/PCR-F: 5′-AAAGATCCTGCAG GTGCGTCA-3′). After adding the adapters, reverse transcription (RT) was carried out with 200 U of M-MLV RTase, 1 µl RTase inhibitor, 1 µl RT-Primer, 4 µl dNTP mix (2.5 mM each), 4 µl 5x buffer and 9 µl of ligation production in a final reaction volume of 20 µl at 42°C for 60 min. Conventional PCR was performed using 5 U of TaKaRa Ex Taq HS, 25 µl 2x PCR Buffer, 5 µl dNTP mix, 1 µl PCR-F, 1 µl PCR-R and 5 µl of RT cDNA in a 50 µl reaction volume on the Mastercycler epgradient system (Eeppendorf, Hamburg, Germany). A 3-step PCR protocol (94)°C for 2 min, then 15 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and 72°C for 3 min) was used. After PCR, an aliquot of 5 µl of the PCR product was analyzed on a 10% polyacrylamide gel. The purified PCR products were cloned into T-Vector (See Fig 1, Takara, Shiga, Japan) and transformed into E coil competent cells-DH5α (Tiangen, Beijing, China) by heat shock. The recombinant plasmids carrying target fragments were isolated from individual colonies and sequenced by 3730xl DNA Analyzer (Applied Biosystems, California, USA).

Sequence analysis: The cloned sequences were scanned in the piRNABank (http://pirnabank.iba b.ac.in/) and NCBI (http://www.ncbi.nlm.nih.gov/). Nucleotide component analysis of those sequences was carried out by MEGA4 software (Tamura et al., 2007).

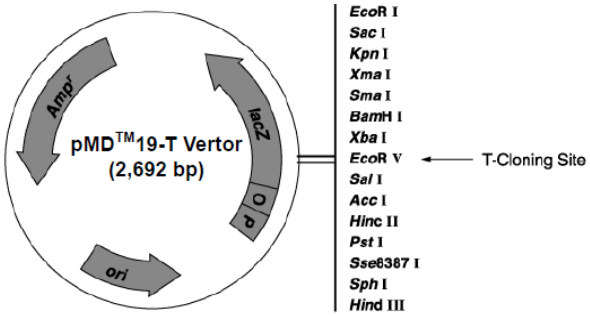

Figure 1 The structure of pMDTM19-T Vector.

Table 1 piRNA-M* sequences of mouse

Name	Sequnce (5'-3')	Size (nt)
piRNA-M1	UACAAAGUGAUACUAUGCCACCAUGUUGC	29
piRNA-M2	UGGUCUUUCUCCUCCCAUCUGGUAUUAACU	30

Note: *M: The testis of mouse, nt: Nucleotide

Table 2 piRNA-Q* sequences of quail

Name	Sequence(5'-3')	Size (nt)
piRNA-Q1	UCUCUGUGCAUUUCCCUUGUUUUACAAA	28
piRNA-Q2	CAGUCCUCAGUCAGGUCUGCAGUAAAUAA	29
piRNA-Q3	UGCACGUGCACCUCGUGAGACCCCA	25
piRNA-Q4	AGUGACUCUGCCCUACUGUGGCUCAGA	27
piRNA-Q5	UGCUGAGCGCCUGCGGUGCCUGCGU	25
piRNA-Q6	ACGUCAGCUGGGUUUAGACCGUCGU	25
piRNA-Q7	UAGUGGUAUCAUGCAAGAUUCCCAUUC	27
piRNA-Q8	UGACUGCAAGUACGAACACUAACCUAU	27

Note: *Q: The testis of quail, nt: Nucleotide

Table 3 piRNA-C* sequences of chicken

Name	Sequence(5'-3')	Size(nt)
piRNA-C1	CCCAGUAAGUGCGGGUCAUAAGCUCGCGUU	30
piRNA-C2	GUUAGUAGCAUCUGCGCUCCUCUUAGCC	28
piRNA-C3	UCGCCGCAAACACAGCAUUGCUGGAAAG	28
piRNA-C4	UCAGCUCCGGACCUCUUCCAAAAUCGC	27
piRNA-C5	UCUGCUCUAAUGCUGUCAGAUGUGUU	26
piRNA-C6	UGCACGGCACACCUUUACAGCCAC	24

Note: *C: The testis of chicken, nt: Nucleotide

Results

Cloning piRNAs from testes of three species: The cloning of sixteen piRNAs sequences of three species is shown in Table 1-3. Of course, these known sequences were just a part of the testis piRNAs library *in vivo.* They are two sequences from mouse, eight sequences from quail and six sequences from chicken. At a glimpse, it seems that piRNAs sequences are less conservative within three species.

