Transcriptome-wide assessment of the m6A methylome of intestinal porcine epithelial cells treated with deoxynivalenol

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Research

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Abstract

Background: Deoxynivalenol (DON) is a cytotoxic compound found in various food and feed products. N\textsuperscript{6}-Methyladenosine (m\textsuperscript{6}A) is a highly abundant epitranscriptomic marker that modifies a wide range of mRNA molecules in mammalian cells. However, the role of the m\textsuperscript{6}A methylome in DON-induced damage remains poorly understood.

Results: In this study, we assessed the transcriptome-wide m\textsuperscript{6}A profile of intestinal porcine epithelial cells (IPEC-J2) treated with 1000 ng/mL DON by m\textsuperscript{6}A sequencing and RNA sequencing. Overall, 5406 new m\textsuperscript{6}A peaks appeared with the disappearance of 2615 peaks in DON-treated IPEC-J2 cells. Genes that were uniquely m\textsuperscript{6}A-modified following DON treatment were found to be associated with the tumor necrosis factor (TNF) signaling pathway. On comparing DON-treated and control cells, we identified 733 differentially expressed mRNAs bearing hyper- or hypomethylated m\textsuperscript{6}A peaks. Further experimental data suggested that METTL3-dependent m\textsuperscript{6}A methylation might also play a role in DON-induced inflammatory response, and CSF2 marker is key functional relevance in the context of DON-induced toxicity.

Conclusions: This is the first study to perform a transcriptome-wide assessment of the m\textsuperscript{6}A methylome of IPEC-J2 cells treated with DON. We believe that our findings should be useful for identifying mechanisms whereby m\textsuperscript{6}A modifications influence the outcomes of DON exposure.

Background

RNA modifications can modulate the stability, function, and metabolic processing of modified RNAs. To date, five primary types of RNA methylation have been described: 5-methylcytosine (\textsuperscript{5}mC), N\textsuperscript{7}-methylguanine (\textsuperscript{7}mG at the 5′-cap), N\textsuperscript{6}-methyladenosine (m\textsuperscript{6}A), N\textsuperscript{1}-methyl adenosine (m\textsuperscript{1}A), and pseudouridine (ψ) [1]. Among these, m\textsuperscript{6}A modifications are the most common and functionally important internal RNA modifications within eukaryotic cells, wherein they serve as key regulators of gene expression [2]. In general, the distribution of m\textsuperscript{6}A modifications is most commonly associated with the stop codon (stopC), transcriptional start site (TSS) sequences, coding sequence (CDS), 3′-untranslated region (3′-UTR) sequences, and 5′-UTR sequences in modified RNAs [3]. These m\textsuperscript{6}A modifications can profoundly impact RNA splicing, nuclear export, translation, and degradation [4]. As such, they are closely linked to normal physiological processes, including gametogenesis and cell division, and to pathological conditions, such as cancer and infertility [5]. There is also evidence that m\textsuperscript{6}A modifications are involved in viral replication [6] and the modulation of host–pathogen interactions [7, 8]. While many studies on m\textsuperscript{6}A modifications have been conducted in humans and mice, few have assessed the relationship among these modifications and vital traits in poultry and livestock. Analyses of m\textsuperscript{6}A methylation in pigs have largely focused on the impact of these modifications on fat deposition [9], lipid metabolism [10], stem cell differentiation [11], and liver development [12]. No comprehensive studies have as yet characterized the transcriptome-wide distribution of m\textsuperscript{6}A modifications in the context of most porcine diseases, underscoring a novel direction for the ongoing epigenetic studies on economically important livestock.

Mycotoxins are secondary metabolites derived from fungi that often contaminate human and animal food supplies and induce significant cytotoxic damage following consumption [13]. Deoxynivalenol (DON) is among the most important mycotoxins associated with impaired intestinal integrity; moreover, it evidently exhibits proinflammatory and immunomodulatory properties [14]. In some previous studies involving an intestinal porcine epithelial cell line (IPEC-J2), exposure to DON was found to induce oxidative stress and inflammation, eventually causing accelerated apoptotic cell death and impaired functionality [15–18]. The m\textsuperscript{6}A status of DON-treated IPEC-J2 cells has not been assessed so far. Thus, we herein conducted an m\textsuperscript{6}A-specific RNA immunoprecipitation assay using the methylated RNA immunoprecipitation sequencing (MeRIP-seq) approach [19] to examine the transcriptome-wide m\textsuperscript{6}A modification profile of control and DON-treated cells. Accordingly, we identified distinct m\textsuperscript{6}A modification patterns associated with DON treatment; we further used bioinformatics and qPCR to identify key signaling pathways and genes related to dysregulated m\textsuperscript{6}A methylation in IPEC-J2 cells. We believe that our findings should serve as a foundation for future studies on the roles of m\textsuperscript{6}A modification in the context of DON-induced cell damage.
Cell Culture

