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ORIGINAL RESEARCH

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HTR7 and its N6-methyladenosine modification: a potential target in cell cycle regulation of cervical cancer

Guo Chen[1,†], Yuting Zhu[1,†], Lu Tian[1], Jie Ying[1], Shuangyue Wu[1], Bin Zhang[1,*], Yang Song[2,*]

[1]Department of Gynecology, Anhui Province Maternity and Child Health Hospital, 230000 Hefei, Anhui, China
[2]Department of Pain Treatment, the First Affiliated Hospital of Anhui Medical University, 230000 Hefei, Anhui, China

***Correspondence**
songyang@ahmu.cn
(Yang Song),
zhangbinshanmu@126.com
(Bin Zhang)

† These authors contributed equally.

Abstract

Pain is highly prevalent among cancer patients. Epidemiological reports indicate that about 30%–50% of cancer patients have varying levels of pain, and about 75–95% of advanced cancer patients have chronic pain. Analgesic is the first-class choice of treatment in the final stage of cervical cancer (CC) therapeutic schedule. Early reports indicated that 5-hydroxytryptamine (5-HT) had improved pain management in cancer, while recent reports indicate that the 5-hydroxytryptamine receptor 7 (HTR7), is closely related to the occurrence and prognosis of various solid tumors. However, few articles have clarified the co-relationship between the 5-HT receptor and CC. Based on RNA-seq re-analysis, we found that HTR7 was increased in CC compared with adjacent tissues. Interestingly, Gene Set Enrichment Analysis and Kyoto encyclopedia of Genes and Genomes (GSEA-KEGG) results indicated that HTR7 could regulate CC cell cycle pathway, suggesting HTR7 as a target oncogene. Further, N6-methyladenosine (m6A) site analysis results showed that *HTR7* had many m6A enzyme binding positions and YTH domain family 2 (YTHDF2), an m6A reader, was positively correlated with *HTR7* in CC. Altogether, this study showed that *HTR7* was elevated in human CC, affected its cell cycle, contributed to tumor procession and was regulated by the m6A enzyme, demonstrating the important mechanisms of epigenetic alteration in CC.

Keywords

5-HT; *HTR7*; N6-methyladenosine (m6A); Cervical cancer; *YTHDF2*

1. Introduction

CC, a notorious type of cancer, represents a serious threat to women's health, especially in developing countries. It accounts for 25% of all female cancers and is the third most common cancer in women [1, 2]. Latest epidemiological reports indicated that 265,672 patients succumb to CC metastasis-induced irreversible injury each year. CC can be treated in its early stages if diagnosed early, but the prognosis for long-term survival decreases as the disease progresses. Hence, a considerable proportion of CC patients have metastasis by the time of diagnosis, with those having recurrent disease suffering from an even worse prognosis [3, 4].

In cancer patients, pain could be the most unbearable symptom, and appropriate pain management may provide great relief to the patients [5]. According to statistics, 30%–50% of cancer patients experience moderate to severe pain [6], and about 75–95% of patients with advanced cancer experience chronic pain that is difficult to control. The first symptom of many cancer patients is pain [7]. Cancer pain has peripheral and central sensitization similar to inflammatory and neuropathic pains. Central sensitization is caused by increased or decreased excitatory functions of central neurons related to pain transmission, which amplifies the response to noxious stimuli [8]. A recent report indicated that inhibiting the 5-HT receptor could attenuate cancer-induced pain, indicating that such could be a new therapy for pain management in cancer patients.

The HTR7, a G-protein-coupling receptor, is widespread in both central and peripheral tissues. In the peripheral tissues, the HTR7 is mainly distributed in small and medium-sized cells of vascular smooth muscle, platelet, lung, gastrointestinal tract, peripheral sensory nerve endings, and dorsal root ganglion (DRG) neurons of humans and animals, and its main function is to regulate the contractions of vascular smooth muscle, bronchus, uterus, and urinary tract. It was discovered that HTR7 receptors are mainly distributed in the forebrain, cerebellum, brainstem nucleus, spinal cord, and other parts of rats. Studies have found that the HTR7 protein plays an important role in inflammatory and neuropathic pains. However, the latest reports have found that HTR7 is closely related to the occurrence and prognosis of various solid tumors, including thyroid cancer, breast cancer and laryngeal cancer, but the role of HTR7 in CC and its mechanism are yet to be clarified. Thus, more research is needed to determine the significance and underlying mechanism of HTR7 in CC due to its diverse

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function in cell signaling networks. In this study, we confirmed that *HTR7* regulated the cell cycle, revealing a potentially promising strategy for CC treatment [9].

2. Materials and methods

2.1 Sample subjects

CC tissues and adjacent non-tumorous cervical tissue data were downloaded from the National Center of Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database [10]. GEO Series 7410 (GSE7410) was chosen and included 40 CC patient cervical tissues and 5 adjacent non-tumorous cervical tissues.

