Comprehensive Analysis of Differences of N⁶-Methyladenosine of Lncrnas Between Atrazine-Inducedand Normal *Xenopus Laevis* Testis

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Abstract

Background: Increasing evidence suggested N^6-methyladenosine (m^6A) plays an important role in RNA stability, degradation, splicing and translation. M^6A is found in different RNA including long non-coding RNA (lncRNA) which has been found possess significant biological functions. Our previous study examined the m^6A profile of mRNAs in testis tissues of *Xenopus laevis* (*X. laevis*) with and without treatment with 100 μg/L atrazine (AZ). The result revealed that m^6A is a highly conserved modification across the species.

Methods: In this study, we apply previous approach to further investigate m^6A modification profile of lncRNAs and predict the potential mechanism. In brief, m^6A was sequenced by MeRIP sequencing using the latest Illumina HiSeq sequencer. Pathway enrichment analysis was used to maps genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

Results: The results showed that m^6A of lncRNAs enriched around intergenic region in testes of *X. laevis*. We further investigated the differential expression of lncRNAs m^6A in testes of AZ-exposed compared with that in animals from control group. The results indicated that up to 198 differentially methylated m^6A sites were detected within 188 lncRNAs, in which 89 sites were significantly up-regulated and 109 sites were significantly down-regulated. Data from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that AZ-affected lncRNAs m^6A sites were mainly involved in 10 pathways in which 3 mutual pathways were found in the result of differentially m^6A-methylated mRNAs.

Conclusions: These findings suggest that differentially m^6A-methylated lncRNAs and these 3 pathways may act on regulatory roles in abnormal testis development of AZ-exposed *X. laevis*. This study for the first time provide insights into the profile of lncRNAs m^6A modifications in amphibian species.

1. Introduction

RNA modifications play crucial roles in gene expression [1]. As the most universal form of post-transcriptional RNA modifications, N^6-methyladenosine (m^6A) modification has become a new research area in epigenetic [2]. Recent studies have shown that the m^6A modification modulates the function of the RNA molecule in multiple ways through its novel functions [3, 4]. The m^6A modification is found in different species of RNA, including tRNA, mRNA, rRNA, and long non-coding RNAs (lncRNAs) [5, 6]. In addition, the m^6A modification can affects many properties of RNA, including gene translation [7], splicing [8], and long non-coding RNA-mediated gene silencing [9].

LncRNAs are non-coding RNAs comprising more than 200 nucleotides without protein coding function and engaged in diverse biological processes across every branch of life [10]. Increasing evidence points that lncRNAs play an important role in regulating multiple processes of gene expression [11]. Studies have also found that regulation of lncRNAs can affect mRNA transcription, splicing, translation and stability [12]. It has been widely recognized that dysregulated lncRNAs play an important part in many diseases [13]. In recent years, m^6A modification of lncRNAs gains great attention and this modification has shown to control mammalian gene expression [14]. To our knowledge, the profile of m^6A modification of lncRNAs in amphibians remain to be explored.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine, AZ) is an environmental endocrine disrupting chemicals (EDCs) used extensively as a herbicide worldwide [15, 16]. AZ has been reported that it can cause endocrine disruption in mammals, birds, reptiles and amphibians by affecting normal reproductive function and development in these organisms [17, 18]. *Xenopus laevis* (*X. laevis*) is a kind of amphibian widely used as an ideal model organism for testing EDCs exposure [19]. Recently, AZ has been shown to cause demasculinization and complete feminization in male *X. laevis* [20]. In our previous studies, we investigated biological response of *X. laevis* exposed to AZ (0.1, 1, 10 or 100 μg/L) for 90 days in the water environment. We found that AZ induced the reduction of gonad weight and gonado somatic index of male *X. laevis*. Meanwhile, AZ induced histological changes in testes of the frogs from all of AZ treatments including irregular shape of seminiferous lobules and large empty spaces [21]. But the mechanism of AZ-induced abnormal development of male *X. laevis* is unclear. Therefore, it is necessary to explore the potential changes of m^6A modification of lncRNAs which maybe play an important role in the abnormal testis of male AZ-exposed *X. laevis*.

