

## ORIGINAL RESEARCH ARTICLE

# MiR-381-3p/TET3 axis promotes cervical cancer: A bioinformatic integrative analysis

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## Abstract

Cervical cancer (CC) is a global public health problem. Epigenetic factors, such as microRNAs, play a key role in the development of CC. Although a previous study reported that miR-381-3p inhibits CC by downregulating fibroblast growth factor 7, its role in CC remains largely unknown. This study aimed to fill the gap by analyzing the role of miR-381-3p in CC. Expression analysis was performed using databases including The Cancer Genome Atlas, Gene Expression Omnibus, and the Human Protein Atlas. Receiver operating characteristics and Kaplan–Meier curves were plotted using GraphPad Prism and Kaplan–Meier Plotter databases. Target messenger RNAs were identified using the miRDB and miRPathDB databases. Kyoto Encyclopedia of Genes and Genomes, Gene Ontology Biological Process, and Gene Set Enrichment Analysis were performed using Enrichr and WebGestalt databases. Mutation analysis was conducted using the cBioPortal database. In this study, we found that miR-381-3p expression is low in CC. Moreover, we identified a total of 1,191 potential targets of miR-381-3p that may be involved in key signaling pathways in this cancer. Interestingly, our results identified ten-eleven translocation 3 (TET3) as a potential target, with evidence suggesting that TET3 expression is increased in CC. This increase in TET3 expression may be useful as a diagnostic and prognostic biomarker. In addition, four mutations were identified in the TET3 protein. TET3 expression increases in patients with International federation of gynecology and obstetrics stage II CC and in those with lymph node metastasis. Mechanistically, TET3 expression may be induced by a gain in copy number, human papillomavirus infection, aberrant methylation, and activation of the transcription factor activator protein 2 $\alpha$ . Finally, we identified 145 genes that are regulated by TET3 in CC, which are involved in key pathways and biological processes associated with CC, such as the cell cycle, DNA replication, mitogen-activated protein kinase signaling pathway, and basal transcription factors. In conclusion, we identified the miR-381-3p/TET3 axis as a key player in the carcinogenic process of CC.

**Keywords:** miR-381-3p; Ten-eleven translocation 3; Cervical cancer; Bioinformatic analysis

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**Citation:** Wences HJ, López PAÁ, Viguri GEC, *et al.* MiR-381-3p/TET3 axis promotes cervical cancer: A bioinformatic integrative analysis. *Cancer Plus*. 2024;6(3):3884. doi: 10.36922/cp.3884

**Received:** June 6, 2024

**Accepted:** August 5, 2024

**Published Online:** September 12, 2024

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## 1. Introduction

Cervical cancer (CC) is the fourth most common cancer among women worldwide, with an estimated 661,021 new cases and 348,189 deaths in 2022.<sup>1</sup> In the same year, the US alone reported approximately 14,100 new cases and 4280 deaths.<sup>2</sup> While human papillomavirus (HPV) infection is the primary risk factor for CC, it is not sufficient by itself to cause this cancer.<sup>3</sup> In this sense, several genetic and epigenetic alterations contribute to the development of CC.<sup>4,5</sup> Some studies have shown that microRNAs (miRNAs)<sup>6</sup> and aberrant DNA methylation promote tumor progression.<sup>7</sup>

MiRNAs are small endogenous RNA molecules, approximately 22 nucleotides in length.<sup>8</sup> MiRNAs negatively regulate the expression of target genes at the post-transcriptional level, including those involved in cellular functions, such as cell growth, differentiation, and apoptosis.<sup>9</sup> Altered expression of miRNAs is associated with several human cancer types, including CC.<sup>6</sup>