2.2 Differential expression of mRNA

The log scale MAS5 software (the Affymetrix microarray suite version 5, Stephen Fodor, State of California, American) was used to normalize the raw files and the mRNAs expression values were obtained. The linear models for microarray data (limma) R package was used to identify the different mRNAs between CC samples and adjacent non-tumorous cervical tissues. Hierarchical clustering was performed using the R scripts.

2.3 GEPIA (Gene Expression Profiling Interactive Analysis)

GEPIA (http://gepia.cancer-pku.cn/index.html), an analysis tool containing RNA sequence expression data of 9736 tumors and 8587 normal tissue samples, is frequently used in bioinformatics analysis [11]. In this study, the "single gene analysis" module of GEPIA was used to analyze mRNA differential expression, pathological staging, and corresponding prognosis of tumor and normal tissues. The p-value cutoff was 0.05. The Student's t-test was used to generate a p-value for expression or pathological stage analysis. Prognostic analysis was performed using a Kaplan-Meier curve. The correlation between two variables was assessed using Pearson's correlation test.

2.4 Gene ontology enrichment analysis

DAVID 6.8 (Visualization and Integrated Discovery 6.8) is a comprehensive, functional annotation website that helps investigators clarify the biological function of targeted genes. First, patients with CC from the GSE7410 dataset were initially divided into a high and a low HTR7 expression group, and the positively and negatively enrichment data were analyzed using DAVID 6.8 for Gene Ontology (GO) enrichment analysis. Bar graphs were visualized using the ggplot2 package in R. Biological processes (BP) were included in the GO enrichment analysis.

2.5 GSEA

To identify signaling pathways significantly influenced by HTR7 in CC patients, we sorted the CC samples in GSE7410 in decreasing order of HTR7 expression values and selected the top 10 (HTR7-high) and bottom 10 (HTR7-low) CC samples for GSEA (version 4.3.0, Broad Institute, American). Gene sets referring to KEGG pathways were obtained from the Molecular Signatures Database (MSigDB), and expression differences among all genes between HTR7-high and HTR7-low HCC samples were used to quantify gene set activity using GSEA version 3.0.

2.6 SRAMP

In this study, the SRAMP (http://www.cuilab.cn/sramp/) software was used to analyze the mRNA sites of m6A. It was also used to predict m6A binding sites from the analysis of RNA sequences. SRAMP (Qinghua Cui, Bei Jing, China) achieved promising performance in cross-validation tests on its training dataset and in rigorous independent tests [12].

2.7 Statistical analysis

The data were analyzed using GraphPad Prism (version 6.0, GraphPad Software, State of California, American). The difference in continuous indexes with normal distribution between two groups was determined using the Student's t-test. The correlation between two genes was measured by the Pearson correlation coefficient. Kaplan-Meier curves were drawn to evaluate the prognostic significance. p-values < 0.05 on both sides were considered statistically significant.

3. Results

3.1 HTR7 was up-regulated in CC samples

In this study, we first collected the RNA-sequencing data of 40 CC tissue and 5 normal samples from NCBI. The Volcano Plot, heatmap and graph bar showed that *HTR7* was up-regulated in CC tissues (Fig. 1A,B1,B2), which was then confirmed by analyzing The Cancer Genome Atlas (TCGA) (Fig. 1C) and GEPIA (Fig. 1D) data. The association between *HTR7* expression and morbidity of CC was also investigated. According to correlation analysis from GEPIA, *HTR7* was found to participate in CC progression. Fig. 1E shows that CC patients with low transcriptional levels of *HTR7* had significantly longer survival than patients with high transcriptional *HTR7* levels.

3.2 Functional GO Analysis of HTR7 in CC Patients

HTR7 neighboring genes were analyzed using DAVID 6.8 and Metascape. The volcano plot (Fig. 2A) showed that *HTR7* enriched 19,922 genes in the CC samples, and the top 10 most highly enriched GO pathways were obtained (Fig. 2B1, B2). Next, we determined the 10 most highly enriched functions in the biological progress and molecular function category regulated by *HTR7* and observed that *HTR7* mainly regulated DNA/RNA processing or cell cycle.

3.3 HTR7 regulates the cell cycle in CC

After determining the GO pathways regulated by *HTR7*, we focused on the CC-related signaling pathways regulated by *HTR7*. The TCGA database was used to determine the potential biological processes using GSEA and KEGG analysis. The positive enrichment results suggested that *HTR7* regulated

