miRNA target prediction of avian Z-linked DMRT1 gene during sex determination in chicken (G. Gallus)

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Abstract. Sex determination in dimorphic animal, such as chicken (G. Gallus), is controlled by the expression of doublesex and mab-3 related transcription factor 1 (DMRT1) gene. This gene act as sex determination switch by critically needed for testis differentiation and as antagonist of ovarian development. miRNA, is belongs to short noncoding RNA which modulate gene expression in specific or board targeted genes. This study was aimed to predict miRNA(s) candidate targeted to DMRT1 expression in chicken. In silico method was employed to mining miRNA targeted to DMRT1 using three online databases namely miRDB, TargetScan, and microT-CDS. Following prediction, clustering was performed to select common miRNA(s) in minimal two databases for gene ontology (GO) analysis. Totally 78 miRNAs were targeted to DMRT1 3′UTR, and eight miRNA(s) were found in minimal two databases. The GO analysis found seven distinct biological functions in membrane, cytoplasm, protein binding, nucleus, integral component of membrane and molecular function, and all are related to the cell growth namely cell proliferation. According to the result, it shows the possibility to use selected candidate of miRNA(s) targeted to DMRT1 to reveal the sex determination mechanism at early stage of chicken development.

1. Introduction

The sex of avian is determined by the inheritance of sex chromosomes ZZ for male and ZW for female. Genes carried on one or both of these sex chromosomes are control sexual differentiation during embryonic life [1]. In chicken, as in other birds, gonads develop into testes or ovaries is triggered by sex-determining genes which involves the Z-linked namely doublesex and mab-3 related transcription factor 1 or DMRT1 gene (ID: 769693, NCBI). The Z-linked DMRT1 gene expression act for testis differentiation and agonist of ovarian development [2]. This gene also plays a key role in testis development by govern the sexual development, together with a role for sex steroid hormones. Earlier report shows high expression level, 1.5 to 2 times higher than females [3], of DMRT1 was found in male gonads during testis development [4]. This high expression then activated other testis development genes namely SOX9, AMH, and HEMGN. Meanwhile, low expression of DMRT1 activated ovarian pathway genes through FOXL2 and CYP19A1 [3].

As generally known, the expression of gene is regulated by short noncoding RNA called miRNA. miRNAs are tiny (18-22 nt long), noncoding RNA molecules which act post-transcriptionally to regulate genes expression [5]. Its act through sequence-specific interactions with the 3′untranslated regions
(UTRs) of target mRNAs [6] and subsequently inducing mRNA destabilization and/or translational repression [7]. According to the bioinformatics analysis, a single miRNA could targeted thousands of genes or vice versa [8]. Due to its broader targeting abilities, then miRNAs are found to be involved in many biological processes and cellular pathways [9].

miRNAs in G. Gallus known as gga-miRNA are also belongs to endogenous small non-coding RNAs, and able to regulate a variety of biological processes [10]. Currently, no specific miRNA(s) have been definitively reported to play a role in embryonic gonadal development in chickens [11] though number of genes have now been identified to control gonadal sex differentiation. For that, this study was aimed to analysis the possibility to find miRNA(s) candidate targeted to DMRT1 gene, using in silico study, which control sex determination in chicken. Selected miRNA candidate then could be used to reveal the mechanism behind that process.

2. Study methods

2.1. Prediction of miRNAs which may target DMRT1 gene

The miRNA target prediction to specific gene, was performed by in silico using three bioinformatics databases namely miRDB (www.mirdb.org), TargetScan (www.targetscan.org/), and microT-CDS (http://www.microrna.gr/microT-CDS). Following target prediction, common miRNAs that were found in at least two databases were selected as candidates. The candidate selection was performed using help of Venn Diagram software (http://bioinformatics.psb.ugent.be/webtools/Venn/). Selected miRNA(s) then proceed to gene clustering of gene ontology (GO) analysis.

2.2. Gene ontology analysis

To investigate the functional characteristics, GO analysis was employed on the genes targeted by the miRNAs candidate. The selected candidate of miRNAs then uploaded to online software for GO classification by using MirPath_v3 (http://snf-515788.vm.okeanos.grnet.gr/). Default threshold analysis of p-value and MicroT were set at 0.05 and 0.8, respectively. Next, GO analysis was build based on the genes which targeted by the miRNAs candidate using genes union way as analyzed by the software. Heatmap figure of the resulted biological process and its clustered miRNAs were then retrieved.