Here, we first elucidated the profile of m^6A modification of lncRNAs in *X. laevis* and dysregulated m^6A methylation of lncRNAs in the AZ-exposed male *X. laevis.* Then, we predicted function classification and involved signaling pathways of dysregulated m^6A methylation of lncRNAs in AZ-exposed male *X. laevis*. Our data will provide the basis for future studies of m^6A methylation of lncRNAs about function and biological significance in amphibians and the exactly mechanism of the abnormal testis development in AZ-exposed male *X. laevis.*

2. Methods

2.1. Sample animals

Reagents, experimental animals and exposure conditions were described in our previous work in our previous study [21]. The *X. laevis* were sacrificed after being exposed to AZ for 180 days. The testis tissues were collected and weight, and then stored at -80°C immediately for further analysis.

2.2. lncRNAs preparation

For each group, at least three biological replicates were setted [22]. Three testes from controls and three ones from 100 μg/L AZ-treated groups were selected randomly for lncRNAs analysis. Then, total RNA of tissue was extracted using TRIzol reagent (Invitrogen Corporation, CA, USA). The concentration and purity of RNA were evaluated by NanoDrop® ND-2000 spectrometer (Thermo, Waltham, MA, USA).

2.3. lncRNAs m^6A MeRIP sequencing

M^6A of lncRNAs was sequenced by MeRIP sequencing using the latest Illumina HiSeq sequencer. Briefly, fragmented RNA was incubated in buffer and the mixture was immune precipitated. Then, bound RNA was eluted from the beads in buffer and then extracted by following the manufacturer's instruction. Both the input sample without immune precipitation and the m^6A IP samples were subjected to 150 bp paired-end sequencing on Illumina HiSeq sequencer. Paired-end reads were harvested and were quality controlled by Q30. Detailed methods were described in our previous study [38].

2.4. Data analysis

After sequencing, quality control of the paired-end reads was performed with Q30, which was subjected to 3' adaptor trimming and low quality reads removing to generate clean reads by Cutadapt software (v1.9.3). Firstly, clean reads of all libraries were aligned to reference genome using bowtie 2 [23] software and mapped to genome by hisat 2 software (v2.04) [24]. Methylated sites on lncRNAs (peaks with a score (-10*log10, *P*-value) of > 3) were identified by MACS software. Differentially m^6A-methylated sites on lncRNAs were detected by diffReps and the identified peaks overlapping with exons of lncRNAs were chosen for further analysis. Pathway enrichment analysis was used to maps genes to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

3. Results

MeRIP-seq analysis of lncRNAs derived from the testes of *X. laevis* revealed that there were 1298 m^6A peaks among 908 lncRNAs in control group. While 1501 m^6A peaks among 1055 lncRNAs were detected inthe testes of AZ exposed *X. laevis*. Importantly, 1100 m^6A recurrent peaks were consistently detected in controls and AZ-exposed groups (Fig. 1).

To further analyze the distribution profiles of m^6A peaks among lncRNAs, these peaks were categorized into 6 groups: bidirectional, exon sense-overlapping, intergenic, intron sense-overlapping, intron sense-overlapping, natural antisense. Particularly, we found that the most m^6A peaks are highly enriched around intergenic region (67.1% in control and 67.8% in AZ-exposed groups) (Fig. 2a and 2b). In the controls, m^6A peak had a highly fold enrichment in bidirectional (95.24%), exon sense-overlapping (95.74%), intergenic regions (96.15%). Meanwhile, in AZ-exposed groups, m^6A peak had a highly fold enrichment in intergenic (86.65%) and intronic antisense (80.47%) (Fig. 2c and 2d).

The results showed that 198 differentially methylated m^6A sites were detected among 188 lncRNAs, in which 89 sites were significantly up-regulated and 109 sites were significantly down-regulated (Table S). The top ten up- and down-methylated m^6A sites of lncRNAs with the highest fold change (FC) values were shown in Tables 1 and 2.