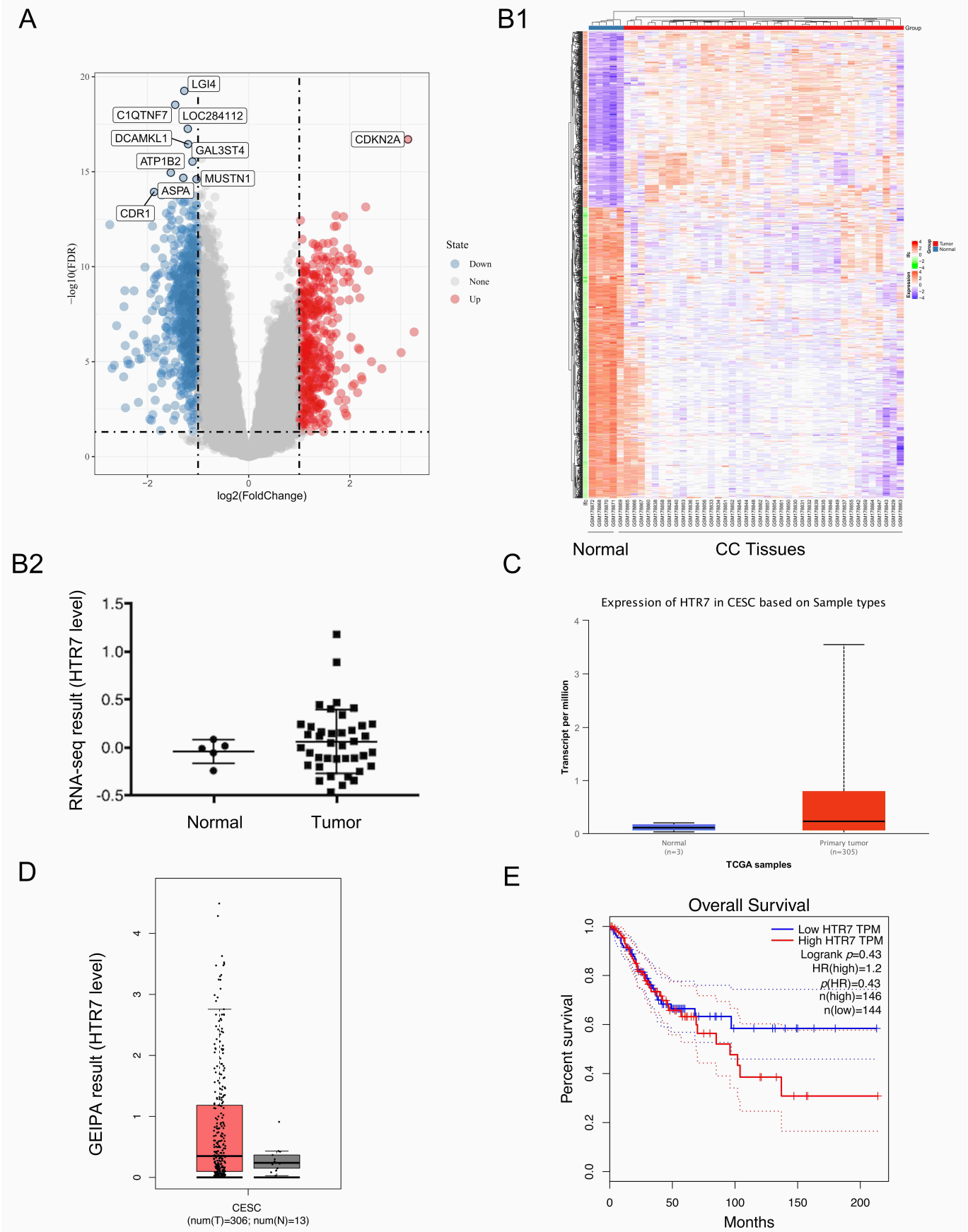
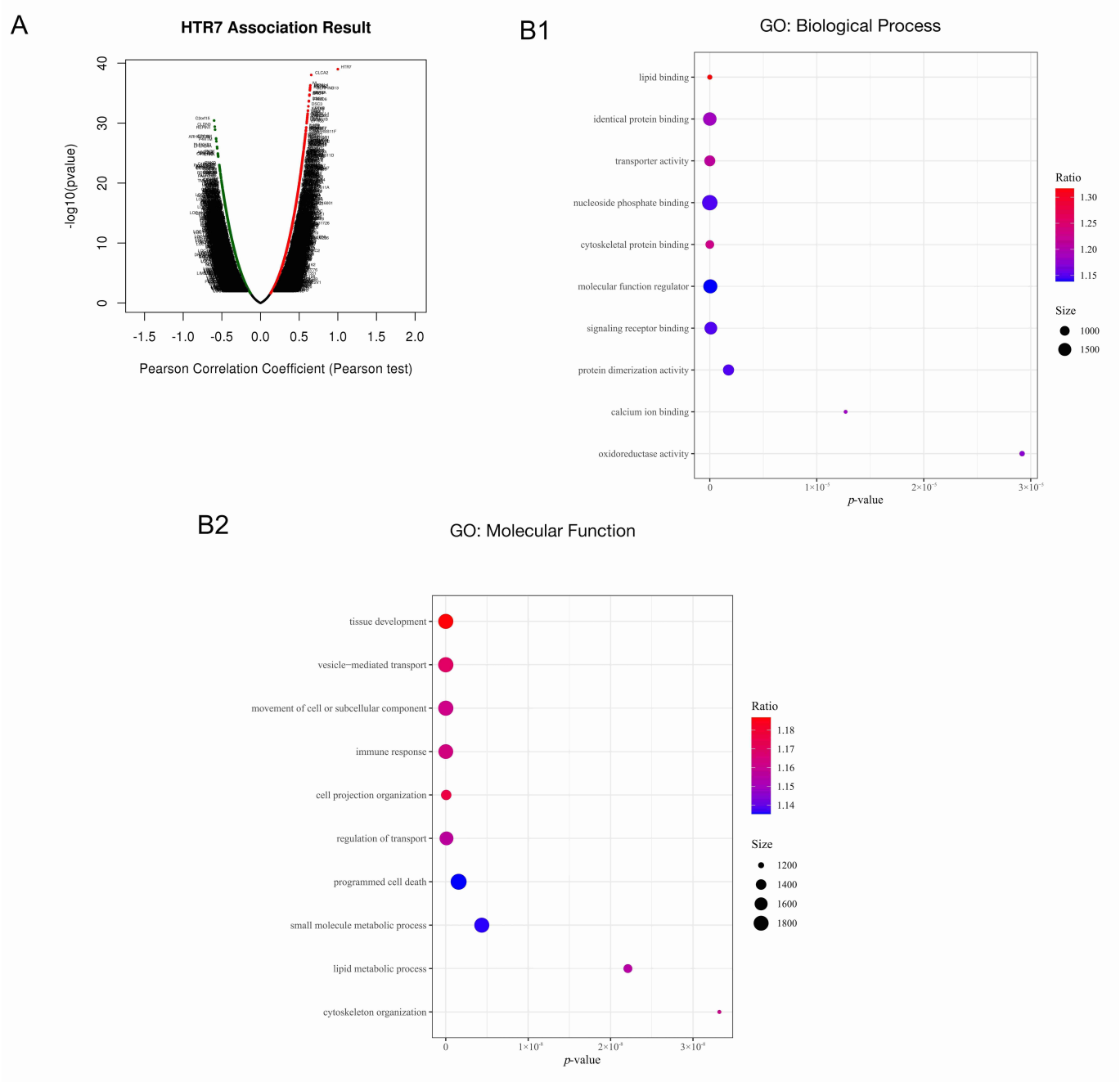