DNA methylation is a reversible epigenetic modification that mainly occurs in cytosines that precede guanine (CpG) at the fifth position of cytosine, 5-methylcytosine (5mC). This reaction is catalyzed by DNA methyltransferases.<sup>10</sup> Over 60% of human gene promoters contain CpG-rich regions known as CpG islands, and about 70 – 80% of CpG sites in the mammalian genome are methylated.<sup>11,12</sup> Aberrant methylation of CpGs in promoters leads to gene silencing through transcription inhibition, particularly affecting tumor suppressor genes; further, aberrant DNA methylation is associated with human malignancies, including CC.<sup>13,14</sup> The deregulation of ten-eleven translocation (TET) family proteins is a key factor in aberrant DNA methylation.<sup>15</sup> The TET family consists of TET1, TET2, and TET3, which are dioxygenases that participate in DNA demethylation by catalyzing the Fe(II)- and  $\alpha$ -ketoglutarate-dependent oxidation of 5mC to 5-hydroxymethylcytosine (5hmC).<sup>16</sup> This is followed by further oxidation to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).<sup>17</sup> Subsequently, 5fC and 5caC are excised and replaced with a new cytosine by the enzyme thymine DNA glycosylase through the base excision repair system.<sup>18–21</sup> Besides, the isocitrate dehydrogenase-dependent catalytic conversion of isocitrate to  $\alpha$ -ketoglutarate is required for TET enzymatic activity.<sup>22</sup>

A previous study reported that miR-381-3p suppresses CC progression by downregulating fibroblast growth factor 7 in CC cell lines;<sup>23</sup> however, the role of this miRNA in CC has not been fully studied. Therefore, the present study aimed to analyze the role of miR-381-3p in CC through an integrative bioinformatic analysis.

## 2. Data and methods

### 2.1. Expression analysis

Expression analysis was performed in CC and normal tissue samples in The Cancer Genomes Atlas (TCGA), Gene Expression Omnibus (GEO)<sup>24</sup> (<https://www.ncbi.nlm.nih.gov/geo/>), and the human protein atlas (HPA) v23.0<sup>25</sup> (<https://www.proteinatlas.org/>) datasets. TCGA dataset was analyzed using databases such as miR-TV<sup>26</sup> (<http://mirtv.ibms.sinica.edu.tw>) for miRNAs, as well as gene expression profiling interactive analysis (GEPIA)<sup>27</sup> (<http://gepia.cancer-pku.cn/>) and cBioPortal<sup>28</sup> (<https://www.cbioportal.org/>) for genes. The differences were determined using the Mann-Whitney *t*-test in miR-TV and cBioPortal databases, and the one-way analysis of variance test in the GEPIA database. A  $P < 0.05$  was considered statistically significant. GEO datasets were downloaded and analyzed using the GEO2R software<sup>24</sup> (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>). The GEO datasets used in this study were GSE86100<sup>29</sup> for miRNAs, GSE7803<sup>30</sup> and GSE26511<sup>31</sup> for messenger RNAs (mRNAs). GSE86100 dataset (Platform: GPL19730 Agilent-046064 Unrestricted\_Human\_miRNA\_V19.0\_Microarray) includes six CC and normal tissue samples each. GSE7803 dataset (Platform: GPL96 [HG-U133A] Affymetrix Human Genome U133A Array) includes 21 CC and 10 normal tissue samples. GSE26511 dataset (Platform: GPL570 [HG-U133\_Plus\_2] Affymetrix Human Genome U133 Plus 2.0 Array) includes 20 and 19 CC tissue samples negative and positive for lymph node metastasis, respectively. Expression was log2-transformed, and differences were determined using the Mann-Whitney *t*-test. A  $P < 0.05$  was considered statistically significant. The HPA dataset includes CC and normal tissue samples, and the TET3 level was analyzed using the HPA050845 antibody. Three representative images of CC (Patient ID: 4223) and normal (Patient IDs: 2451 and 2102) tissue samples are shown (<https://www.proteinatlas.org/ENSG00000187605-TET3/tissue/cervix#img> and <https://www.proteinatlas.org/ENSG00000187605-TET3/pathology/cervical+cancer#img>).

Differentially expressed genes (DEGs) were identified in CC tissue samples compared with normal tissue samples from the GSE7803 dataset using GEO2R software<sup>24</sup> (<https://www.ncbi.nlm.nih.gov/geo/geo2r/>). Genes with a fold change  $>0.5$  or  $>-0.5$  and a  $P < 0.05$  were considered as DEGs.

