High-throughput sequencing reveals the epitranscriptome-wide landscape of m6A-modified circRNA in oral squamous cell carcinoma

Wei Zhao

Tianjin Medical University

Jingwen Liu

Tianjin Medical University

Jie Wu

Tianjin Medical University

Xiaozhou Ma

Tianjin Medical University

Xi Wang

Tianjin Medical University

Leyu Zhang

Tianjin Medical University

Jianming Yang

Tianjin Medical University

Xin Hu

Tianjin Medical University

Yameng Cui

Tianjin Medical University Cancer Institute and Hospital

Jiayin Deng (✉ jdeng@tmu.edu.cn)

Tianjin Medical University

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Abstract

Emerging transcriptome high throughput screenings reveal the landscape and functions of RNAs, such as circular RNAs (circRNAs), in human cancer. In addition, the post-transcriptional internal modifications, especially N⁶-methyladenosine (m⁶A), greatly enrich the variety of RNAs metabolism. However, the m⁶A modification on circRNAs has yet to be addressed. Here, we report an epitranscriptome-wide mapping of m⁶A-modified circRNAs (m⁶A-circRNA) in oral squamous cell carcinoma (OSCC). Utilizing the data of MeRIP-seq and m⁶A-circRNAs epitranscriptomic microarray analysis, we found that m⁶A-circRNAs exhibited their particular modification style in OSCC, which was independent of m⁶A-mRNA. Besides, m⁶A modification on circRNAs frequently occurred on the long exons in the front part of the coding sequence (CDS), which was distinct from m⁶A-mRNA that in 3'-UTR or stop codon. In conclusion, our work preliminarily demonstrates the traits of m⁶A-circRNAs and probed into the association of m⁶A-circRNAs and m⁶A-mRNA, which may bring enlighten for the roles of m⁶A-circRNAs in OSCC.

Highlights

1. m⁶A-circRNAs exhibited their particular modification style in OSCC, which was independent of m⁶A-mRNA.
2. m⁶A on circRNAs frequently occurred on the long exons in the front part of CDS, which was distinct from m⁶A-mRNA that in 3'-UTR or stop codon.

Introduction

Oral squamous cell carcinoma (OSCC) acts as the most common cancers of oral region, accounting for 90% of oral malignant tumor(1). In addition to local hyperplasia, tissues erosion and dysfunction, OSCC frequently results in lymphatic metastasis to the neck area. Although comprehensive therapeutic schedules have made remarkable progress, including surgical excision, chemotherapy and radiotherapy, the survival and prognosis of OSCC sufferer remain poor(2). The latest research shows that the tumorigenesis of OSCC is a complicated process involved in diverse alterations of genetic and epigenetic. Therefore, unremitting exploration targeting the initiation and oncogenesis of OSCC could provide valuable directions for precise treatment.

Circular RNAs (circRNAs) are groups of covalently closed loop transcripts in noncoding transcriptome(3). CircRNAs are characterized by a continuous loop generated from the back-splicing of linear RNA, which is different from linear RNA. Benefited from covalently-bonded RNA molecule, circRNAs could resist the digestion of RNA enzyme and stably exist in the cellular microenvironment, which is responsible for their high abundance in different species. In OSCC, the functions of circRNAs have been preliminarily uncovered. Certain circRNAs regulate the OSCC cellular glycolysis metabolism, e.g. circ_0000140(4) and hsa_circRNA_100290(5). Certain circRNAs regulate the epithelial-mesenchymal transition (EMT) of OSCC, e.g. hsa_circ_0009128(6) and circEPSTI1(7). Moreover, some circRNAs serve as potential diagnostic predictor or biomarkers for OSCC, e.g. hsa_circ_0008309(8) and hsa_circ_0003829(9). Therefore, the evidence illustrates the critical roles participating in OSCC initiation and progression.