FIGURE 1. ***HTR7*** **was up-regulated in cervical cancer samples.** (A) The volcano plot shows the overall differential expression landscape in cervic cancer (CC) samples (n = 40) compared with matched surgical margin (n = 5) from TCGA (The Cancer Genome Atlas). (B1) Heatmap illustrates expression values of *HTR7* and genes in surgical margin and matched CC tissues from TCGA. (B2) RNA-seq result of *HTR7* mRNA level. The transcriptional levels of *HTR7* in CC tissues were significantly elevated according to (C) UALCAN (The University of ALabama at Birmingham CANcer data analysis Portal) and (D) GEIPA (Gene Expression Profiling Interactive Analysis) database. (E) Overall survival curves of CC samples were stratified by the median *HTR7* expression value generated by the Kaplan-Meier method.



F I G U R E 2. Functional GO(Gene Ontology) analysis of *HTR7* in patients with CC. The enrichment analysis of differentially expressed *HTR7* and most frequently altered GO in CC (DAVID 6.8). (A) Volcano Plot of *HTR7* enriched genes. Bar plot of GO enrichment in biological process terms (B1) and molecular function (B2).

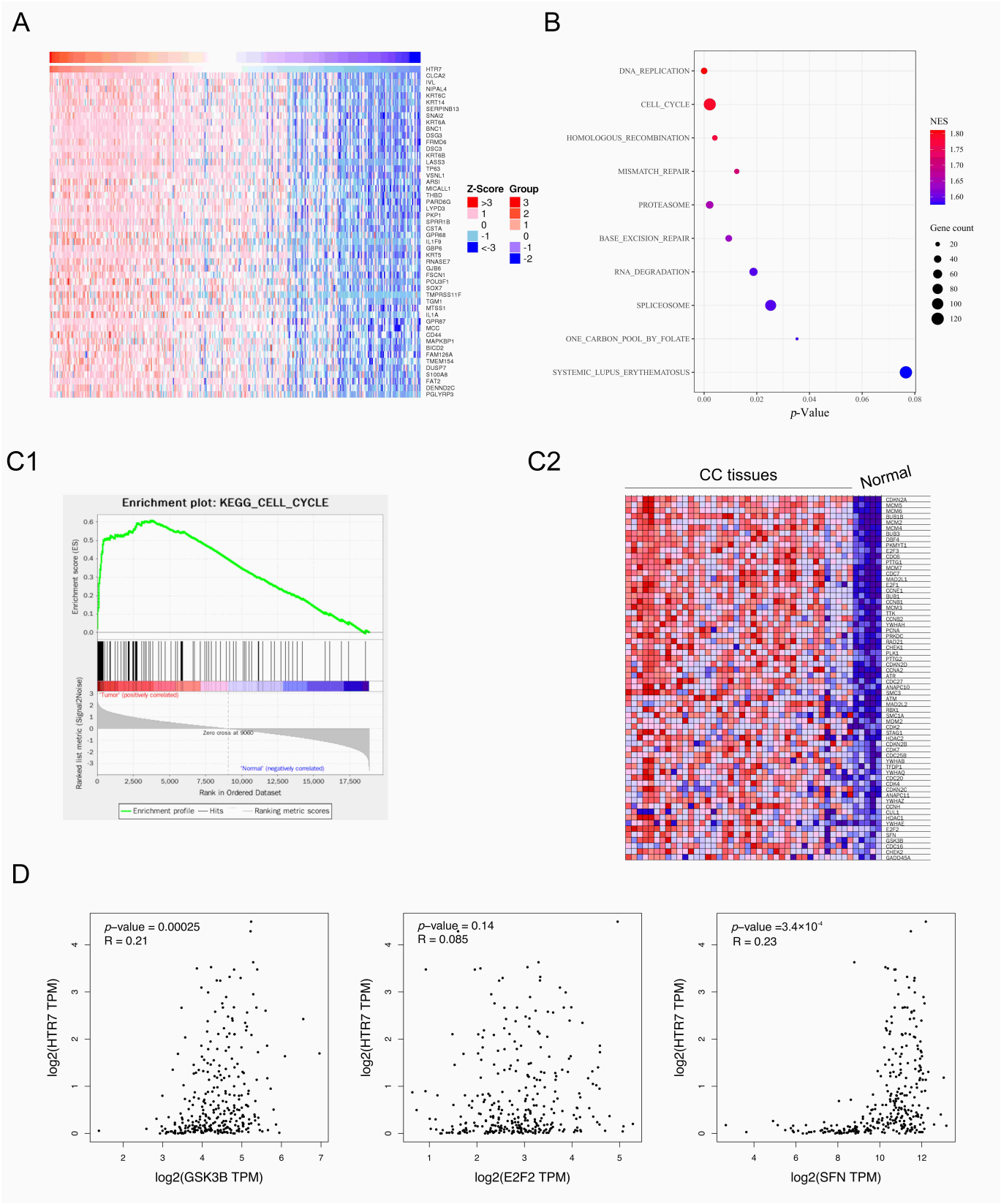


FIGURE 3. *HTR7* regulates the cell cycle in CC. (A) Heatmap shows the positively related genes with *HTR7* in CC. (B) GSEA-KEGG (Gene Set Enrichment Analysis and Kyoto encyclopedia of Genes and Genomes) analysis: *HTR7* positively related signal pathways in CC. (C1) KEGG enrichment plot: *HTR7* regulates the cell cycle pathway. (C2) Heatmap shows the genes in the cell cycle that *HTR7* may regulate. (D) Correlation analysis of *HTR7* and cell cycle pathway-related genes (GEPIA).