IPEC-J2 cells were obtained from the University of Pennsylvania (PA, USA) and cultured in DMEM/F12 containing 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. For viability analyses, the cells were added to 96-well plates (2 × 10^4/well) for 12 h, and then treated with 1000 ng/mL DON (Sigma, Germany) for 24, 48, or 72 h. The Cell Counting Kit-8 (Dojindo, Japan) assay was performed to examine cell viability, as per manufacturer instructions. To assess apoptosis, the cells were treated with DON (1000 ng/mL) for 48 h, followed by staining using the Annexin V-FITC/PI Apoptosis Detection Kit (Solarbio, Beijing, China). For RNA-based experiments, IPEC-J2 cells were added to 6-well plates (1 × 10^5/well) and incubated until 80% confluence; the cells were subsequently treated with DON (1000 ng/mL) for 48 h. They were then collected for downstream analyses.

m^6^A MeRIP-seq and RNA-seq

For RNA-seq, three pairs of control and DON-treated cell samples were harvested. TRIzol (Invitrogen, CA, USA) was used for total RNA extraction, and RNA quality was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). MeRIP-seq and RNA-seq were performed by CloudSeq Biotech Inc. (Shanghai, China) using previously reported methods [19]. Briefly, the GenSeq™ m^6^A-MeRIP Kit (GenSeq Inc., China) was used, as per manufacturer instructions, to conduct m^6^A RNA immunoprecipitation. The immunoprecipitated (IP) and control input samples were then used for RNA-seq library preparation with the NEBNext® Ultra II Directional RNA Library Prep Kit (New England Biolabs, Inc., USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA) was used to confirm the quality of the prepared libraries, and an Illumina HiSeq 4000 sequencer (Illumina, Inc.) was used for library sequencing with 150 bp paired-end reads. The raw high-throughput m^6^A RNA-seq data have been uploaded to the Gene Expression Omnibus database (accession no.: GSE156078).

Sequencing data analysis

After sequencing, the samples were subjected to Q30-based quality control. Following 3′-adaptor-trimming, low-quality reads were removed using Cutadapt v1.9.3 [20]. Clean library reads were aligned to a reference genome (Sscrofa11.1, https://www.ncbi.nlm.nih.gov/genome/?term=pig) with Hisat2 v2.0.4 [21]. MACS2 v.2.1.2 was then used to identify m^6^A peaks in MeRIP-seq data [22]. m^6^A peaks that overlapped with transcript exons were selected for further evaluation. Differences in m^6^A methylation rates between DON-treated and control cells were compared using diffReps [23]. Peaks that were significantly different between the groups met the following criteria: log2[fold change] ≥ 1 and FDR ≤ 0.05. These peaks were then subjected to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses.

Sequencing data analysis

The m^6^A peak distribution patterns associated with different functional regions (TSS, CDS, stopC, 3′-UTR, 5′-UTR) were assessed, and m^6^A peaks along the entire transcript were assessed using the Integrative Genomics Viewer tool [24]. Significant motifs among these peak sequences were detected using MEME suite (http://meme-suite.org/) and DREME (http://memesuite.org/tools/dreme). The HTSeq program (v0.9.1) was used to obtain raw mRNA-seq read data, which were normalized using edgeR. GO (www.geneontology.org) and KEGG pathway (www.genome.jp/kegg) enrichment analyses of differentially expressed mRNAs and differentially methylated genes were then performed.

qPCR analysis

TRIzol (Invitrogen) was used for total RNA isolation from IPEC-J2 cells, as stated earlier, and cDNA was obtained using the PrimeScript RT-PCR Kit (TaKaRa, Beijing, China). qPCR was performed on an ABI7500 instrument (Applied Biosystems, CA, USA) with the following thermocycler settings: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. The expression levels of the following genes were assessed: CSF2, KLF6, CCR7, CCL5, KLF10, PIGR, CD86, DUSP6, TLR3, STAT2, IL-6, IL-12, IL-1β, TNF-α, METTL3, METTL14, WTAP, FTO, ALKBH5. GAPDH was used as the housekeeping gene. The primers used in this study are listed in Supplementary Table S1.

Western blotting analysis

Total proteins were extracted using a NE-PER kit (Nuclear and Cytoplasmic Extraction Reagents, Thermo Fisher Scientific) according to the manufacturer’s protocol. BCA kit (Nanjing Keygen Biotech) was used to normalize protein levels. Proteins were transferred to PVDF membranes, which were then immunoblotted with relevant primary detection antibodies [METTL3 (1:1,000, EPR18810, Abcam, China), WTAP (1:1,000, EPR18744, Abcam, China), ALKBH5 (1:1,000, EPR18958, Abcam, China), GAPDH
followed by secondary detection antibody [horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (Abcam, UK, 1:5,000)].