Table 1

The top ten up-methylated m^6A sites of lncRNAs

Chromosome	txStart	txEnd	lncRNA	FC
NC_030726.1	167623498	167623720	LOC108707576	143.6
NC_030729.1	96905501	96905826	LOC108713215	140.5
NC_030729.1	42037587	42037701	LOC108712839	119.9
NC_030737.1	37948621	37948839	LOC108697950	107.9
NC_030726.1	78976541	78976920	LOC108708210	101.9
NC_030730.1	122514814	122514835	LOC108713571	99.0
NC_030727.1	93713360	93713391	LOC108708955	96.1
NC_030727.1	83393506	83393693	LOC108708939	90.1
NC_030724.1	53570461	53570705	LOC108699628	87.1
NC_030730.1	105478624	105478680	LOC108713666	87.0

txStart/txEnd: Start/end position of the differentially methylated RNA peaks.

Table 2

The top ten down-methylated m^6A sites of lncRNAs

Chromosome	txStart	txEnd	lncRNA	FC
NC_030730.1	56714441	56715000	LOC108713510	108.0
NC_030737.1	3748163	3748254	LOC108697148	93.6
NC_030727.1	30334141	30334313	LOC108709317	82.1
NC_030727.1	141936052	141936238	mmp8.S	79.6
NC_030741.1	23682418	23682540	LOC108702712	77.2
NC_030731.1	10726561	10726740	LOC108715064	77.2
NC_030733.1	59942967	59943104	LOC108717426	72.3
NC_030725.1	4717538	4717900	LOC108706487	71.8
NC_030736.1	79214125	79214280	LOC108695842	71.8
NC_030736.1	43073001	43073560	LOC108696274	70.0

txStart/txEnd: Start/end position of the differentially methylated RNA peaks.

Further analysis showed that according to the positional relationships of lncRNAs near the coding gene transcripts, most differentially methylated m^6A sites of lncRNAs were assigned to intergenic (Fig. 3a and 3b). Besides, among the up-methylated sites, those within the intergenic had the highest mean of FC.

While among the down-methylated sites, those within the intron sense-overlapping had the highest mean of FC (Fig. 3c).

To further explore the roles of differentially m^{6}A-methylated lncRNAs in the abnormal development of testis from AZ-exposed *X. laevis*, we performed KEGG pathway analysis of differentially m^{6}A-methylated lncRNAs-related genes to look for the potential key pathways. The result of pathway analysis indicated that 2 pathways with highly enrichment score (-log10(P-value)) were acquired in up-regulated sequencing data. The two signaling pathways, as "SNARE interactions in vesicular transport and Ubiquitin mediated proteolysis", were shown in Fig. 4a. Meanwhile, 8 pathways were found in down-regulated sequencing data, including "Terpenoid backbone biosynthesis, GnRH signaling pathway, Cell cycle, AGE-RAGE signaling pathway in diabetic complications, Vascular smooth muscle contraction, Wnt signaling pathway, Autophagy-animal, NOD-like receptor signaling pathway" (Fig. 4b).

4. Discussion

M^{6}A modification is characterized by wide existence, unique distribution, and dynamic reversibility [25, 26]. It has also been found that enhancer RNAs, non-coding transcripts produced from enhancer regions are highly m^{6}A modified [27]. M^{6}A has been shown to be the abundant internal modification in eukaryotic mRNAs [28]. Emerging findings have shed light on the involvement of m^{6}A modification of lncRNAs [29, 30]. A recent study showed that m^{6}A methylation regulatory network regulates RNA processing and participates in various cellular biological processes, such as biological rhythm, immune modulation, fat metabolism, reproductive development [31]. To explore the m^{6}A modification profile of lncRNAs in the testis of *X. laevis* and the changes of m^{6}A modification of lncRNAs in AZ-exposed *X. laevis*, we examined the transcriptome-wide m^{6}A modification of lncRNAs distribution in the testis of AZ-exposed *X. laevis.* Meanwhile, the changes of m^{6}A modification of lncRNAs distribution were analyzed by exogenous stimulation.

The profiles of m^{6}A modification of lncRNAs in mammals were identified in recent years, such as mouse, rat and human [32–35]. Dominissini et al. suggested that m^{6}A in exonic regions was preferentially found in longer exons in human and mouse [36]. Further work has identified integrated m^{6}A modification sites of lncRNAs mainly enriched in exonic regions in Arabidopsis and Fruitfly [37, 38]. In our study, the patterns of the m^{6}A modification of lncRNAs were identified in *X. Laevis*. Our results showed that the m^{6}A peak mainly enriched around the intergenic region in *X. Laevis*. Additionally, most of differentially expressed m^{6}A modification sites of lncRNAs in AZ-exposed *X. Laevis* also enriched around the intergenic region. Our results suggested that m^{6}A modification of lncRNAs in amphibian species had unique modification sites.