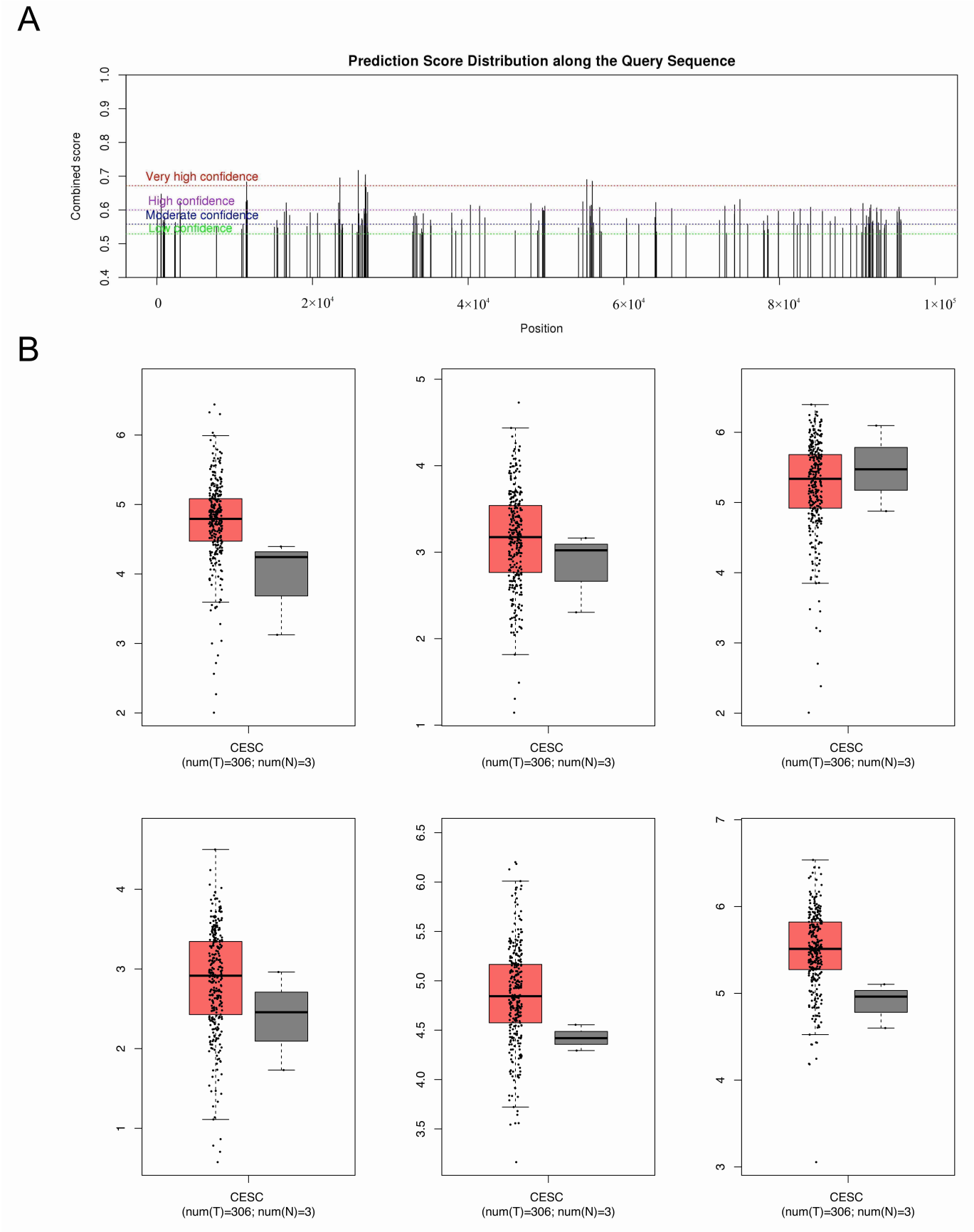


FIGURE 4. m6A modification m6A regulates HTR7. (A) SRAMP predicts m6A modification sites on the *HTR7* mRNA sequences (SRAMP). (B) The transcriptional levels of m6A genes in CC tissues were significantly elevated (GEPIA).

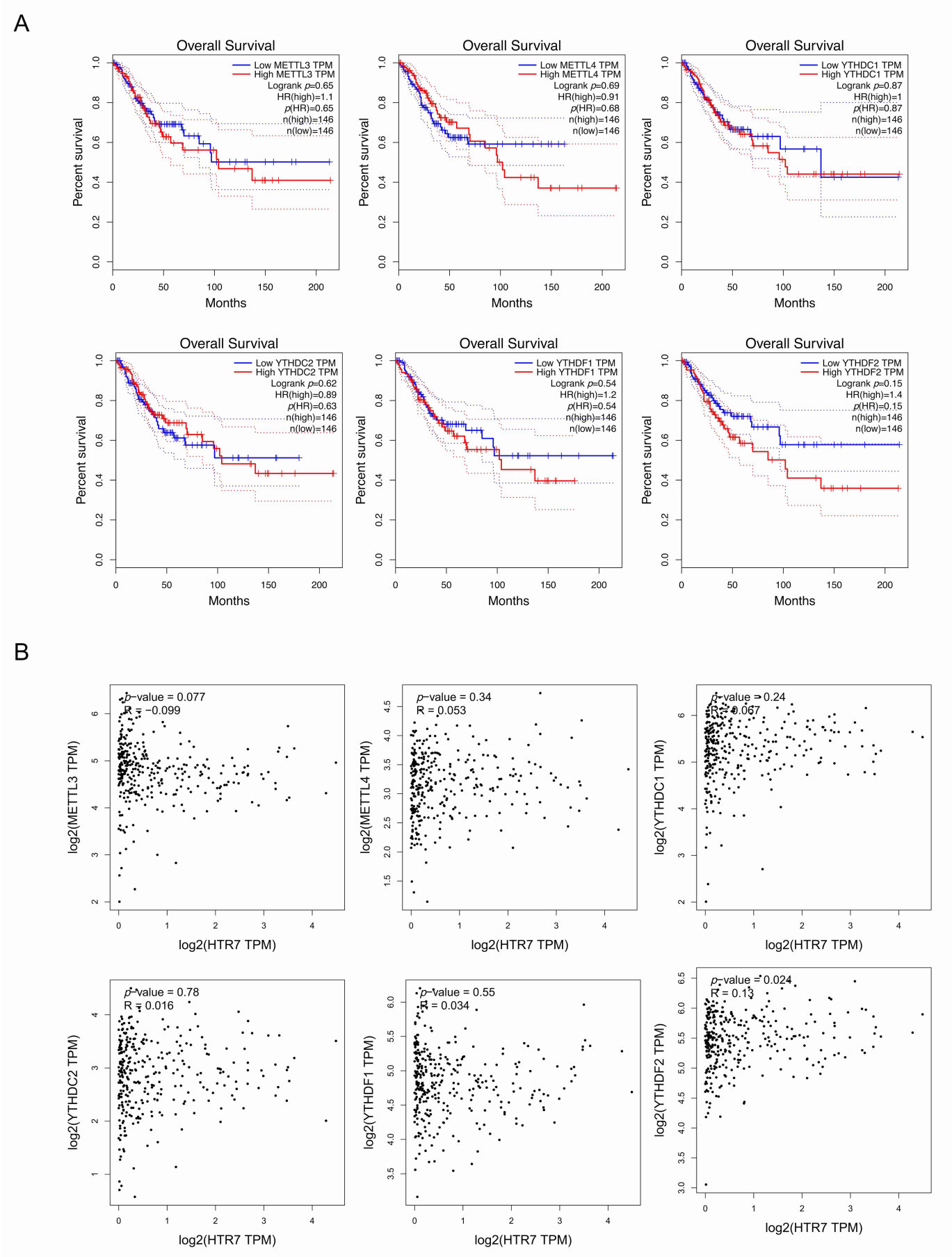


FIGURE 5. The prognostic value of m6A in patients with CC. (A) Overall survival and disease-free survival curves of CC samples were stratified by the median m6A expression value generated by the Kaplan-Meier method. (B) Correlation analysis of *HTR7* and m6A writers, erasers, and readers (GEPIA).