Statistical analyses

The $2^{-\Delta\Delta Ct}$ method [25] was used to assess relative gene expression. SPSS 18.0 (SPSS, Inc., IL, USA) was used for statistical analyses. Data are expressed as mean ± standard deviation (SD) and were compared using Student's $t$-test. $P<0.05$ indicated statistical significance.

Results

Effects of DON on IPEC-J2 cell viability, inflammation, apoptosis

In this study, DON treatment was associated with a marked reduction in the viability of IPEC-J2 cells (Fig. 1a). In comparison with the control group, cell viability significantly decreased upon DON treatment for 48 h ($P<0.01$, Fig. 1a); moreover, cell confluency of the DON-treated group was lower than that of the control group, and cell morphology showed an obvious alteration (Fig. 1b). We also found that DON-treated cells exhibited significantly higher expression levels of IL-6, IL-12, IL-1β, and TNF-α relative to control cells ($P<0.05$; Fig. 1c). Consistent with these findings, DON-treated cells showed significantly higher rates of apoptotic cell death relative to control cells, as assessed via flow cytometry ($P<0.01$; Fig. 1d-e).

Transcriptome-wide assessment of DON-related m6A methylation profiles

Transcriptome-wide m6A-sequencing (m6A-seq) and RNA-seq of DON-treated and control cells were performed. These two datasets yielded 33.62–43.03 million and 42.17–43.31 million clean reads, respectively, with the RNA-seq dataset also serving as the m6A-seq input library in this study (Table S2). Of these clean reads, 80.22% and 85.58% mapped to the reference genome (Sscrofa11.1), respectively.

Model-based ChIP-seq (MACS) analyses led to the identification of 15,879 and 13,088 m6A peaks in DON-treated and control samples, corresponding to the transcripts of 7265 and 6354 genes, respectively (Fig. 2a-b). Ben diagram analyses revealed that 10,473 m6A peaks and 5649 m6A-modified genes were detectable in both the groups. In comparison with the control group, the DON-treated group exhibited 2615 new peaks and 5406 peaks that were no longer detectable, confirming that there were substantial differences in global m6A modification patterns between the groups (Fig. 2c). In addition, m6A peaks were found to be characterized by the canonical GGACU motif in the groups (Fig. 2d).

We next sought to understand m6A-modified peak distributions on a per-gene basis and determined that approximately 30% of all modified genes (2321/7970) exhibited a unique m6A modification peak. Most modified genes (5077/7970) exhibited between one and three m6A-modified sites (Fig. 2e), while genes unique to the DON-treated group typically exhibited only one m6A-modified site relative to m6A-modified genes that were unique to the control group (Fig. 2f).

In addition, for both the groups, we assessed m6A peak distributions throughout the entire transcriptome to determine the presence of any treatment-specific sites of preferential modification. The m6A peaks were thus separated into the start codon (startC), 5'-UTR, CDS, stopC, and 3'-UTR peaks based on their transcript locations. A Metagene profile of peak density revealed these m6A modifications to be specifically enriched in startC and stopC regions (Fig. 2g). Total and unique m6A peaks were particularly enriched in the vicinity of CDS and start/stopC regions (Fig. 2h–j).

Enrichment of m6A-modified genes in signaling pathways

We next compared the differences in m6A peak abundance between the DON-treated and control groups. Of the 10,473 m6A peaks that were evident in both the groups, we selected 3871 differentially methylated sites for further analyses. Relative to the control group, 2156 significantly hypermethylated m6A peaks (Table S3) and 1715 significantly hypomethylated m6A peaks (Table S4) were detected in the DON-treated group (fold change ≥ 2 and $P<0.05$; Fig. 3a). The Integrative Genomics Viewer tool was used to visualize these methylated sites, which revealed that the GGACU motif surrounded the corresponding m6A peaks in the DON-treated and control groups (Fig. 3b).
To assess the potential biological impact of m\(^6\)A modifications on DON-treated cells, we performed GO and KEGG pathway enrichment analyses of differentially methylated genes. We found that the genes harboring hypermethylated m\(^6\)A sites in DON-treated samples were enriched in many signaling pathways, including the TNF, TGF-\(\beta\), and NF-\(\kappa\)B signaling pathways (Fig. 3c-d, Table S5-S6). Moreover, the transcripts with DON-specific m\(^6\)A peaks were found to be involved in several biological processes, including the TNF signaling pathway (Fig. 3e-f).

**Identification of differentially expressed genes (DEGs) in DON-treated cells**

The aforementioned m\(^6\)A-seq input library was utilized as an RNA-seq dataset for elucidating global mRNA expression patterns in DON-treated and control cells. Scatter plots were constructed and hierarchical clustering analyses were performed using these RNA-seq data (Fig. 4a-b). In total, 1776 DEGs were identified upon comparing DON-treated and control samples; 728 genes were upregulated (Table S7) and 1048 were downregulated (Table S8) (fold change > 2 and \(P < 0.05\)) in DON-treated samples. GO enrichment analyses indicated that upregulated genes in DON-treated cells were involved in processes such as gene expression and RNA metabolism (Fig. 4c), while KEGG pathway enrichment analyses revealed these genes to be significantly enriched in the TNF, NOD-like receptor, and MAPK signaling pathways (Fig. 4d-e, Table S9-S10).