N⁶-methyladenosine (m⁶A) is one of the most abundant internal modifications in eukaryotic messenger RNA (mRNA) that exerts essential roles in mRNA fate, including mRNA stability, splicing and translation(10). RNA m⁶A modification is a well-known chemical modification occurred in the sixth nitrogen. The specific transcriptome modifications have been proved to influence the cellular pathophysiology, thereby regulating tumorigenesis. For example, RNA modification enzyme methyltransferase-like 3 (METTL3) is upregulated in OSCC cohorts and the high expression of METTL3 is associated with poor prognosis. Functionally, METTL3 promotes cell proliferation, migration, invasion and self-renewal through promoting BMI1 translation under the cooperation with IGF2BP1 in OSCC(11). Besides, m⁶A demethylase fat mass and obesity-associated protein (FTO) knockdown induces the downregulation of m⁶A-contained eIF4G1, which is captured by YTHDF2, and enhances the autophagic flux, thus inhibiting OSCC tumorigenesis. With the release of new research work about m⁶A, there are reasons to believe that the mechanism by which m⁶A regulates OSCC could be identified.

Given that m⁶A modification regulates the fate of RNA, while circRNAs are a group of covalent closed-loop RNA, there could be a pivotal connection between m⁶A and circRNAs(12). In fact, current researches do indeed prove this hypothesis in human cancers. However, the role of m⁶A on the function of circRNAs has yet to be addressed. Here, our team utilized the data of MeRIP-seq and m⁶A-circRNAs epitranscriptomic microarray analysis to investigate the epitranscriptome-wide mapping of m⁶A-modified circRNA in OSCC. Our work focused on the traits of m⁶A-circRNAs and probed into the association of m⁶A-circRNAs and m⁶A-mRNA, which may bring enlighten for the roles of m⁶A-circRNAs in OSCC.

Materials And Methods

Human m⁶A-circRNA Epitranscriptomic microarray analysis

The m⁶A-circRNA Epitranscriptomic microarray analysis was performed by Aksomics Inc (KangChen Bio-tech, Shanghai, China). Total RNA from cell samples (SCC25 cells, HNOK cells) was quantified using the NanoDrop ND-1000. The microarray hybridization was performed based on the Arraystar's standard protocol. In brief, total RNAs were immunoprecipitated with anti-m⁶A rabbit polyclonal antibody (Synaptic Systems, 202003). The unmodified RNAs were recovered from the supernatant as 'Sup'. The m⁶A-modified RNAs were eluted from the immunoprecipitated magnetic beads as the 'IP'. Then, 'Sup' and 'IP' RNAs were administrated with RNase R (Epicentre, Inc.), and subsequently labeled with Cy3 and Cy5 respectively as cRNAs in separate reactions by Arraystar Super RNA Labeling Kit (Arraystar, AL-SE-005). The cRNAs were combined and hybridized on Arraystar Human circRNA Epitranscriptomic Microarray (8x15K, Arraystar). Slides were washed and the arrays were scanned by an Agilent Scanner G2505C in two-color channels.

Array images were analyzed by Agilent Feature Extraction software (version 11.0.1.1). Raw intensities of Sup (Supernatant, Cy3-labelled) and IP (Immunoprecipitated, Cy5-labelled) were normalized with average of log2-scaled Spike-in RNA intensities. After Spike-in normalization, the probe signals having Present (P) or Marginal (M) QC flags in at least 3 out of 6 samples were retained for further 'm⁶A methylation level' and 'm⁶A quantity' analyses.

'm⁶A methylation level' was calculated for the percentage of modification based on the IP (Cy5-labelled) and Sup (Cy3-labelled) normalized intensities. 'm⁶A quantity' was calculated for the m⁶A methylation amount based on the IP (Cy5-labelled) normalized intensities. Differentially m⁶A-methylated circRNAs between two comparison groups were identified by filtering with the fold change and statistical significance (p-value) thresholds. Hierarchical Clustering was performed to show the distinguishable m⁶A-methylation pattern among samples.

Workflow of m⁶A-circRNA epitranscriptomic microarray analysis

The workflow of human m⁶A-circRNA microarray analysis in OSCC cells was presented in the graphical representation, including RNA extraction, quality control (QC), library construction and data analysis. There were 3 pairs of samples for the m⁶A-circRNA epitranscriptomic microarray analysis, including OSCC cells (3 independent samples, SCC25 cells) and normal cells (3 independent samples, HOK cells). After the m⁶A immunoprecipitation, the m⁶A-modified RNAs were eluted from the immunoprecipitated magnetic beads were marked as 'IP' group (immunoprecipitated RNAs), and the unmodified RNAs were recovered from the supernatant and marked as 'Sup' group (supernatant unmodified RNAs) (**supplementary Figure S1A**). Regarding the raw data, the statistic analysis was performed from two aspects, including m⁶A methylation level and m⁶A quantity (**supplementary Figure S1B**). The data from m⁶A-circRNA epitranscriptomic microarray analysis reveals the landscape of m⁶A-modified circRNAs in OSCC.