the cell cycle (Fig. 3A,B), and abnormal expression of *HTR7* was associated with the occurrence and progression of CC. We also found that HTR7 regulated the cell cycle *via* target genes such as glycogen synthase kinase 3 beta (*GSK3B*), E2F Transcription Factor 2 (*E2F2*) and Sulforaphane (*SFN*) (Fig. 3C1,C2). Pearson analysis showed that only *GSK3B* was significantly associated with *HTR7* (Fig. 3D). Altogether, these results indicated that *HTR7* regulated CC occurrence and progression *via* the cell cycle pathway.

3.4 m6A modification regulates HTR7

Here, we focused on gene alteration rather than the protein structure of HTR7. To investigate whether m6A methylation was involved in *HTR7* gene alteration, the SRAMP software was used to predict the m6A sites of *HTR7*. According to Fig. 4A, m6A enzymes could specifically bind to the front of the *HTR7* gene. To investigate the correlation between *HTR7* and m6A, we analyzed the m6A expression and evaluated the expression of genes that coordinated m6A modifications, including m6A writers, erasers and readers, using GEPIA data. The results showed that methyltransferase-like 3 (*METTL3*), methyltransferase-like 4 (*METTL4*), YTH domain containing 2 (*YTHDC2*), YTH domain family 1 (*YTHDF1*) and YTH domain family 2 (*YTHDF2*) were up-regulated in tumoral tissues compared with normal tissues (Fig. 4B).

3.5 The prognostic value of m6A in patients with CC

Previous data suggested that m6A might regulate *HTR7* gene mRNA. To further investigate the m6A functions in CC progression, Kaplan-Meier curves were plotted by GEPIA. Analysis of the overall survival curves suggested that m6A enzymes played critical roles in CC progression (Fig. 5A). Pearson analysis results indicated that *YTHDF2* had a positive relationship with *HTR7* (Fig. 5B). Together, these data showed that inhibiting m6A enzymes' activity, especially *YTHDF2*, might significantly enhance the prognosis of CC patients.

4. Discussion

Currently, the 5-year survival rate of CC remains unsatisfactory, at only about 52% [13]. Pain is one of the most common and unbearable symptoms of advanced-stage CC. It is mainly associated with the tumor compressing or eroding the ureter, narrowing and obstructing the duct and leading to hydronephrosis, which is then manifested as back pain or even severe pain on one side. About 70% to 90% of patients have ineffective cancer pain control [14], which is easy to form peripheral and/or central sensitization and can turn into intractable pain. Hence, analgesics are widely used in the comprehensive treatment of CC [15]. In this study, we were surprised to find that HTR7, a member of the 5-HT family of analgesia, had promising potential in inhibiting CC progression.

5-HT, a peripheral nociceptive transmitter with strong nociceptive effects, promotes the contraction of visceral smooth muscle and participates in the regulation of various physiological functions such as nociception, emotion and body temperature. 5-HT receptors are the famous member of the G protein-coupled receptors (GPCRs) family [16]. Presently, seven classes of 5-HT receptors have been identified, including 5-HT1 to 5-HT7 receptors, of which 5-HTR2 and 5-HTR7 were shown to be involved in pain modulation and demonstrated different analgesic or nociceptive effects in different peripheral and central sites [16]. In primary sensory neurons, 5-HTR has the ability to mediate transient receptor potential vanilloid-1 (TRPV1nociceptive sensitization. It has been shown that in response to stimuli, such as capsaicin, protons or injurious heat, 5-HTR2A and 5-HTR7 expression levels increase in the dorsal root ganglion, sensitizing it by increasing intracellular Ca^{2+} concentrations and inward currents *via* the Phospholipase C/protein kinase C(PLC/PKC) and Cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathways, leading to depolarization of the cell membrane and phosphorylation of TRPV1 [17]. However, HTR7 was found to not only play an important role in inflammatory and neuropathic pains but was also closely related to tumorigenesis and prognosis. It was previously reported that spinal 5-HT levels were elevated in bone cancer pain and that increased levels of 5-HT and receptors were involved in bone cancer pain [18]. 5-HT receptor subtype agonists and antagonists were found to be more likely used as new non-opioid analgesic drugs in clinics, such as for cancer treatment. HTR7 protein has been proved as a potential oncogene target. For instance, HTR7 correlated with progression-free survival (PFS), overall survival (OS), and disease-free survival (DFS) in breast cancer [19]. Cell proliferation assay and animal models confirmed that HTR7 overexpression activated the AKT pathway to promote laryngeal cancer proliferation and growth [20]. The above shows that HTR7 could be used not only as an analgesic target for advanced cancer treatment but also as a therapeutic target to inhibit tumor growth and prognosis and could prolong the survival of patients with intermediate and advanced cancer. The results also supported that *HTR7* was up-regulated in CC and was an unfavorable factor affecting the prognosis of CC patients. GO enrichment analysis was performed to determine the function of high *HTR7* expression, which showed that it was mainly related to metabolism and tissue development pathways. These data suggest that the *HTR7* is highly expressed in the CC and might be a potential target.