**Conjoint analysis of m\(^6\)A-RIP-seq and RNA-seq data**

Conjoint analyses of our m\(^6\)A-RIP-seq and RNA-seq datasets revealed a strong positive correlation (\(R = 0.93, P < 0.0001\)) between differential m\(^6\)A methylation and mRNA expression (Fig. 5a). Of the 2156 hypermethylated m\(^6\)A sites detected via m\(^6\)A-seq, 364 genes were also upregulated (“hyper-up”) and 10 were downregulated (“hyper-down”) at the mRNA level. In contrast, only three genes exhibiting hypomethylated m\(^6\)A sites were upregulated (“hypo-up”), while 356 were downregulated (“hypo-down”) at the mRNA level (Fig. 5b). Notably, 364/367 (99%) upregulated mRNA transcripts in DON-treated samples were associated with hypermethylated m\(^6\)A peaks. In addition, substantially more “hyper-up” and “hypo-down” genes were detected relative to “hyper-down” and “hypo-up” genes (Fig. 5b). Together, these data suggested that m\(^6\)A hypermethylation is typically associated with the upregulation of gene expression in DON-treated cells.

We then selected 41 candidate genes associated with DON-related pathways (TNF, NF-\(\kappa\)B, NOD-like receptor, and MAPK signaling pathways) that exhibited significant changes in both m\(^6\)A abundance and mRNA transcript expression in DON-treated cells relative to control cells (Table 1, Fig. 5c). Next, m\(^6\)A peak locations in RNA transcripts were assessed by analyzing the overall expression levels of m\(^6\)A-methylated and non-m\(^6\)A-methylated transcripts in DON-treated and control samples (Fig. 5d). We further analyzed the transcripts that had been classified into subgroups (start\(\text{C}\), 5′-UTR, CDS, stop\(\text{C}\), and 3′-UTR) based on their m\(^6\)A modification sites. All m\(^6\)A peaks were analyzed, as were those that were unique to DON-treated and control samples. We found that the transcripts with m\(^6\)A modifications proximal to CDS were typically expressed at significantly lower levels relative to those harboring stop\(\text{C}\) modifications (\(P < 0.01\), Fig. 5e). Different genes exhibit variable numbers of m\(^6\)A-modified sites, as mentioned earlier. We found that a higher number of m\(^6\)A-modified sites per gene was associated with upregulated gene expression (Fig. 5f). While gene expression is controlled by many complex mechanisms, differential m\(^6\)A modifications seem to clearly impact gene expression; further studies are thus warranted.
Table 1
List of 41 genes that exhibited a significant change both at the m⁵A level and in mRNA transcript abundance in DON-treated IPEC-J2 cells as compared with control cells