### 2.2. Analysis of mutations in TET3 protein

Identification of mutations in TET3 protein was performed in CC tissue samples from the TCGA dataset using the cBioPortal<sup>28</sup> (<https://www.cbioportal.org/>) database. The sample IDs are TCGA-EK-A2PG-01 (L173M), TCGA-IR-A3LK-01 (L189F), TCGA-IR-A3LK-01 (F886L), and TCGA-BI-A0VS-01 (E912K).

### 2.3. Analysis of copy number of *TET3* gene

Putative copy number alterations in the *TET3* gene, such as deletions and gains, were analyzed in CC tissue samples from the TCGA dataset using the cBioPortal database<sup>28</sup> (<https://www.cbioportal.org/>).

### 2.4. Analysis of diagnostic and prognostic value

The diagnostic value of miR-381-3p and TET3 expression was analyzed in CC and normal tissue samples from GSE86100 and GSE7803 datasets through receiver operating characteristic (ROC) curves using GraphPad Prism v8.2 software (GraphPad Software, USA). The area under the curve (AUC) with a confidence interval (CI) of 95% was calculated, and an AUC  $\geq 0.8$  with a  $P < 0.05$  was considered acceptable.<sup>32</sup> The sensitivity and specificity were determined according to the optimal cut-off using the Youden index.<sup>33</sup>

The prognostic value of miR-381-3p and TET3 expression was evaluated as overall survival (OS) and analyzed in tissue samples of patients with CC from the TCGA dataset using the Kaplan–Meier Plotter database<sup>34</sup> (<http://kmplot.com>). The median expression for each transcript was considered the cutoff value, based on which the patients were grouped into high- or low-expression groups. The hazard ratio and log-rank  $P$ -value were calculated, and a  $P < 0.05$  was considered statistically significant.

### 2.5. miRNAs target gene identification

MiR-381-3p targets were identified in miRDB<sup>35</sup> (<https://mirdb.org/>) and miPathDB V2.0<sup>36</sup> (<https://mpd.bioinf.uni-sb.de/>) databases. In these two databases, the targets in common were selected using a Venn diagram (<https://bioinformatics.psb.ugent.be/webtools/Venn/>).

### 2.6. Methylation analysis

The *TET3* promoter (−2000–+2000 bp relative to transcription start site [TSS]) was downloaded from Eukaryotic Promoter Database<sup>37</sup> (<https://epd.expasy.org/epd>) using the Expasy portal<sup>38</sup> (<https://www.expasy.org/>). The prediction of CpG islands in the *TET3* promoter was performed with the MethPrimer software<sup>39</sup> (<https://www.urogene.org/methprimer/>) considering a window >200, shift: 1, Obs/Exp >0.6, and GC% >50. The methylation level on the *TET3* promoter (2 kb upstream of TSS to 0.5 kb downstream) was analyzed in CC and normal tissue samples with the array-based technology (450k Illumina Infinium HumanMethylation450 BeadChip) in the DiseaseMeth v3.0 database<sup>40</sup> (<http://diseasemeth.edbc.org/>). Differences were determined with the Student's  $t$ -test, and a  $P < 0.05$  was considered statistically significant.

The prognostic value of methylation in the *TET3* gene was analyzed in CC tissue samples from the TCGA dataset in the MethSurv database<sup>41</sup> (<https://biit.cs.ut.ee/methsurv/>). The median was selected as the cut-off value. A hazard ratio with a 95% CI was calculated, and the differences were determined with the likelihood ratio test, and a  $P < 0.05$  was considered significant.

### 2.7. Correlation analysis

Correlation analysis between mRNA expression and DNA methylation was performed in CC tissue samples from the TCGA dataset using the cBioPortal database<sup>28</sup> (<https://www.cbioportal.org/>), while the correlation between TET3 and activator protein 2 $\alpha$  (AP-2 $\alpha$ ) expressions was analyzed using GEPIA<sup>27</sup> (<http://gepia.cancer-pku.cn/>) database. The correlation was analyzed using Pearson, Spearman, and  $R^2$  coefficients, and a  $P < 0.05$  was considered significant.