In adults, peripheral organs are the main "working area" of 5-HT receptors, but they could also be seen in some restricted areas of the brain. There is a strong link between activated 5-HTR2B and cellular proliferation. Activated 5-HTR2B may regulate the α subunit of the G protein/GTP binding protein (Gq GTP)-binding protein to activate the phospholipase C (PLC) signaling pathway [21]. 5-HTR2B may also activate the extracellular regulated protein kinases (ERK) pathway *via* a G protein- and the p21Ras-dependent manner in mouse fibroblasts [22]. Interestingly, the famous protein p60Src could also be regulated by 5-HTR2B to further activate cyclin D1 and cyclin E. Here, we focused on these cyclins because they were shown to activate retinoblastoma protein and the transcription factor E2F for DNA replication [23]. However, the regulatory roles of HTR7 in the cell cycle in CC were not yet reported. In this study, GSEA identified the signaling pathways *via* which *HTR7* regulated cervical cancer, among

which the cell cycle was the most significant.

In general, most signaling pathways, which may constrain the proliferative response in normal cells, are perturbed in cancer. Tumor development requires a class of mutations that help somatic cells to achieve external mitogenesis. Such mutations are easily found in human cancers. For instance, c-Myc expression is monitored tightly under normal cells, preventing its overexpression. However, it could overcome this control during tumorigenesis and was thus reported to be overexpressed in cancer cells [24]. In this study, GSEA analysis confirmed that *HTR7* could regulate the gene levels of *GSK3B*, *E2F2* and *SFN*. Interestingly, Pearson correlation analysis showed that only *SFN* and *GSK3B* had a positive correlation with HTR7 (correlation value reaches 0.2). These results confirmed that HTR7 regulated CC *via* the cell cycle pathway.

Recently, epigenetics has been highlighted in gene research [25]. Hence, we implemented mRNA modification methods to investigate the effects of *HTR7* in CC. The following results support our hypothesis that m6A enzymes combined tightly with the *HTR7* gene. m6A enzymes could significantly regulate mRNA decay, translation, and processing [26]. Recent studies have suggested the critical implications of m6A deregulation in different disease models [27]. Through in-depth studies on the mechanism of action related to m6A methylation, it was found that the process of m6A methylation modification occurring in mRNA was reversibly regulated and acted upon by effectors of m6A, including encoders, readers and decoders. Encoders (*i.e.*, METTL3, METTL14, *etc.*) add methyl to the target RNA *via* S-adenosylmethionine transferase to form m6A methylation sites. Decoders (*i.e.*, Fat mass and obesity-associated protein (FTO) and m6A Demethylase (ALKBH5)) catalyze the removal of m6A modifications in an Fe(II)/α-ketoglutarate-dependent manner. Readers (*i.e.*, YTHDF1-3, YTHDC1-2, *etc.*) specifically recognize and bind RNAs with m6A modifications to control their fate and regulate downstream functions [28, 29]. Extensive studies suggest that m6A is involved in tumor cell initiation, proliferation, differentiation, metastasis and other life processes by regulating the expression of tumor-related genes. An up-regulation of METTL3 was reported to be negatively correlated with DFS and OS in patients with cervical squamous carcinoma [30]. Moreover, tissue assays revealed that YTHDF2 expression was significantly increased in cervical cancer tissues, and *in vitro* assays revealed that the knockdown of YTHDF2 significantly inhibited the proliferation of CC cells, increased apoptosis and blocked the cell division cycle [31]. In this study, we confirmed that *METTL3, METTL4, YTHDC2, YTHDF1* and *YTHDF2* were up-regulated in different solid tumors and served as an oncogene in CC. More importantly, Pearson's correlation analysis showed that only *YTHDF2* positively correlated with *HTR7* in CC. These results confirmed that m6A enzymes had a high co-relationship with *HTR7* modification and regulated CC progression.

5. Conclusion

In this study, we identified *HTR7* as an analgesic target, which was also associated with the survival of CC. We also found that potential target genes of *HTR7*, such as *GSK3B* and *SFN*, regulated cancer cell development, and m6A enzymes, especially *YTHDF2*, might regulate the expression level of *HTR7*. However, the mechanism of these genes and drugs in CC patients is still unclear, and further research is required to fully elucidate these mechanisms.

AUTHOR CONTRIBUTIONS

YS, GC and BZ—designed the research study. GC—performed the research. YTZ, LT, JY and SYW—analyzed the data. GC and YTZ—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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