| Gene    | Pattern | Chr | m⁵A level change | mRNA level change |
|---------|---------|-----|------------------|-------------------|
|         |         |     | Peak region      | Peak start        | Peak end | Fold change | p-value | Strand | Fold change | p-value |
| DUSP3   | Hyper-Up| chr12| stopC            | 19346441          | 19346980 | 2.7687      | 1.84E-11 | +       | 2.4357      | 5.00E-05 |
| TNFAIP3 | Hyper-Up| chr1 | stopC            | 26477694          | 26478020 | 2.8919      | 3.82E-09 | -       | 2.5494      | 5.00E-05 |
| GADD45A | Hyper-Up| chr6 | startC           | 144940801         | 144941475| 2.9203      | 1.24E-08 | -       | 3.1914      | 5.00E-05 |
| CSF2    | Hyper-Up| chr2 | stopC            | 134356721         | 134357200| 85.6210     | 3.87E-09 | +       | 41.0167     | 0.00265   |
| KITLG   | Hyper-Up| chr5 | 3'UTR            | 94105866          | 94106700 | 6.0816      | 1.49E-08 | +       | 5.1153      | 5.00E-05 |
| BIRC3   | Hyper-Up| chr9 | startC           | 33044581          | 33045625 | 2.7765      | 2.27E-10 | -       | 2.3343      | 5.00E-05 |
| PDPK1   | Hyper-Up| chr3 | stopC            | 39434321          | 39434920 | 2.3270      | 8.72E-11 | -       | 2.3343      | 5.00E-05 |
| CRK     | Hyper-Up| chr12| CDS              | 47657981          | 47658440 | 2.8193      | 2.40E-10 | -       | 2.0419      | 5.00E-05 |
| KLF6    | Hyper-Up| chr10| CDS              | 66491750          | 66492174 | 2.6507      | 4.47E-08 | +       | 2.1633      | 5.00E-05 |
| PPM1A   | Hyper-Up| chr1 | 5'UTR            | 189279752         | 189280610| 2.5586      | 7.38E-09 | +       | 2.1644      | 5.00E-05 |
| GADD45B | Hyper-Up| chr2 | stopC            | 76107781          | 76108656 | 9.7123      | 1.07E-06 | -       | 10.2064     | 5.00E-05 |
| VCAM1   | Hyper-Up| chr4 | CDS              | 117507887         | 117508199| 20.0952     | 2.36E-12 | -       | 9.9608      | 5.00E-05 |
| DDIT3   | Hyper-Up| chr5 | stopC            | 22785381          | 22786030 | 3.7200      | 2.98E-09 | -       | 3.1181      | 0.0009    |
| RAP1A   | Hyper-Up| chr4 | startC           | 108676581         | 108677320| 3.5678      | 2.95E-11 | -       | 2.3491      | 5.00E-05 |
| RELB    | Hyper-Up| chr6 | CDS              | 51479541          | 51480160 | 2.8405      | 1.06E-10 | +       | 2.5145      | 5.00E-05 |
| KLF10   | Hyper-Up| chr4 | startC           | 34429922          | 34430291 | 4.4327      | 1.82E-09 | +       | 6.7444      | 5.00E-05 |
| NFKBIE  | Hyper-Up| chr7 | stopC            | 39255074          | 39255380 | 5.0043      | 1.59E-11 | -       | 5.0155      | 5.00E-05 |
| CCR7    | Hyper-Up| chr12| stopC            | 21868261          | 21869000 | 17.6960     | 4.03E-12 | +       | 11.8906     | 5.00E-05 |
| CLCF1   | Hyper-Up| chr2 | stopC            | 5151801           | 5152580  | 5.7717      | 1.91E-11 | +       | 4.1889      | 5.00E-05 |
| DUSP10  | Hyper-Up| chr10| stopC            | 10867141          | 10867691 | 8.7014      | 2.65E-09 | -       | 4.6017      | 5.00E-05 |
| CCL5    | Hyper-Up| chr12| stopC            | 39658801          | 39659121 | 5.5442      | 1.10E-09 | +       | 3.9232      | 5.00E-05 |
To comprehensively elucidate the mechanisms regulating DON-induced cellular damage, we constructed a protein–protein interaction (PPI) network of m^6^A-modified genes using the STRING database. The resultant network contained 41 nodes and 152 relationship pairs (Supplementary Table S11), and it was visualized using Cytoscape [26]. CSF2 was the most central protein in