Differentially m^{6}A modification of lncRNAs were identified by comparing AZ-exposed testes of *X. laevis* to controls. Here, this result revealed a potential role of m^{6}A modification sites of lncRNAs in testes of *X. laevis* induced by environmental agents such as AZ. Interestingly, we found lncRNA "XR_001933134" which was up-regulated in the testis of *X. laevis* exposed to AZ in our previous study was down-regulated in m^6A modification [39], which may that m^6A modification may negatively regulate the expression of lncRNA "XR_001933134". Wu et al. demonstrated that m^6A modification of lncRNAs may increase lncRNA RP11 expression [29]. Ban et al. indicated that dysregulation of m^6A modification might account for aberrant expression of LNCAROD in HNSCC [40]. Consequently, our results suggested that the negative regulatory relationship between m^6A modification of lncRNAs and the expression of lncRNAs in abnormal testis development of *X. laevis* exposed to 100 μg/L AZ. Meanwhile, it has been shown that m^6A modification was highly enriched on lncRNA MALAT1 and can increase its RNA stability in mammal [41]. Therefore, we predicted that m^6A modification of lncRNAs may regulate their expression which involved in abnormal testis development of AZ-exposed *X. laevis*.

Up to now, no data has been reported about the pathway analysis of m^6A-methylated lncRNA-associated target genes in AZ-treated *X. laevis*. Therefore, in the present study, we used KEGG pathway annotation method to analyze the m^6A-methylated lncRNA-associated target genes in the testes of *X. laevis* exposed to100 μg/L AZ. The results of KEGG pathway analysis indicated that 10 pathways were involved in the current sequencing data.

In current study, the top one term was "SNARE interactions in vesicular transport" signaling pathway. SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins could drive vesicle fusion between endosomal compartments in eukaryotic cells [42]. SNARE proteins establish the core membrane fusion machinery of intracellular transport and intercellular communication, which contribute to cell growth, cell expansion, pathogen defense and homeostasis [43, 44]. Additionally, acrosome assembly in spermatogenesis and acrosome reaction in the interaction between sperm and oocyte are unique processes of vesicle synthesis, transportation and fusion, which are the basis of sperm fertilization [45, 46]. The previous study has also shown that SNARE syntaxin was associated with the acrosome inspermatids during sperm development in the testis [47]. Hence, we predicted that m^6A-methylated lncRNAs included in SNARE interactions in vesicular transport signaling pathway may play an important role in the abnormal testis tissues of AZ-exposed *X. laevis*.

It is known that ubiquitin mediated proteolysis possesses many biological processes in controlling cell signaling, regulating cell proliferation, apoptosis, and immune responses [48, 49]. Ubiquitination is also a kind of the versatile cellular regulatory mechanisms and ubiquitin binds to protein playing a crucial role in substrate specificity [50]. In particular, several evidences have demonstrated that *X. laevis* offers the ability to generate soluble proteins, which capable to carry out the biochemical modifications of protein ubiquitylation [51]. Ubiquitylation usually occurs lysine residues and the residues could bond with ubmolecules and then target proteins for destruction [52]. Moreover, ubiquitin is highly expressed in mammalian gametes and embryos at any particular stage of development and ubiquitin ligasesare very active in the testis [53]. However, the study on ubiquitin mediated of gametogenesis in amphibian is sketchy. Our present study indicated that "ubiquitin mediated proteolysis pathway" regulated by m^6A-methylated lncRNAs may involve in the abnormal testis development of *X. laevis* exposed to AZ.