### 2.8. Identification of potential binding sites to transcription factors

The analysis of identification of potential binding sites to transcription factors in *TET3* promoter (−100 to +100 bp relative to TSS) was performed in ALGGEN-PROMO v8.3<sup>42</sup> ([https://alggen.lsi.upc.es/cgi-bin/promo\\_v3/promo/promoinit.cgi?dirDB=TF\\_8.3](https://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)) and Alibabab v2.1 (<http://gene-regulation.com/pub/programs/alibabab2/>) programs using the parameters by default. The common potential binding sites to transcription factors between these two programs were identified using a Venn diagram (<https://bioinformatics.psb.ugent.be/webtools/Venn/>).

### 2.9. Gene ontology (GO) and pathway analysis

GO and pathway analysis were carried out using the GO Biological Process 2023 and Kyoto encyclopedia of genes and genomes (KEGG) 2021 Human libraries from the Enrichr database<sup>43</sup> (<https://maayanlab.cloud/Enrichr/>). A  $P < 0.05$  was considered significant and  $-\log_{10}$ -transformed.

Gene Set Enrichment Analysis was performed with the DEGs using the WEB-based Gene Set Analysis Toolkit (WebGestalt) database<sup>44</sup> (<https://www.webgestalt.org/>) with the parameters by default. The enrichment score and normalized enrichment score were calculated, and the enriched gene sets with a  $P < 0.05$  and a false discovery rate of <0.05 were considered statistically significant.

## 3. Results

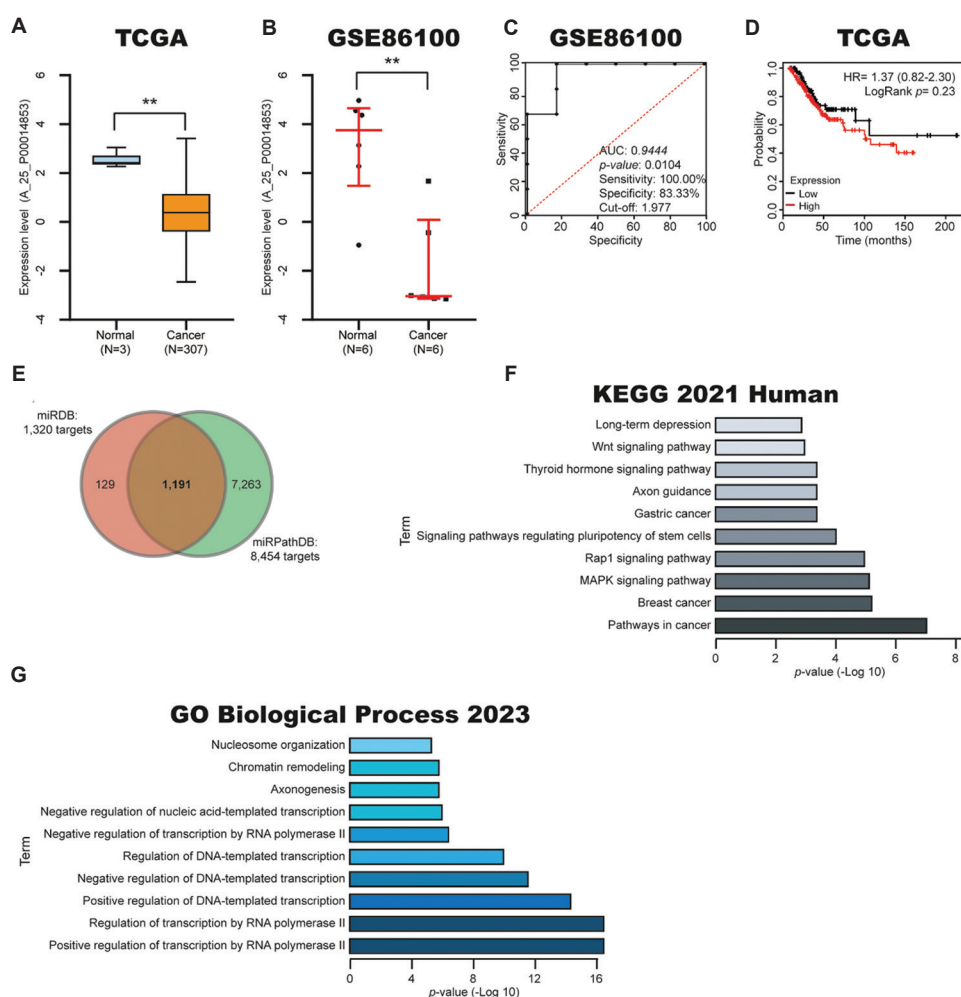
### 3.1. Decreased MiR-381-3p expression levels in CC