| Gene   | Pattern | Chr | m^6^A level change | mRNA level change |
|--------|---------|-----|--------------------|-------------------|
|        |         |     | Peak region       | Peak start | Peak end   | Fold change | p-value | Strand | Fold change | p-value |
| ICAM1  | Hyper-up| chr2 | stopC              | 69106756   | 69107600   | 2.9456      | 2.86E-09 | +       | 2.0062      | 5.00E-05 |
| PRKCA  | Hyper-up| chr12 | 3'UTR              | 13262181   | 13263100   | 3.0166      | 5.13E-11 | +       | 2.5048      | 5.00E-05 |
| TGFB1  | Hypo-down| chr6 | 3'UTR              | 49333561   | 49334280   | 11.6198     | 1.01E-08 | -       | -2.4351     | 5.00E-05 |
| BCL3   | Hypo-down| chr6 | stopC              | 51265021   | 51265354   | 2.0353      | 2.17E-08 | +       | -2.0036     | 5.00E-05 |
| TEC    | Hypo-down| chr8 | CDS                | 38051181   | 38051723   | 2.5395      | 1.38E-11 | -       | -4.3544     | 5.00E-05 |
| STAT2  | Hypo-down| chr5 | stopC              | 21733029   | 21733480   | 5.1305      | 2.77E-09 | -       | -4.5569     | 5.00E-05 |
| PIGR   | Hypo-down| chr9 | 5'UTR              | 67553941   | 67554580   | 30.4601     | 1.66E-10 | -       | -23.3819    | 5.00E-05 |
| RFX5   | Hypo-down| chr4 | stopC              | 97904201   | 97904663   | 2.0043      | 4.20E-09 | +       | -2.3931     | 5.00E-05 |
| DAXX   | Hypo-down| chr7 | CDS                | 29680121   | 29680480   | 3.6822      | 4.98E-13 | -       | -2.2014     | 5.00E-05 |
| DUSP6  | Hypo-down| chr5 | stopC              | 93307561   | 93308040   | 58.5000     | 2.30E-06 | +       | -3.3500     | 5.00E-05 |
| TRIM25 | Hypo-down| chr12 | stopC            | 33187481   | 33187871   | 2.2950      | 1.12E-08 | -       | -3.2655     | 5.00E-05 |
| PRLR   | Hypo-down| chr16 | stopC              | 20637556   | 20638360   | 6.0164      | 1.41E-10 | -       | -6.8650     | 0.0005     |
| FOS    | Hypo-down| chr7 | stopC              | 98451361   | 98452400   | 7.4379      | 2.59E-09 | +       | -8.2442     | 5.00E-05 |
| CD86   | Hypo-down| chr13 | 3'UTR             | 138427841  | 138429760  | 2.5790      | 8.54E-12 | -       | -2.5451     | 5.00E-05 |
| IFNAR1 | Hypo-down| chr13 | stopC              | 196949349  | 196950532  | 2.0459      | 3.90E-10 | +       | -2.3086     | 5.00E-05 |
| IL4R   | Hypo-down| chr3 | stopC              | 19528281   | 19528874   | 2.4307      | 1.23E-09 | -       | -2.1698     | 5.00E-05 |
| ELK4   | Hypo-down| chr9 | CDS                | 66315867   | 66316374   | 2.6963      | 1.43E-11 | -       | -2.3659     | 5.00E-05 |
| DDX58  | Hypo-down| chr10 | CDS               | 33931532   | 33932040   | 52.9034     | 1.21E-08 | +       | -51.1088    | 5.00E-05 |
| TNFRSF21 | Hypo-down| chr7 | CDS                | 42066848   | 42067440   | 2.9831      | 1.26E-09 | -       | -3.5779     | 5.00E-05 |
| TLR3   | Hypo-down| chr15 | startC            | 46969005   | 46969457   | 2.1686      | 2.51E-10 | +       | -3.1485     | 5.00E-05 |
this network (Fig. 6), and CSF2 was a DEM and DMG in RNA-seq and m^6^A-seq analyses, respectively, exhibiting a significant difference in its expression level in DON-treated cells (Table 1). To further validate our sequencing results, we performed qPCR to assess the expression level of several hyper- and hypomethylated genes (CSF2, KLF6, CCR7, CCL5, KLF10, PIGR, CD86, DUSP6, TLR3, and STAT2), and we found the expression levels to be similar to those detected using RNA-seq (Fig. 7). Notably, the expression of CSF2 was markedly upregulated in DON-treated cells ($P<0.01$).

### Expression change of m^6^A methyl marks in DON-treated IPEC-J2 cells

To gain insights into how the m^6^A methyl marks regulated the DON-induced damage, we analyzed the mRNA and protein expression profiles of three m^6^A writers (METTL3, METTL14, and WTAP) and two m^6^A erasers (ALKBH5 and FTO) in DON-treated IPEC-J2 cells. qPCR detection showed (Fig. 8a), the expression of ALKBH5 was significantly upregulated in DON-treated cells ($P<0.05$), while the expression of METTL3 and WTAP were significantly downregulated in DON-treated cells ($P<0.05$). Further validation of western blot showed, the protein expression of METTL3 was markedly downregulated in DON-treated cells, but the change of ALKBH5 and WTAP expression were not obvious.

### Discussion

The m^6^A modification is the most common internal epitranscriptomic marker within mammalian mRNAs. The MeRIP-seq approach has been widely used to characterize m^6^A methylation profiles in a range of species. Dominissini et al. (2012) reported the successful construction of human and murine m^6^A methylation profiles and investigated transcriptome-wide m^6^A methylation patterns in these species [3]. Some other studies were able to characterize m^6^A methylation profiles in *Arabidopsis thaliana* [27], *Oryza sativa* [28], and *Saccharomyces cerevisiae* [29]. However, only few studies have assessed porcine m^6^A methylation profiles to date. In this study, we characterized transcriptome-wide m^6^A modification patterns in IPEC-J2 cells using the MeRIP-seq approach. We found that the m^6^A modification sites were primarily enriched in CDS and start/stopC regions, with relatively few sites in 3′-UTR and 5′-UTR, and this result was consistent with that of an earlier study involving porcine liver tissues [12]. Similarly, m^6^A peaks have been reported to be primarily concentrated around TSS and stopC regions in human cells. In mice, these peaks were more abundant throughout the internal region of modified mRNAs, with the following distribution frequencies: 37% CDS, 30% stopC, 20% 3′-UTR, and 13% TSS [30]. Overall, these findings demonstrate that at a high level, m^6^A modification profiles in mammalian transcriptomes are similar across species. In contrast, in case of *A. thaliana*, the m^6^A modification sites were primarily enriched in 3′-UTR and stop/startC regions, and distinct motifs in m^6^A-modified startC regions were noted in *A. thaliana* relative to those observed in mammals [27]. Further, in case of *O. sativa*, the majority of the m^6^A modification sites were found to be distributed in intergenic regions, with 70% of such sites being found in CDS and 3′-UTR, and 20% in intronic regions and 5′-UTR [28]. These findings suggest that m^6^A methylation patterns are species specific. In general, most m^6^A methylation sites in mammals and plants were significantly enriched in stopC regions and 3′-UTR, suggesting that these modification patterns are consistent with the typical topological m^6^A patterning of mature mRNAs [27, 28, 30]. The m^6^A enrichment in these terminal regions seems to be associated with RNA stability and signal transduction, and may further regulate the recruitment of specific factors necessary for mRNA transport or translation (Wang et al., 2014; Li et al., 2014). The RRACH consensus sequence is characteristic of the canonical conserved domains at which m^6^A methylation occurs [31–33], and this was confirmed via our m^6^A high-throughput sequencing results [34–36]. No such RRACH sequence, however, was detected in *O. sativa* mRNAs [28]. Further studies should be conducted to explore differences in species-specific m^6^A consensus sequences.