Gonadotropin-releasing hormone (GnRH) was synthesized in hypothalamic neurons and binded to specific G-protein coupled receptors on the gonadotrope cell surface. Itcould regulate the biosynthesis and secretion of gonadotropin such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) which are required for the testis to produce both mature sperm [54, 55]. Recent reports have shown that active immunization against GnRH could inhibit synthesis or secretion of gonadotropins, and thereby induced the termination of gametogenesis, inhibited reproductive behavior, and finally caused infertility of both male and female animals [56]. Moreover, orexin receptors type 1 (OX1R) was G protein-coupled receptors whose receptor expression was found in the pituitary of *X. laevis* [57]. The expression level was regulated by gonadal GnRH [54]. Therefore, m^6A-methylated lncRNAs involved in "GnRH signaling pathway" may play an important role in damaged testis of AZ-exposed *X. laevis.*

Interestingly, "SNARE interactions in vesicular transport", "NOD-like receptor signaling pathway" and "GnRH signaling pathway" were also found in the results of KEGG of differentially m^6A-methylated mRNAs in *X. laevis* exposed to 100 μg/L AZ in our previous study [58]. The results showed that 3 mutual pathways may play important regulatory roles and possibly induce testes damage in AZ-exposed *X. laevis*.

5. Conclusion

We examined the m^6A modification profile of lncRNAs in testis tissues of *X. laevis* withand without treatment with 100 μg/L AZ through m^6A sequencing analysis using the latest Illumina HiSeq sequencer. The results indicated that AZ leaded to alter expression profile in 198 m^6A modification sites of lncRNAs (89 up-regulatedand 109 down-regulated). KEGG pathway analysis indicated that the "SNARE interactions in vesicular transport", "GnRH signaling pathway" and "NOD-like receptor signaling pathway" may be closely associated with abnormal testis development of *X. laevis* due to exposure to AZ. Analysis results showed a negative correlation between m^6A modification of lncRNA and lncRNA abundance, suggesting a regulatory role of m^6A of lncRNAs in amphibious gene expression. Our study provide a fundamental contribution to possible molecular mechanisms underlying the reproductive system toxicity of AZ on male *X. laevis*.

However, in our study the first m^6A transcriptome-wide map of lncRNAs of an amphibian species *X. laevis* presented here provides a starting roadmap for uncovering the role of m^6A modification of lncRNAs that may affect/control amphibian testis development in the future. Meanwhile, our study characterized the differential m^6A methylome of lncRNAs in the testis of *X. laevis* exposed to 100 μg/L AZ relative to the controls, suggesting a strong association between m^6A methylation and the regulation of developmental metabolism in the testis of *X. laevis* exposed to 100 μg/L AZ, thereby providing a fundamental contribution to future studies aimed to gain deeper insights.

Abbreviations

m^6A: N^6-methyladenosine; lncRNA: long non-coding RNA; *X. laevis*: *Xenopus laevis*; AZ: Atrazine; KEGG: Kyoto Encyclopedia of Genes and Genomes; FC: Fold Change; SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor; GnRH: Gonadotropin-releasing hormone; FSH: follicle-stimulating hormone; LH: luteinizing hormone; OX1R: orexin receptors type 1 Authors' contributions

Declarations

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Authors' contributions

Xuejie Qi: Data curation, Methodology, Software, Roles/Writing-original draft. Xiao Geng: Conceptualization, Data curation, Validation, Visualization. Juan Zhang: Validation, Software. Mingming Han: Formal analysis, Software. Qiang Jia: Project administration, Validation. Binpeng Qu: Methodology, Project administration. Qiming Guo: Methodology, Supervision. Wenhui Yin: Formal analysis, Software. Cunxiang Bo: Data curation, Methodology, Validation. Linlin Sai: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing-review&editing. Ling Li: Data curation, Formal analysis, Supervision. Cheng Peng: Writing-review&editing.

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Availability of data and materials

All relevant data generated or analyzed during this study are included in this manuscript.

Consent for publication

All authors have approved the publication.

Competing interests

The authors declare that they have no competing interests.