To analyze the miR-381-3p expression in CC, we compared its expression levels in CC tissue samples with those in normal tissue samples from the TCGA and GSE86100 datasets using the miR-TV and GEO databases,

respectively. The results showed that miR-381-3p expression is decreased in CC (Figure 1A and B).

To investigate the potential diagnostic and prognostic value of miR-381-3p expression in CC, we performed ROC curve analysis and Kaplan–Meier survival analysis using miR-381-3p expression data from the GSE86100 and TCGA datasets, analyzed with GraphPad Prism software and the Kaplan–Meier database. As shown in Figure 1C, the ROC curve revealed an AUC of 0.9444 with a  $P < 0.05$ , as well as a sensitivity and specificity of 100.00% and 83.33%, respectively (using a cut-off of 1.977). On the other hand, the Kaplan–Meier curve suggested that miR-381-3p expression is not associated with OS in patients with CC (Figure 1D).

To explore the potential role of miR-381-3p in CC, we first identified the target mRNAs of this miRNA in CC using miRDB and miRPathDB v2.0 databases. A total of 1,320 targets were identified in the miRDB database, while 8,454 targets were identified in the miRPathDB v2.0 database. Interestingly, we identified 1,191 common target mRNAs using a Venn diagram (Figure 1E). These mRNAs include transcripts from genes such as *ZNF318* and *ZNF619*, which encode transcription factors, and *TET2* and *TET3*, which encode demethylation enzymes. Next, we performed GO and pathway analyses on these 1,191 target mRNAs using KEGG 2021 Human and GO Biological Process 2023 libraries in the Enrichr database. As shown in Figure 1F, these target mRNAs participate



**Figure 1.** Decreased miR-381-3p expression in CC. MiR-381-3p expression was analyzed in CC tissue samples versus normal tissue samples from the (A) TCGA and (B) GSE86100 datasets, using the miR-TV database and GEO2R software. Significance was determined using the Mann–Whitney t-test.  $**P < 0.01$ . (C) Receiver operating characteristic curve and (D) Kaplan–Meier curve analysis of miR-381-3p expression in normal and CC tissue samples from GSE86100 and TCGA datasets, analyzed using GraphPad Prism software and Kaplan–Meier database. (E) Identification of miR-381-3p target mRNAs using the miRDB and miRPathDB databases. (F) KEGG pathway analysis and (G) GO Biological Process analysis using the 1191 target mRNAs identified in the KEGG 2021 Human and GO Biological Process 2023 libraries in the Enrichr database. The  $P$ -values were  $-\log_{10}$ -transformed. Abbreviations: CC: Cervical cancer; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.



in key pathways implicated in CC, such as the pathways in cancer, the mitogen-activated protein kinase (MAPK) signaling pathway, signaling pathways regulating the pluripotency of stem cells, and the Wnt signaling pathways. Interestingly, the GO analysis revealed that the identified 1191 target mRNAs participate in processes related to DNA transcription, such as positive regulation of transcription by RNA polymerase II, regulation of transcription by RNA polymerase II, positive regulation of DNA-templated transcription, and negative regulation of DNA-templated transcription (Figure 1G).