m⁶A-circRNA data analysis

The 'm⁶A methylation level' for a transcript was calculated as the percentage of modified RNA (modified %) in all RNAs based on the IP (Cy5-labelled) and Sup (Cy3-labelled) normalized intensities:

$$\begin{aligned}\text{modified\%} &= \frac{\text{modified RNA}}{\text{Total RNA}} = \frac{\text{IP}}{\text{IP} + \text{Sup}} \\ &= \frac{IP_{\text{Cy5 normalized intensity}}}{IP_{\text{Cy5 normalized intensity}} + Sup_{\text{Cy3 normalized intensity}}}\end{aligned}$$

The "m⁶A quantity" was calculated for the m⁶A methylation amount of each transcript based on the IP (Cy5-labelled) normalized intensities.

$$\text{Sample m6A quantity} = \text{Sample } IP_{\text{Cy5 normalized intensity}}$$

Cells and culture

OSCC cells (SCC25 cells) were provided by ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in DMEM Medium supplemented with fetal bovine serum (FBS, 10%), 100 U/ml penicillin, 100 µg/ml streptomycin. Normal cells (Human Oral Keratinocytes, HOK, Catalog No. 2610, ScienCell) were provided by ScienCell (San Diego, California, USA). HOK cells were cultured in Oral Keratinocyte

Medium (OKM, Cat. No. 2611, ScienCell) recommended by ScienCell in vitro. Cells were incubated in a 37 °C humidified incubator with 5% CO₂.

MeRIP-Seq

The total RNA was extracted from cell samples, and then divided into two groups, including Input control sample and immunoprecipitation (IP) sample. The RNAs were firstly spliced into ~100nt fragments. IP samples provide unbiased measurements of methylated RNA fragments with specific m⁶A antibodies. Meanwhile, the Input control sample reflected the abundance of basic RNA enrichment. The reference genome was hg38 gencode. Through library construction, high-throughput sequencing and bioinformatics analysis, mapping of whole transcriptome m⁶A location was generated by Jiayin Biotechnology Ltd. (Shanghai, China).

Statistical analysis

Differentially m⁶A-methylated RNAs between two comparison groups were identified by filtering with the fold change (>1.5 fold) and statistical significance (p-value<0.05) thresholds. Data were shown as means ± standard deviation (SD).

Results

m⁶A-circRNA epitranscriptomic microarray analysis revealed the m⁶A-circRNAs profile in OSCC

m⁶A-circRNA epitranscriptomic microarray analysis was performed according to the workflow using SCC25 cells and HOK cells. As regarding to the microarray raw data, the differentially m⁶A-methylated circRNAs were expressed as 'm⁶A methylation level' and 'm⁶A quantity', which was different from conventional circRNA microarray analysis. Thus, in our m⁶A-circRNA epitranscriptomic microarray analysis, the differentially expressed m⁶A-circRNAs were presented by two aspects, including 'm⁶A-circRNA Methylation Level' and 'm⁶A-circRNA Quantity'. The screening threshold was set to p-value <0.05 and fold change >1.5 fold. Based on this threshold, there were 104 up-regulated m⁶A-circRNA and 145 down-regulated m⁶A-circRNA on the basis of 'm⁶A-circRNA Methylation Level', meanwhile, there were 2586 up-regulated m⁶A-circRNA and 472 down-regulated m⁶A-circRNA on the basis of 'm⁶A-circRNA Quantity'. The raw data of 'm⁶A-circRNA Methylation Level' and 'm⁶A-circRNA Quantity' were presented by Heat Map (**Figure 1A, 1B**), Volcano Plot (**Figure 1C, 1D**), and Scatter Plot t (**Figure 1E, 1F**). Taken together, m⁶A-circRNA epitranscriptomic microarray analysis revealed the m⁶A-circRNAs profile in OSCC.