3. Results and discussion

The prediction of miRNA which may targeted to DMRT1, resulting 78 unique miRNAs from three database as resumed in Table 1. TargetScan, miRDB and microT-CDS are database which provide the prediction data for miRNA in chicken, gga-miRNA, and they can be accessed online using URL mentioned in the study method. Following prediction, we then clustering the predicted gga-miRNA using the help of Venn diagram (Figure 1), common gga-miRNAs that were found in minimum two databases were then selected as candidates. As the result, eight gga-miRNAs were selected (Table 2).

| Table 1. Predicted miRNAs targeted to DMRT1 in selected online database |
|-----------------------------|------------------|
| Database                  | Number of predicted miRNAs |
| TargetScan                | 40                |
| miRDB                     | 11                |
| microT-CDS                | 27                |
| Total                      | 78                |
Figure 1. Venn diagram of miRNA candidate targeted to DMRT1

Table 2. List of unique miRNAs in selected database

| Database                  | Total Prediction | miRNA name                                                                 |
|---------------------------|------------------|-----------------------------------------------------------------------------|
| TargetScan - miRDB        | 6                | gga-miR-6562-3p; gga-miR-138-2-3p; gga-miR-1781-5p; gga-miR-1580; gga-miR-153-5p; gga-miR-1728-3p |
| miRDB - microT-CDS        | 9                | gga-miR-6558-5p; gga-let-71-3p; gga-miR-1754-3p; gga-miR-6683-3p; gga-miR-103-3p; gga-miR-107-3p; gga-miR-1453; gga-miR-1746; gga-miR-757 |
| TargetScan - microT-CDS   | 31               | gga-miR-6684-5p; gga-miR-367; gga-miR-1630; gga-miR-1591-3p; gga-miR-92-3p; gga-miR-6588-3p; gga-miR-6570-3p; gga-miR-6545-5p; gga-miR-1715-3p; gga-miR-1769-5p; gga-miR-6680-3p; gga-miR-1581; gga-miR-32-5p; gga-miR-1687-3p; gga-miR-194; gga-miR-6706-5p; gga-miR-6641-3p; gga-miR-6581-5p; gga-miR-20b-3p; gga-miR-6649-3p; gga-miR-1654; gga-miR-6633-5p; gga-miR-30e-3p; gga-miR-6574-3p; gga-miR-1564-5p; gga-miR-200a-3p; gga-miR-1457; gga-miR-7469-5p; gga-miR-1687-5p; gga-miR-1811; gga-miR-6663-5p |
| miRDB                     | 20               | gga-miR-6549-3p; gga-miR-7466-3p; gga-miR-6548-5p; gga-miR-7481-5p; gga-miR-1550-5p; gga-miR-551-5p; gga-miR-203a; gga-miR-1795; gga-miR-6552-3p; gga-miR-1695; gga-miR-302d; gga-miR-1753; gga-miR-1676-3p; gga-miR-6608-5p; gga-miR-7479-3p; gga-miR-6594-5p; gga-miR-219b; gga-miR-302b-3p; gga-miR-302c-3p; gga-miR-1458 |
The selected gg-miRNA candidate then uploaded to an online database for gene target clustering for GO analysis. Each individual gg-miRNA shows has different total of targeted gene as listed in Table 3. In here, we found that gga-miR-6701-3p has the most gene targets compared to the other listed gg-miRNA. Following the gene target collection, then GO analysis shows seven biological processes that are in molecular function, intracellular, integral component of membrane, protein binding, nucleus, cytoplasm, and membrane. The heatmap (Figure 2) shows the function of each individual gg-miRNA, by its log p-value, to each specific biological function. gg-miRNAs which have darker red indicates a higher degree in relative comparison to the light yellow. Seven gg-miRNAs, except gga-miR-138-2, shows low degree of its influence in molecular function. Moreover, all the gg-miRNA in Table 3, are having high degree on its influence in membrane, cytoplasm, protein binding, and nucleus.