Overall, these results suggest that decreased miR-381-3p expression in CC inhibits key pathways and biological processes, indicating its potential utility as a diagnostic biomarker.

### 3.2. Increased TET3 expression in CC

Alterations in DNA demethylation play a key role in cervical carcinogenesis,<sup>45</sup> and previous studies have shown low expressions of TET1 and TET2 enzymes in CC.<sup>46,47</sup> However, the expression and role of TET3 in CC remain largely unknown. To analyze TET3 expression in CC, we compared its expression in CC tissue samples with normal tissue samples from the TCGA and GSE7803 datasets using the GEPIA and GEO databases, respectively. As shown in Figure 2A and B, TET3 expression is elevated in CC. To confirm this result, we analyzed TET3 protein expression using the HPA database, and similar results were obtained (Figure 2C). In addition, four missense mutations (L173M, L189F, F886L, and E912K) were identified in the TET3 protein in CC tissue samples from the TCGA dataset using the cBioPortal database (Figure 2D). Interestingly, the F886L and E912K mutations are located in the J-binding protein domain of the TET3 protein.

To investigate the potential role of TET3 in the progression of CC, we analyzed its expression according to the International Federation of Gynecology and Obstetrics (FIGO) stages and lymph node metastasis in CC tissue samples from the TCGA and GSE26511 datasets using the cBioPortal and GEO databases, respectively. The results revealed that TET3 expression is elevated in patients with stage II CC (Figure 2E) and those with lymph node metastasis (Figure 2F).

To evaluate the potential diagnostic and prognostic value of TET3 expression in CC, we performed ROC curve analysis and Kaplan-Meier survival analysis using TET3 expression data from the GSE7803 and TCGA datasets, analyzed with GraphPad Prism software and the Kaplan-Meier database, respectively. As shown in Figure 2G, we observed an AUC of 0.8762 with a  $P < 0.05$ , along with a sensitivity of 95.24% and specificity of 80.00%, using

a cut-off of 3.088 (Figure 2G). In addition, the Kaplan-Meier analysis demonstrated that high TET3 expression is associated with shorter OS in CC patients (Figure 2H).

Collectively, these results suggest that TET3 expression is increased in CC and may promote CC progression, making it a potential diagnostic and prognostic biomarker in patients with CC.