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Figures

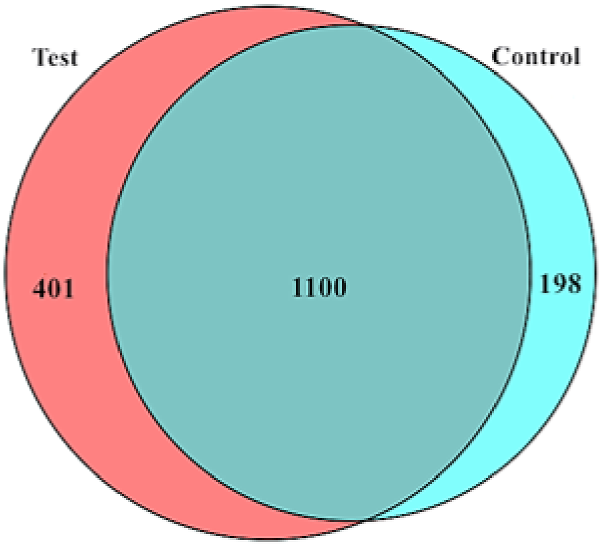

Figure 1

Venn diagram showing the overlap of m6A peaks within lncRNAs in AZ-exposedand control groups. Test represented AZ-exposed samples. Control represented control samples.