The significantly expressed m⁶A-circRNAs

Given that m⁶A-circRNA epitranscriptomic microarray analysis discovered hundreds or thousands of circRNAs with different 'm⁶A methylation level' or 'm⁶A quantity', further analysis focused on their intersection to determine the potential m⁶A-methylated circRNAs with significant expression. Circos plot showed the locations of m⁶A-circRNAs on human chromosomes, including 'm⁶A methylation level' or 'm⁶A quantity' (**Figure 2A, 2B**). In the schematic, the outermost layer was chromosome map of human genome. The inner green layer indicated all the m⁶A-circRNAs detected by m⁶A-circRNA epitranscriptomic microarray. The inner blue layer indicated the m⁶A-circRNAs from OSCC cells (SCC25), and the innermost red layer indicated the m⁶A-circRNAs from normal control cells (HOK) cells.

In consideration of the fact that m⁶A-modified circRNAs were categorized and detected based on 'm⁶A methylation level' and 'm⁶A quantity', our team took the intersection of 'm⁶A methylation level' and 'm⁶A quantity' to zoom out the scope, thereby ascertaining the up-regulated or down-regulated m⁶A-circRNAs. Venn diagram showed the up-regulated m⁶A-circRNAs (100) (**Figure 2C**) and down-regulated m⁶A-circRNAs (90) (**Figure 2D**). Besides, several up-regulated or down-regulated m⁶A-circRNAs were randomly chosen and listed in the diagram. Moreover, among these screened m⁶A-circRNAs, the significantly up-regulated (Top 10) m⁶A-circRNAs were shown in **Table 1** and down-regulated m⁶A-circRNAs were shown in **Table 2**.

The genomic characteristic of m⁶A-circRNAs in OSCC

An essential literature by Dr. Alan C Mullen(13) (2017) has raised an enlightening point that m⁶A-modified circRNAs exhibit characteristic rules, thus, we investigate the genomic characteristic of m⁶A-circRNAs in OSCC with the help of MeRIP-Seq and m⁶A-circRNA epitranscriptomic microarray. The differentially expressed m⁶A-modified circRNAs, including up-regulated (100 m⁶A-circRNAs) and down-regulated (90 m⁶A-circRNAs), were incorporated into the research. Firstly, exons quantity of up-regulated (**Figure 3A**) and down-regulated (**Figure 3B**) m⁶A-modified circRNAs were counted. Results illustrated that the exons of m⁶A-circRNAs mainly concentrated on 1-3 exons. Moreover, the length (spliced length, not genomic length) was counted by stages (100 bp). Results indicated that the length of m⁶A-circRNAs

primarily distributed at 200-500 bp (**Figure 3C, 3D**). In other words, the shorter circRNAs with a small number of exons were more easily methylated. Our previous research performed MeRIP-seq to detect the m⁶A profile in OSCC cells(14). Taking MeRIP-seq as reference, we could find the difference and connection between m⁶A-mRNA and m⁶A-circRNAs. Metagene profiles showed the enrichment of m⁶A modification on mRNA and circRNA across RNA transcriptome. In up-regulated (**Figure 3E**) and down-regulated (**Figure 3F**) m⁶A-circRNAs, the m⁶A modification sites were primarily located in the front part of CDS, which was distinct from m⁶A-mRNA that in 3'-UTR or stop codon. Taken together, the data of m⁶A-circRNA epitranscriptomic microarray revealed the genomic characteristic of m⁶A-circRNAs in OSCC.

The association of m⁶A-circRNAs and m⁶A-mRNA in OSCC

To date, the major field of m⁶A epitranscriptome research focused on mRNA m⁶A modification. In previous published literature, our team had performed the MeRIP-seq on OSCC prior to present m⁶A-circRNAs epitranscriptomic microarray analysis(14). Utilizing the MeRIP-seq data and m⁶A-circRNAs epitranscriptomic microarray data, we found several traits of m⁶A-circRNAs and probed into the association of m⁶A-circRNAs and m⁶A-mRNA in OSCC. Combined with the existing literature and our findings, we confirmed that exons of certain gene could generate both pre-mRNA transcripts with m⁶A modification and exon-derived circRNAs with m⁶A modification (**Figure 4A**). Moreover, the m⁶A modification was installed by identical m⁶A methyltransferase complex. Indeed, there might be other types of m⁶A-modified circRNAs, e.g. intron-derived circRNAs or intron/exon-derived circRNAs, which were elusive and will be investigate in our further research.