Analysis of piRNAs sequences from three species: From table 4, we can see that the size of mouse piRNAs is from 29 to 30 nucleotides, the probability of Uridine in 5' position is 100% and the average contents of bases U, C, A, G are 35.6%, 27.1%, 22.0% and 15.3%, respectively. The proportion of base composition shows that mouse piRNAs are relatively lack of G and the average content of A+T (57.6%) is higher than that of C+G (42.4%). In quails, the size of piRNAs is from 25 to 29 nucleotides, 62.5% of 5' end is Uridine and the average contents are 27.7% for base U, 27.7% for base C, 21.6% for base A and 23.0% for base G, which indicates that these four bases are equal in the sequences of quail piRNAs. Likewise, the size of chicken piRNAs is from 24 to 30 nucleotides, the percentage of Uridine in 5' position is 66.7% and the average contents of bases U, C, A, G are 25.2%, 31.3%, 21.5% and 22.1% respectively, suggesting that base C is relatively higher than the others and the average content of C+G (53.4%) is higher than that of A+T (46.7%). It is clear that the proportion of base composition of chickens is opposite to that of mice.

Discussion

piRNAs are a novel class of non-coding small RNAs and found in the mouse testes interacting with PIWI protein. Previous researches showed that the length of piRNAs was between 26 and 31 nucleotides and an overwhelming bias for uracil was at the first position, which was different from miRNAs and siRNAs (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Lau et al., 2006). Girard et al. (2006) confirmed that there were piRNAs only in the sperm cells which accumulated in the beginning of meiosis and disappeared in mature sperm cells by Northern blotting. Grivna et al. (2006) found that piRNAs existed only in testis cells while detecting different tissues from mice using denaturing PAGE and Northern blotting. In consideration of tissue specificity, we only cloned some piRNAs from testes by constructing small RNA cDNA libraries in mice, quails and chickens. In this study, the length of mouse piRNAs is from 29 to 30 nucleotides, which is consistent with the result of previous studies. Within table 4, the length of piRNAs is from 25 to 29 nucleotides in quails and from 24 to 30 nucleotides in chickens. Compared with the mammal, we concluded that avian piRNAs might have a similar length, which still needs to be confirmed by further experiments such as investigating lots of avain piRNA sequences. The probabilities of Uridine in 5' position are 100% for mice, 62.5% for quails and 66.7% for chickens respectively, which suggests that the bias for uridine at 5′ end likewise exists in our investigations. The analysis of the proportion of base composition suggests that piRNAs variations are very widespread in either one species or different species.

Table 4 Analysis of piRNAs sequences from different species

piRNAs	Size(nt)	U(%)	C(%)	A(%)	G(%)	Uridine in 5' position (%)
Mouse	29-30	35.6	27.1	22.0	15.3	100
Quail	25-29	27.7	27.7	21.6	23.0	62.5
Chicken	24-30	25.2	31.3	21.5	22.1	66.7

Note: nt: Nucleotide

As piRNABank only provides related information on piRNAs in these animals namely human, mouse, rat, zebrafish, platypus and a fruit fly excluding poultry, we can't carry out to search the known piRNAs from quails and chickens. Although the two mouse piRNAs from our study have already existed in piRNABank database, piRNA-M1 lost a Cytimidine in 3'-end comparing with mmu_piR_009607 and piRNA-M2 contained a more Uridine than mmu_piR_034084 in 3'-end. The mmu_piR_009607 and mmu_piR_034084 are located on chromosome 6 and 17 in rats, respectively, which may participate in the sperm development. For the piRNAs sequences from quails and chickens, we found that piRNA-C1 matched completely with the gene (GeneID: 769505) similar to neuropeptide in the corresponding region by BLAST in the whole chicken genome, likewise piRNA-C3 matched with CHCHD3 (GeneID: 418435)and the gene (GeneID: 427940) similar to Phf7 protein. Neuropeptides are peptides transmitting information in vivo, which may play a different role as neurotransmitters, modulators or hormone-like things. Since most studies on piRNAs have been limited to the role of regulating the gametogenesis, we speculated that piRNA-C1 is likely to regulate neurodevelopmental development in organisms. The CHCHD3 gene (another name FLJ20420 gene) is named for its coding protein with coiled-coil-helix-coiled-coil-helix domain containing 3. Schauble et al. (2007) reported that FLJ20420 gene expressed in plastosomes and served as a substrate of PKA participating in the oxidative phosphorylation of bodies. Moreover, the expression of FLJ20420 gene changes in human diseases such as cancers, coronary heart diseases and multiple scleroses according to available studies which implied that the FLJ20420 gene is likely to take part in the pathological process of these diseases. It is possible that piRNA-C3 has a relationship with the FLJ20420 gene such as regulating gene expression by silencing. Phf7 protein exists in the nucleus and mainly involved in the transcription of gene regulation and the DNA-dependent processes of life. This study can provide basic foundation for the study of genetic characters and molecular mechanisms of small RNAs in avain and transgenic chicken.

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