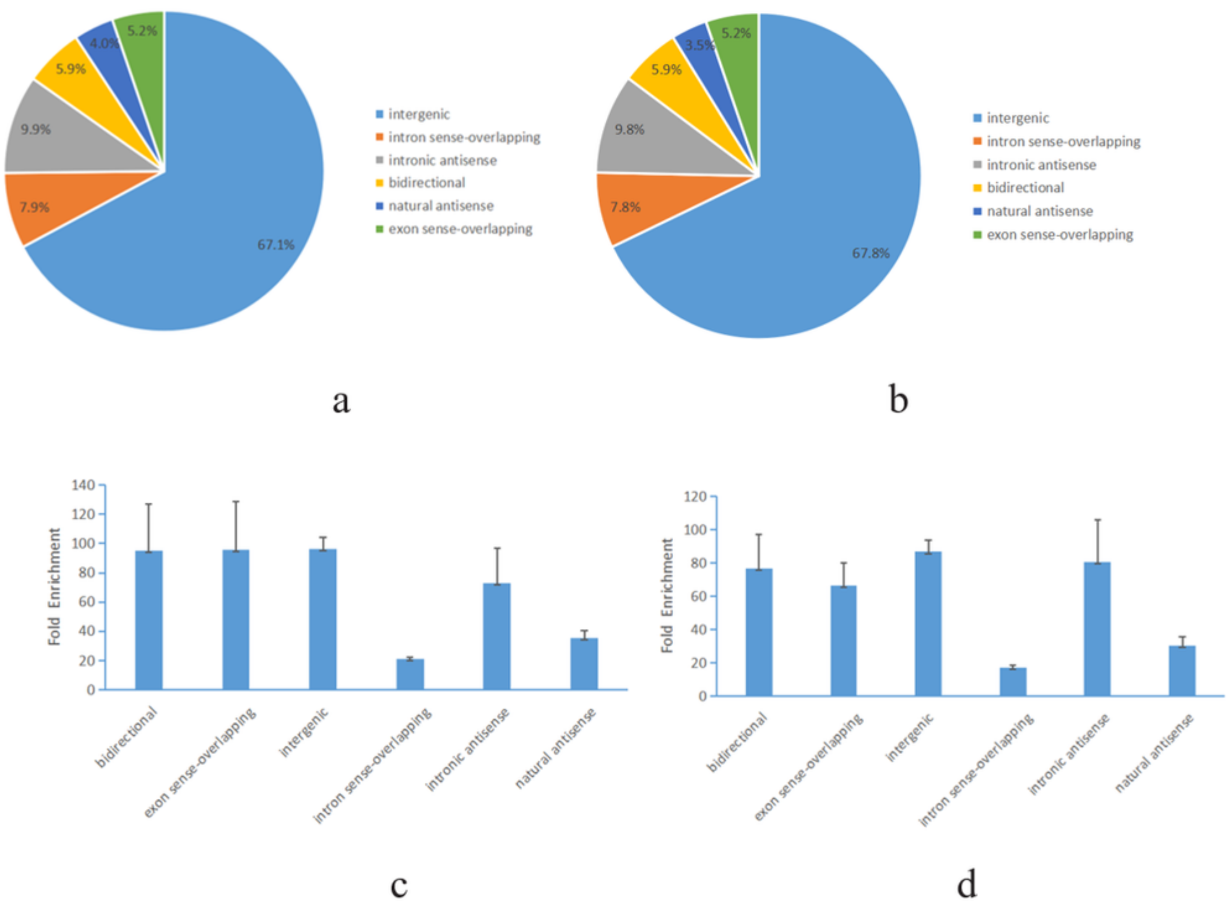

Figure 2

Overview of m6A methylome profiles of lncRNAs in the testes of X. laevis from control and AZ-exposed groups. a: Pie charts showing the percentage of m6A peaks in the genomic organization of lncRNAs in control group. b: Pie charts showing the percentage of m6A peaks in the genomic organization of lncRNAs in AZ-exposed group. c: Distributions of mean fold enrichment of m6A peaks in six segments in control group. d: Distributions of mean fold enrichment of m6A peaks in six segments in AZ-exposed group. Error bars represent the standard error of the mean. The mean fold enrichment in the intergenic segments was the largest both in the control and AZ-exposed group with lower standard error of the mean.