At a functional level, m^6^A modifications modulate diverse processes, including metabolism, differentiation, and disease pathology [11, 37]. m^6^A methylation patterns vary among tissues and in response to specific environmental stimuli. Dominissini et al. (2012) observed variances in these patterns in human hepatoma (HepG2) cells following treatment with a range of factors, including heat shock, interferons, and ultraviolet radiation [3]. Environment- and stimuli-specific m^6^A methylation patterns have also been studied using murine embryonic stem cells, embryonic fibroblasts, and preadipocytes [38, 39]. In this study, on comparing differences in m^6^A methylation patterns between DON-treated and control IPEC-J2 cells, genes that were both upregulated and more frequently m^6^A modified were found to be associated with various immunological signaling pathways, such as the TNF and MAPK pathways. Wang et al. (2018) revealed that DON treatment induces toxicity and apoptotic cell death...
in piglet hippocampal nerve cells via the MAPK signaling pathway [40]. Further, Zhang et al. (2020) reported that in IPEC-J2 cells, DON treatment induced the production of proinflammatory factors by activating p38 and ERK1/2 [18], while Wang et al. (2019) showed that treating IPEC-J2 cells with DON caused inflammatory injury via activation of the NF-κB signaling pathway [41]. With regard to the m6A-modified genes identified in this study, our RNA-seq and qPCR analyses indicated that CSF2 was differentially expressed the most significantly in DON-treated cells, and CSF2 was the most central protein in the constructed PPI network (Fig. 6). CSF2 is involved in the TNF signaling pathway and thus regulates human disease pathology [42–44], which suggests that CSF2 is of key functional relevance in the context of DON-induced toxicity. Few studies to date, however, have analyzed porcine CSF2, indicating it is pivotal to systematically assess the association between CSF2 expression and DON treatment via Cas9-mediated knockout and overexpression experiments.

Generally, m6A modification is deposited to RNAs by the m6A methyltransferase complex (METTL3, METTL14, WTAP) [45], and reversed by demethylases (FTO, ALKBH5) [46, 47]. Deoxynivalenol (DON), as mycotoxin, does not encode any known methyltransferase or demethylases, host methyltransferases and demethylases appear to play a critical role in m6A methylation. To explore the role of m6A methyltransferase and demethylases in DON-induced damage, we evaluated the expression patterns of METTL3, METTL14, WTAP, FTO and ALKBH5 in DON-treated IPEC-J2 cells. Among these, METTL3 showed the most obvious difference between normal cells and DON-treated cells. METTL3, as a key enzyme of m6A methylation modification, can attenuate LPS induced inflammatory response in macrophages through NF-κB signaling pathway [48]. METTL3 knockdown inhibits osteoblast differentiation and activates the inflammatory response by regulating MAPK signaling in LPS-induced inflammation [49]. Moreover, the m6A methylome analysis showed that the upregulated m6A-modified genes in DON-treated IPEC-J2 cells mainly participate in the TNF and MAPK signaling pathways. Accordingly, the similar expression pattern of the m6A methyltransferase METTL3 and m6A-modified genes suggested that METTL3 might play a functional role in DON-induced inflammatory response, but further verification will be required in the future.

Conclusions

To summarize, this study characterized m6A modification patterns in IPEC-J2 cells, and provide evidence that CSF2 seems to play key roles in modulating inflammatory responses in the presence of DON. Further studies should be conducted by utilizing RNA methylases (METTL3) to comprehensively analyze the role of m6A methylation in the regulation of responses of porcine cells to DON treatment.

Abbreviations

DON: Deoxynivalenol; m6A: N6-Methyladenosine; IPEC-J2: intestinal porcine epithelial cells; TNF: tumor necrosis factor; TSS: transcriptional start site; CDS: coding sequence; UTR: untranslated region; MeRIP-seq: methylated RNA immunoprecipitation sequencing; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: differentially expressed genes

Declarations

Ethics approval and consent to participate

The Institutional Animal Care and Use Committee (IACUC) of the Yangzhou University Animal Experiments Ethics Committee approved the animal study proposal, with the permit number: SYXXK(Su) IACUC 2012-0029.