All the profound analysis was performed based on our MeRIP-seq data and m⁶A-circRNAs epitranscriptomic microarray data. According to the difference of methylated location, we concluded several subgroups of m⁶A-modified circRNAs (**Figure 4B**). Firstly, a representative non-circRNA-derived mRNA (c-Myc mRNA) with m⁶A modification was selected as negative control (**Figure 4C**). The m⁶A modification site was located in the 3'-UTR of c-Myc mRNA. Besides, a circRNA-derived gene (HIPK3) was selected as positive control, whose exon-2 was cyclized to be circHIPK3 (hsa_circ_0000284) (**Figure 4D**). Our MeRIP-seq data revealed that there wasn't any remarkable m⁶A modified site in the HIPK3 mRNA. The subgroup of circRNA, such as circHIPK3, is a more common type of transcripts without m⁶A modification neither in circRNA nor host gene.

Furthermore, we investigated the interaction of m⁶A modification with circRNAs or their host genes. We notice a feature that whether circRNAs are m⁶A-modified isn't related to their host genes methylated or not. For example, circFOXM1 (hsa_circ_0025039) was a non-m⁶A-modified circRNA, however, its host gene (FOXM1) was remarkably m⁶A-modified at 3'-UTR (**Figure 4E**). In addition to the circRNAs derived from m⁶A-modified host genes, another group of m⁶A-modified circRNAs was cyclized from unmethylated host genes. For example, circKRT5 (hsa_circ_0026457) was a m⁶A-modified circRNA, however, there wasn't any m⁶A site in its host gene (KRT5) (**Figure 4F**). Moreover, the m⁶A modification could be installed both circRNA transcript and host genes. For instance, circIPO9 (hsa_circ_0015936) was a m⁶A-modified circRNA, besides, its host gene (IPO9) was m⁶A-modified at 3'-UTR (**Figure 4G**).

Overall, based on MeRIP-seq and m⁶A-circRNAs epitranscriptomic microarray data, our findings illustrated that m⁶A-circRNAs exhibited their particular modification style in OSCC, which was independent of m⁶A-mRNA.

GO and KEGG Pathway Analysis

To investigate the mechanisms correlated to m⁶A-circRNAs in OSCC, Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the host genes of differentially expressed circRNAs were performed. GO terms included the biological process (BP), cellular component (CC), and molecular function (MF) categories. The top 10 enriched GO terms in BP, CC and MF were 'keratinization', 'Keratin filament' and 'glutathione binding' respectively (**Figure 5A**). The top 10 KEGG pathways were shown as following (**Figure 5B**) and the host genes of differentially expressed circRNAs were mainly associated with necroptosis.

ceRNA network of potential circRNA-miRNA-mRNA in OSCC

Given that circRNAs could act as miRNA sponge to harbor their downstream miRNAs, thereby releasing the fettered target mRNAs to modulate the cancer progression. The regulation pattern was regarded as competing endogenous RNA (ceRNA). Here, our lab utilized the m⁶A-circRNA epitranscriptomic microarray analysis to analyze the potential circRNA-miRNA-mRNA in OSCC. A great number pairs of circRNA-mRNA and miRNA-mRNA were found in the results. For a better exhibition, we chose two circRNA (hsa_circRNA_101017, hsa_circRNA_406747) to show the ceRNA network in OSCC (**Figure 6**).

Discussion

As an indispensable part of epigenetic regulation, m⁶A modification is suggested as a crucial regulator participating in human cancer tumorigenesis. Chemical modification of m⁶A on RNAs is an efficient way of regulating molecular function, which influences the downstream pathways. Increased knowledge of m⁶A modification of noncoding RNA highlighted their effect on gene expression. Modifications on RNA contribute to post-transcriptional regulation of RNA fate. Here, our research focused on the m⁶A-modified circRNA (m⁶A-circRNA) epitranscriptome-wide mapping in OSCC, which may provide valuable references for tumor epigenetics research.