**Table 3.** Listed candidate of miRNA and its target gene for GO analysis

| miRNA name       | Genes targeted | GO Analysis                                      |
|------------------|----------------|--------------------------------------------------|
| gga-miR-6701-3p  | 473            | molecular function (GO:0003674)                  |
| gga-miR-7438-5p  | 459            | intracellular (GO:0005622)                       |
| gga-miR-6562-3p  | 81             | integral component of membrane (GO:0016021)      |
| gga-miR-138-2-3p | 257            | protein binding (GO:0005515)                     |
| gga-miR-1781-5p  | 138            | nucleus (GO:0005634)                             |
| gga-miR-1580     | 7              | cytoplasm (GO:0005737)                           |
| gga-miR-153-5p   | 392            | membrane (GO:0016020)                            |
| gga-miR-1728-3p  | 21             |                                                  |

**Figure 2.** Heatmap graph of the function of selected gga-miRNA
Cluster analysis using dendrogram of gg-miRNA shows three clusters of gg-miRNA in this study (Figure 3). Six gg-miRNA have similar function, while the other two have different function. The clear function of those miRNA also shows three distinct pathways as illustrated in Figure 4. The first pathway is in molecular function, the second is in intracellular and integral component of membrane, then the third is in membrane, cytoplasm, protein binding and nucleus. All these pathways are related to the biological function of the genes targeted by selected candidate of gg-miRNA in Table 3.

![Figure 3. gg-miRNA cluster dendrogram](image)

![Figure 4. Pathway cluster dendrogram](image)

DMRT1 is a transcript that work as regulator of male differentiation which required for testicular development. The transcript indirectly activates genes related receptor and repress the pluripotent regulator of the cell [12], play key in sex determination by controlling testis development and male germ cell proliferation, then control the sperm production. In the other study, DMRT1 was reported exhibit a gonad-specific and sexually dimorphic expression pattern to maintain the fate or determined the further testes or ovaries development [13], therefore the expression pattern of DMRT1 is only restricted in the gonad and upregulated when the testis development occur [14]. The deficient of DMRT1 gene display problems with cell differentiation and cell survival in the postnatal testis. At this point, it could be said that DMRT1 involved in the development of the sexual organ by control the cell growth (proliferation) in embryonal stages. This statement is supported by the result of GO analysis, which shows gene pathway related to the cellular growth, cell communication and its interaction (Figure 2 and 4). Cell growth cover the function of nucleus, cytoplasm, protein binding, membrane, and cell to cell communication which positively regulates the proliferation process [15].
Cell proliferation is an increase in cell number due to cell division. It is a complex, tightly controlled, well-defined process, and necessary for normal tissue development. This process requires chemical communication which occurs at various levels in an integrated exchange of information such as intercellular signaling, cellular communication [16], and cellular metabolism as the foundation of all biological activities. During cell proliferation, extensive metabolic must be occurred in order for cells to acquire sufficient nutrients which are necessary to support cell growth [17].

Of the in silico analysis of gg-miRNA targeted to DMRT1 in the current study, the present of miRNA in chicken and its function have been reported in the previous study. For example, especially its function in chicken reproduction area, it is reported that gga-miR-1684a and gga-miR-1434 were able to enhance or reduce the production of miRNAs in pituitary and hypothalamic which responsible for chicken egg production rate [10]. In the egg production traits gga-miR-34b, gga-miR-34c, and gga-miR-216b, were reported to regulate processes such as proliferation, cell cycle, apoptosis and metastasis and were expressed differentially in ovaries of high-rate egg production chickens. According to that, these gg-miRNA are suggested to have important role in ovary development and reproductive management of chicken [18].

In male chicken, the upregulation of gga-miR202* in male gonads suggest its function to regulate testicular development [19], and might have connection to DMRT1 gene as critical regulator in sex determination. Another study also shows that gga-miR-215 has influence in the molecular profile of testis and genes that may determine sperm motility in chickens [20]. Moreover, the gga-miR-155 and gga-miR-7480-5p are reported to be two candidates that affect to chicken sperm motility [21].

Those ample evidence of the present and the possible function of gg-miRNA in chicken would open other possibility to use miRNA for further functional study. The manipulation of miRNA action could improve and/or decrease the gene expression which lead to its biological function. If the use of miRNAs in chicken are promising to enhance the productivity either in local or commercial bird production, this will have a huge impact to the livestock industry.

4. Conclusions
According to the result, we found eight gg-miRNA as candidate targeted to DMRT1 gene. The GO analysis reveals seven distinct pathways of gene or biological function of the DMRT1 regulation during sex determination in chicken which related to cell proliferation process. Further study is required to validate those gg-miRNA targeted to DMRT1 to exactly know the sex differentiation in chicken during embryonal stage.

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