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**Authors’ contributions**

WBB conceived and designed the experiments. ZCW, CX and FHW performed the experiments, including cell treatment, qPCR assay, and wrote the manuscript. ZCW performed the statistical analyses. SG and SLW helped to revise the manuscript. All authors read and approved the final version of this manuscript.

**Availability of data and materials**

The raw high-throughput m^6^A and RNA-seq data have been uploaded to the Gene Expression Omnibus (GEO) database (GEO accession No.: GSE156078). The other datasets analysed in the current study are available from the corresponding author upon reasonable request.

**Consent for publication**

Not applicable.

**Competing interests**

All authors declare that they have no competing interests.

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Figures
Figure 1

Effects of DON on IPEC-J2 cell viability, inflammation, and apoptosis. (a) IPEC-J2 cells were treated with DON for 24, 48, or 72 h, and the effect of DON on cell viability was assessed using the Cell Counting Kit-8 assay. (b) Morphology of IPEC-J2 cells. The control group was treated with an equal volume of solvent; the DON-treated group was treated with 1000 ng/mL DON. (c) Detection of inflammation-associated gene expression in DON-treated cells for 48 h. (d-e) Apoptotic cell death was assessed in DON-treated cells for 48 h. The rate of apoptosis was analyzed using flow cytometry. Data are expressed as mean ± SD from triplicate experiments. *P < 0.05, **P < 0.01.
m6A-RIP-seq-mediated assessment of the m6A methylome of DON-treated IPEC-J2 cells. (a) Numbers of group-specific and common m6A peaks. (b) A Venn diagram was constructed to highlight m6A peaks present in DON-treated and control samples. (c) m6A-modified genes identified via m6A-seq. (d) A sequence diagram highlighting the conserved RRACH motif in m6A-containing peak regions. (e) Numbers of m6A-modified peaks per gene. (f) m6A-modified peak distributions on a per-gene basis in genes that were uniquely modified in DON-treated and control samples. (g) m6A peak density along a metagene. (h–j) Pie charts highlighting m6A peak distribution in different gene regions in DON-treated and control samples. DON, DON-treated IPEC-J2 cells; Control, untreated IPEC-J2 cells.
Figure 3

Global changes in m6A modification patterns in DON-treated IPEC-J2 cells relative to control cells. (a) Volcano plots highlighting distinct m6A peaks (fold change ≥ 2 and P < 0.05). (b) Visualization of TLR3 and STAT2 mRNA m6A modifications in DON-treated and control samples. (c-d) Pathway analyses of transcripts with significantly up- or downregulated m6A modification rates in DON-treated samples relative to control samples. (e) KEGG pathway analysis of transcripts with DON-specific m6A peaks. (f) GO analysis of biological processes associated with transcripts exhibiting DON-specific m6A peaks.
Figure 4

Identification of DEGs between DON-treated and control IPEC-J2 cells via RNA-seq. (a) Scatter plots showing DEGs in DON-treated and control cells (fold change ≥ 2 and P < 0.05). (b) Hierarchical clustering analysis of RNA-seq data from DON-treated and control cells. (c) GO analysis of biological processes enriched for genes upregulated in DON-treated samples. (d-e) KEGG pathway analysis of genes that were up- and downregulated in DON-treated samples relative to control samples.
Figure 5
Conjoint analysis of m6A-RIP-seq and RNA-seq data. (a) Dot plots showing a positive correlation between overall m6A methylation and mRNA expression levels. (b) A four-quadrant graph highlighting DEGs containing differentially methylated m6A peaks. (c) Hierarchical clustering analysis of DEGs containing differentially methylated m6A peaks. (d) Overall expression levels of m6A-methylated and non-m6A-methylated transcripts in different groups. (e) m6A peak locations in RNA transcripts and relative gene expression levels in all m6A peaks, as well as in peaks unique to control and DON-treated cells. **P < 0.01 compared with the “stop codon” region. (f) Relative mRNA expression levels for transcripts containing different numbers of m6A peaks. *P < 0.05 relative to the first column (m6A peak = 1).
Figure 6

PPI network constructed with dysregulated m6A-modified genes. Cytoscape was used to visualize a network incorporating m6A-modified genes/proteins based on the conjoint analysis of m6A-RIP-seq and RNA-seq data.
Figure 7

qPCR validation of dysregulated m6A-modified genes. Relative mRNA expression levels of 10 representative genes were assessed using qPCR. Bars correspond to mean values of four technical replicates. **P < 0.01.
Figure 8

Expression detection of RNA methyltransferases and demethylases. (a) mRNA expression levels of RNA methyltransferases (METTL3, METTL14, WTAP) and demethylases (ALKBH5, FTO) were detected by qPCR. (b) western blot assay. *P < 0.05, **P < 0.01.

Supplementary Files

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