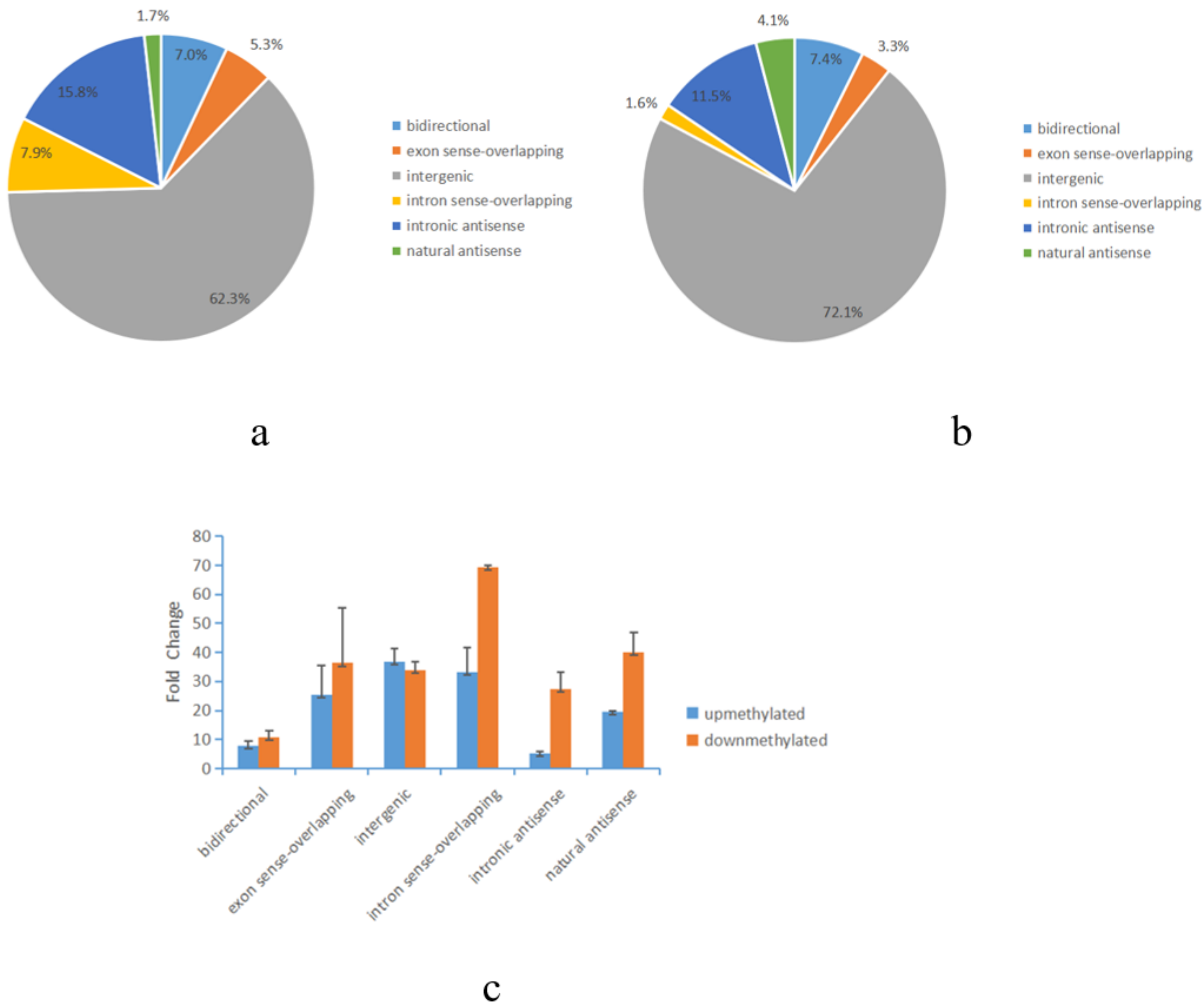

Figure 3

Distribution of differentially methylated m6A sites of lncRNAs. a: Pie charts showing the percentage of up-methylated m6A peaks in six segments. b: Pie charts showing the percentage of down-methylated m6A peaks in six segments. c: Statistics of mean of m6A peaks in six segments in up- and down-methylated sites. Error bars represent the standard error of the mean.

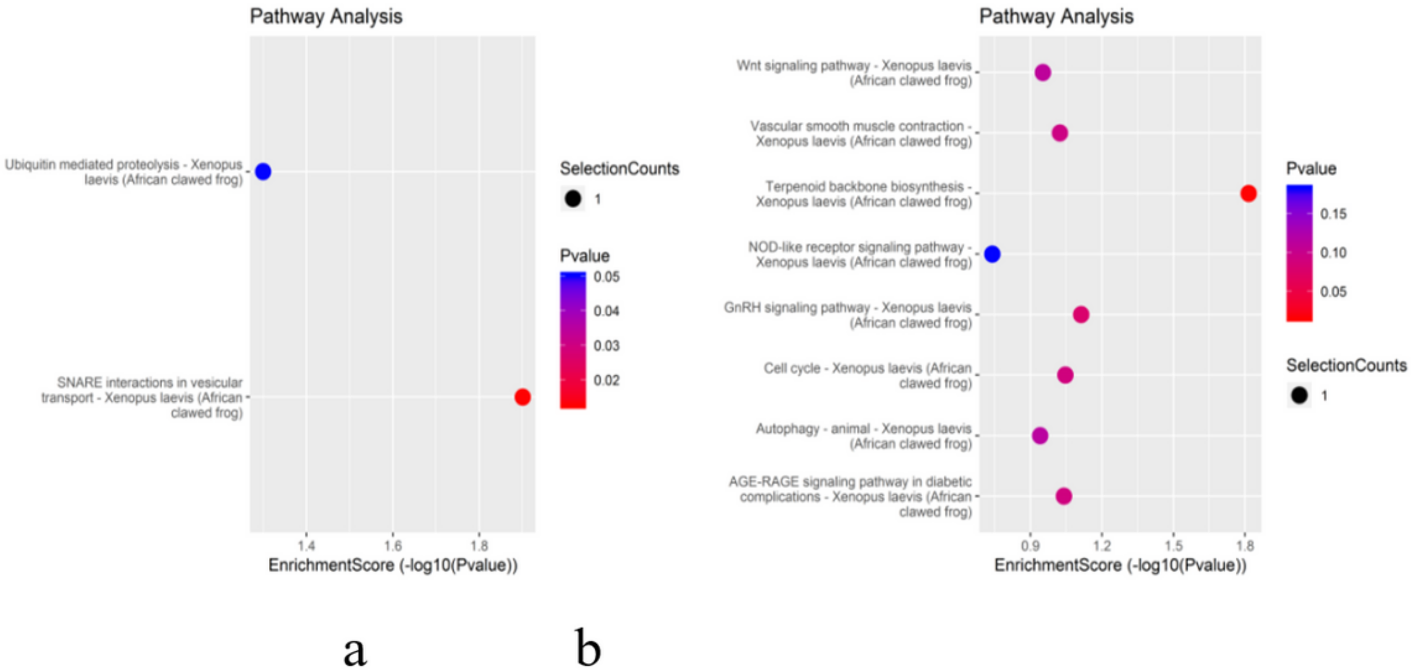

Figure 4

The annotated significant pathways targeted by the enrichment score of the differentially m6A-methylated (up-regulated (a) and down-regulated (b)) lncRNAs-related genes in testis of X. laevis exposed to 100 μg/L AZ. The horizontal axis is the -LogP (logarithm of P-value) for the pathway and the vertical axis is the pathway category.

Supplementary Files

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