CircRNAs have been identified to play important roles in tumorigenesis by virtue of their stability and enzyme-resistance. High-throughput sequencing for circRNAs reveals that many genes, previously considered as protein-coding genes, can produce circRNAs by back-splicing. In a variety of tumor subgroup, circRNAs participate in the cell differentiation, proliferation, energy metabolism and chemoradiotherapy resistance. As the research of circRNA progressed, the functions of circRNAs have been gradually investigated. The achievement is acquired not only in solid cancers, but also in OSCC. Our previous research found that, in OSCC, circUHRF1 up-regulated in OSCC and accelerated the tumorigenesis(15). Up to now, the major regulatory type of circRNAs is competing endogenous RNA (ceRNA), by which circRNAs sponge micro RNAs to relieve the activity restrain of target proteins, e.g. circHIPK3(16), circFOXO3(17) and circIGHG(18). In addition to ceRNA, more regulation manners of circRNAs have been reported. For instance, circ-ZNF609 is associated with heavy polysomes and could be translated into protein via splicing-dependent and cap-independent manner(19). Thus, with the further research, the modes of circRNAs will be reported more and more.

Alan C. Mullen et al (2017) firstly reported the profile of m⁶A modifications on circRNAs through a computational pipeline (AutoCirc) tool. In this study, researchers defined thousands of m⁶A-circRNAs with cell-type-specific expression. Besides, m⁶A-circRNAs share identical m⁶A readers and writers with mRNAs, however, m⁶A-circRNAs are frequently derived from exons that unmethylated in mRNAs. Moreover, this study proposed another critical trait that the same exons methylated both on mRNA and m⁶A-circRNAs exhibit less stability. Overall, this groundbreaking work makes a valuable contribution to the field of m⁶A-modified circRNAs.

Here, our study performed the newly-developed m⁶A-circRNA epitranscriptomic microarray to detect the profile of m⁶A-modified circRNAs in OSCC. In our results, the differentially expressed m⁶A-circRNAs were presented by two aspects, including 'm⁶A-circRNA Methylation Level' and 'm⁶A-circRNA Quantity' based on threshold of p-value <0.05 and fold change >1.5 fold. Finally, there were 104 up-regulated m⁶A-circRNA and 145 down-regulated m⁶A-circRNA on the basis of 'm⁶A-circRNA Methylation Level', meanwhile, there were 2586 up-regulated m⁶A-circRNA and 472 down-regulated m⁶A-circRNA on the basis of 'm⁶A-circRNA Quantity'. In previous study, our team had performed the MeRIP-seq on OSCC and thus we analyzed the genomic characteristic of m⁶A-circRNAs in OSCC utilizing the data of MeRIP-Seq and m⁶A-circRNA epitranscriptomic microarray. The differentially expressed m⁶A-modified circRNAs were selected into the research, including up-regulated (100 m⁶A-circRNAs) and down-regulated (90 m⁶A-circRNAs).

As regarding to the genomic characteristic of m⁶A-circRNA in OSCC, we found that the exons of m⁶A-circRNAs mainly concentrated on 1-3 exons, and the length primarily distributed at 200-500 bp. Moreover, shorter circRNAs with a small number of exons were more easily methylated. Then, we analyzed the enrichment of m⁶A modification on mRNA and circRNA across RNA transcriptome. Results indicated that the m⁶A modification sites of m⁶A-circRNAs were primarily located in the front part of CDS, which was distinct from m⁶A-mRNA that in 3'-UTR or stop codon. Taken together, the genomic characteristic of m⁶A-circRNAs in OSCC was preliminarily investigated with the help of MeRIP-Seq and m⁶A-circRNA epitranscriptomic microarray.

To date, emerging literatures report the cross-talking of m⁶A and circRNA in human cancer(20). Within the scope of epigenetics, RNA modifications and circRNAs are two rapidly expanding fields, and increasing number of researchers are beginning to turn their attention in this direction. For example, the m⁶A modification of circNSUN2 increases its export to the cytoplasm and circNSUN2 enhances the stability of HMGA2 mRNA to promote colorectal carcinoma metastasis progression, forming a circNSUN2/IGF2BP2/HMGA2 RNA-protein ternary complex(21). In another example, METTL3 and YTHDC1 control the circ-ZNF609 accumulation and back-splicing reaction(22). Moreover, in colorectal cancer, METTL3 induced the overexpression of circ1662 by binding the flanking sequences through installing its m⁶A modification(23). In spite of the evidence showing essential roles of m⁶A and circRNA on human cancer, the comprehensive analysis of m⁶A-circRNA at epitranscriptome-wide is still absent. Actually, the direct evidence that in support of the function of m⁶A-circRNA are worth looking forward.

In conclusion, this research reveals the epitranscriptome-wide mapping of m⁶A-modified circRNA in OSCC. The findings expand our understanding of circRNAs and enrich the roles of m⁶A-circRNAs in OSCC, providing potential resolution strategy for OSCC targeted therapy.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the authors agree with the publication.

Availability of data and material

The sequencing raw data could be available for researchers based on reasonable ground.

Conflict of interests

Authors declare no conflict of interests.

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Author contributions:

Wei Zhao, Jingwen Liu, and Jie Wu perform the experiments and write the paper. Xi Wang, Leyu Zhang, Xiaozhou Ma, Jianming Yang act as the assists. Jiayin Deng, Xin Hu and Cui Yameng are responsible for the designing and funding. All authors read and approved the final manuscript.

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Tables

Table 1. Top 10 up-regulated m⁶A-circRNAs in OSCC.

dysregulated	ArrayStar ID	circBase ID	p-value	Log ₂ FC	Host gene	Chromosome	Length (bp)	Location
Up	hsa_circRNA_026358	hsa_circ_0026358	6.559E-06	2.037	KRT7	chr12	534	52628938-52635420
Up	hsa_circRNA_025042	hsa_circ_0025042	4.266E-05	1.688	FOXM1	chr12	549	2983142-2983691
Up	hsa_circRNA_101929	hsa_circ_0004931	6.379E-05	1.642	NXN	chr17	460	722678-729318
Up	hsa_circRNA_000756	hsa_circ_0000756	8.923E-05	1.644	ATAD5	chr17	1366	29170930-29196664
Up	hsa_circRNA_007923	hsa_circ_0007923	7.750E-05	1.632	RRM1	chr11	631	4123222-4133292
Up	hsa_circRNA_101081	hsa_circ_0000408	2.511E-05	1.503	TIMELESS	chr12	378	56824664-56826308
Up	hsa_circRNA_104564	hsa_circ_0083444	8.186E-05	1.549	MTUS1	8hr8	2441	17601112-17613470
Up	hsa_circRNA_103069	hsa_circ_0060516	2.781E-04	1.560	PABPC1L	chr20	235	43547546-43547918
Up	hsa_circRNA_407080	None	4.701E-03	1.629	CHD7	chr8	-	61707544-61714152
Up	hsa_circRNA_403287	None	9.186E-05	1.544	LPCAT1	chr5	-	1488505-1501718

Table 2. Top 10 down-regulated m⁶A-circRNAs in OSCC

dysregulated	circRNA	circBase ID	p-value	Log ₂ FC	Host gene	Chromosome	Length	Location
Down	hsa_circRNA_001819	hsa_circ_0000658	2.829E-07	0.362	MCTP2	chr15	1502	94847149-94848651
Down	hsa_circRNA_104898	hsa_circ_0088249	5.702E-06	0.381	PAPPA	chr9	994	119106821-119130033
Down	hsa_circRNA_001484	hsa_circ_0000701	8.072E-06	0.465	CHD9	chr16	3095	53188358-53191453
Down	hsa_circRNA_000094	hsa_circ_0000247	9.871E-05	0.025	MCU	chr10	792	74474868-74475660
Down	hsa_circRNA_101122	hsa_circ_0027842	6.493E-05	0.283	ANKS1B	chr12	535	100200181-100219167
Down	hsa_circRNA_008421	hsa_circ_0008421	9.522E-05	0.374	FBN1	chr15	374	48888479-48905289
Down	hsa_circRNA_000403	hsa_circ_0000403	2.563E-05	0.433	ZNF385A	chr12	80	54764057-54764137
Down	hsa_circRNA_092561	hsa_circ_0001612	2.605E-05	0.636	SENP6	chr6	498	76331247-76357517
Down	hsa_circRNA_022382	hsa_circ_0022382	9.482E-04	0.493	FADS2	chr11	411	61605249-61608197
Down	hsa_circRNA_008786	hsa_circ_0008786	8.307E-04	0.485	POLE2	chr14	424	50120707-50122529

Figures

Figure 1

m⁶A-circRNA epitranscriptomic microarray analysis revealed the m⁶A-circRNAs profile in OSCC. (A, B) Heat Map showed the differentially expressed m⁶A-circRNAs, including 'm⁶A-circRNA Methylation Level' and 'm⁶A-circRNA Quantity'. OSCC cells were SCC25 cells and normal cells were HOK cells. (C, D) Volcano Plot showed the differentially expressed m⁶A-circRNAs. Based on this threshold (p-value < 0.05, fold change > 1.5 fold), there were 104 up-regulated m⁶A-circRNA and 145 down-regulated m⁶A-circRNA on the basis of 'm⁶A-circRNA Methylation Level' (C). And, there were 2586 up-regulated m⁶A-circRNA and 472 down-regulated m⁶A-circRNA on the basis of 'm⁶A-circRNA Quantity' (D). (E, F) Scatter Plot displayed the correlation distribution of m⁶A-circRNA in SCC25 cells and HOK cells.

Figure 2

The significantly expressed m⁶A-circRNAs. (A, B) Circos plot showed the locations of m⁶A-circRNAs on human chromosomes, including 'm⁶A methylation level' or 'm⁶A quantity'. The outermost layer was chromosome map of human genome. The inner green layer indicated all the m⁶A-circRNAs detected by m⁶A-circRNA epitranscriptomic microarray. The inner blue layer indicated the m⁶A-circRNAs from OSCC cells (SCC25). The innermost red layer indicated the m⁶A-circRNAs from normal control cells (HOK) cells. (C, D) Venn diagram showed the up-regulated 100 m⁶A-circRNAs and 90 down-regulated m⁶A-circRNAs. Several up-regulated or down-regulated m⁶A-circRNAs were randomly chosen and listed in the diagram.

Figure 3

The genomic characteristic of m⁶A-circRNAs in OSCC. (A, B) The exons quantity of up-regulated and down-regulated m⁶A-modified circRNAs were counted in differentially expressed m⁶A-modified circRNAs, including up-regulated (100 m⁶A-circRNAs) and down-regulated (90 m⁶A-circRNAs). (C, D) The length (spliced length, not genomic length) was counted by stages count (~100 bp). (E, F) Metagenome profiles of enrichment of m⁶A modification sites on mRNA and circRNA of gene locus across RNA transcriptome. 5'-untranslated regions (5'-UTR), coding sequences (CDS), 3'-untranslated regions (3'-UTR).

Figure 4

The association of m^6A -circRNAs and m^6A -mRNA in OSCC. (A) Schematic diagram illustrated the biogenesis of m^6A -circRNAs and m^6A -mRNA in OSCC cells. The m^6A modification was installed by identical m^6A methyltransferase complex (m^6A writers). Red A indicated the m^6A modification site. (B) Schematic diagram presented the genomic structure of m^6A -mRNA, unmethylated circRNAs and m^6A -circRNAs. (C) c-Myc mRNA acted as a representative for m^6A -mRNA, which was methylated at 3'-UTR and without circRNA derived. (D) circHIPK3 (hsa_circ_0000284) represented the group of circRNAs without m^6A modification in neither circular transcripts nor host gene. (E) CircFOXM1 (hsa_circ_0025039) represented the group of circRNAs without m^6A modification in circRNA transcript, however, their host genes were m^6A -modified at 3'-UTR. (F) CircKRT5 (hsa_circ_0026457) represented the group of circRNAs only m^6A -modified in circRNA transcript, instead of host genes. (G) CircIPO9 (hsa_circ_0015936) represented the group of circRNAs m^6A -modified both in circRNA transcript and their host genes.

[illegible]

Figure 5

GO and KEGG Pathway Analysis. (A) Gene Ontology (GO) enrichment analysis of biological process (BP), cellular component (CC), and molecular function (MF). The vertical axis stands for the enrichment score of GO terms. (B) Bulb map displayed the top 10 KEGG pathways for host genes of differentially expressed circRNAs. The size of the dot indicates the gene counts enriched in the pathway. The of the dot indicates the significance (p value) of the enriched pathway.

Figure 6

ceRNA network of potential circRNA-miRNA-mRNA in OSCC. A great number pairs of circRNA-mRNA and miRNA-mRNA were found in the results. For a better exhibition, we chose two circRNA (hsa_circRNA_101017, hsa_circRNA_406747) to show the ceRNA network in OSCC.

Supplementary Files

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- [supplementaryFigureS1.tif](#)