### 3.3. Promotion of TET3 expression in CC by copy number alterations and HPV infection

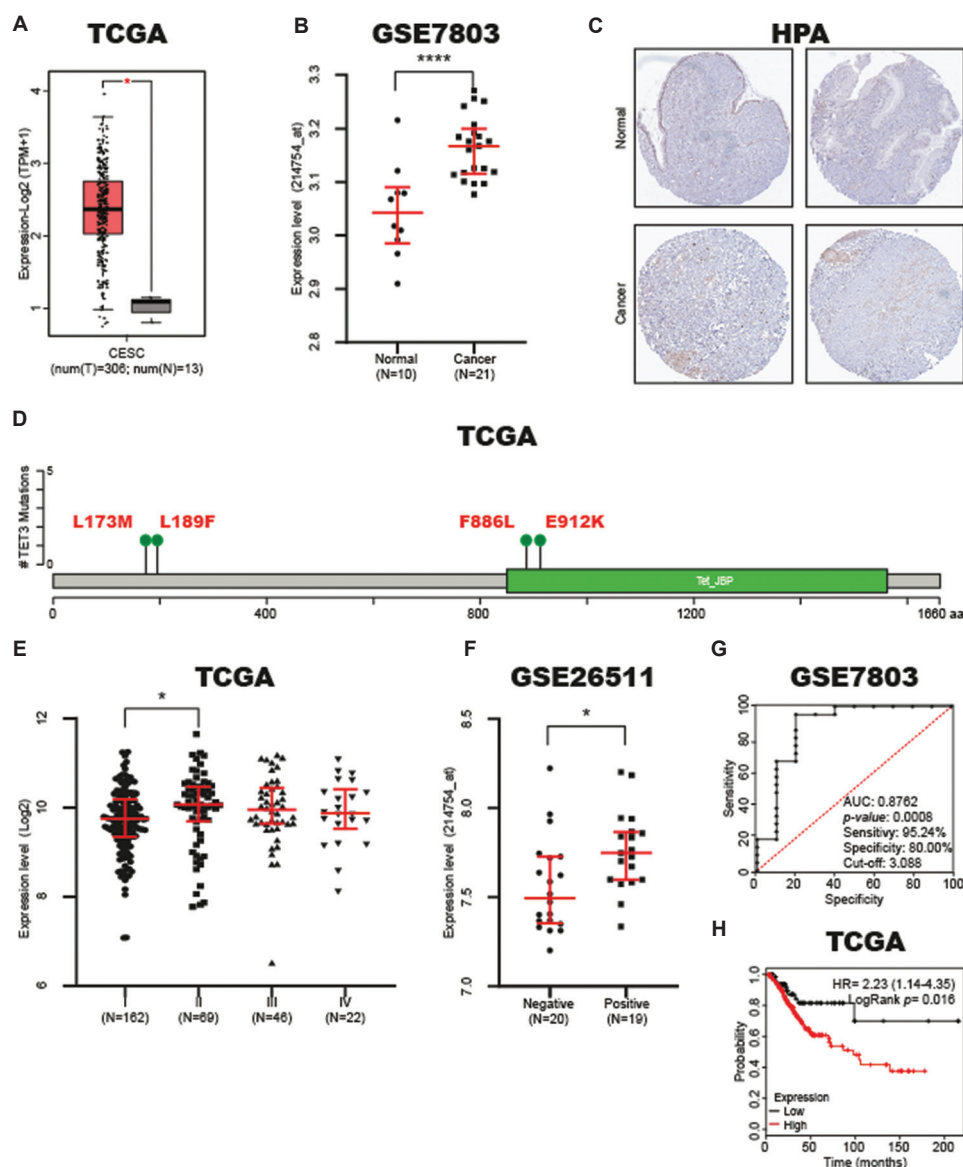
To investigate whether copy number alterations of the *TET3* gene contribute to its overexpression in CC, the CC tissue samples from the TCGA dataset were classified into three groups according to the copy number: Deletion, diploid, and gain. Interestingly, as shown in Figure 3A, 70.9% of CC tissue samples were included in the gain group. Next, we analyzed TET3 expression in these three groups and found that TET3 expression was significantly higher in the gain group (Figure 3B).

Given that high-risk HPV infection is the main risk factor for developing CC, we analyzed TET3 expression in CC tissue samples with HPV infection (16, 18, 16/18, and other genotypes) and compared them with normal tissue samples without HPV infection from the TCGA dataset using the cBioPortal database. The results indicate that TET3 expression is elevated in CC samples with HPV infection (Figure 3C).

These results suggest that both a gain in the copy number of the *TET3* gene and HPV infection may promote TET3 expression in CC.

### 3.4. Induction of TET3 expression by AP-2α transcription factor through aberrant methylation in its promoter in CC

Previous studies have identified that aberrant methylation in the *TET3* promoter inhibits its expression in melanoma and ovarian cancer<sup>48,49</sup>; however, whether aberrant methylation in the *TET3* promoter is associated with its overexpression in CC remains unknown. To investigate this, we first searched for the presence of a CpG island in the *TET3* promoter using MethPrimer software (Figure 4A). Next, we performed a correlation analysis between TET3 expression and methylation level in its promoter in CC tissue samples from the TCGA dataset using the cBioPortal database and found a negative correlation (Figure 4B). In addition, we found that the methylation level is decreased in CC tissue samples compared to normal tissue samples from the TCGA dataset, as analyzed in the DiseaseMeth database (Figure 4C). Interestingly, we identified four regions related to CpG island (Open\_Sea)—specifically three genomic regions in 3'UTR (cg13808088, cg02237855,

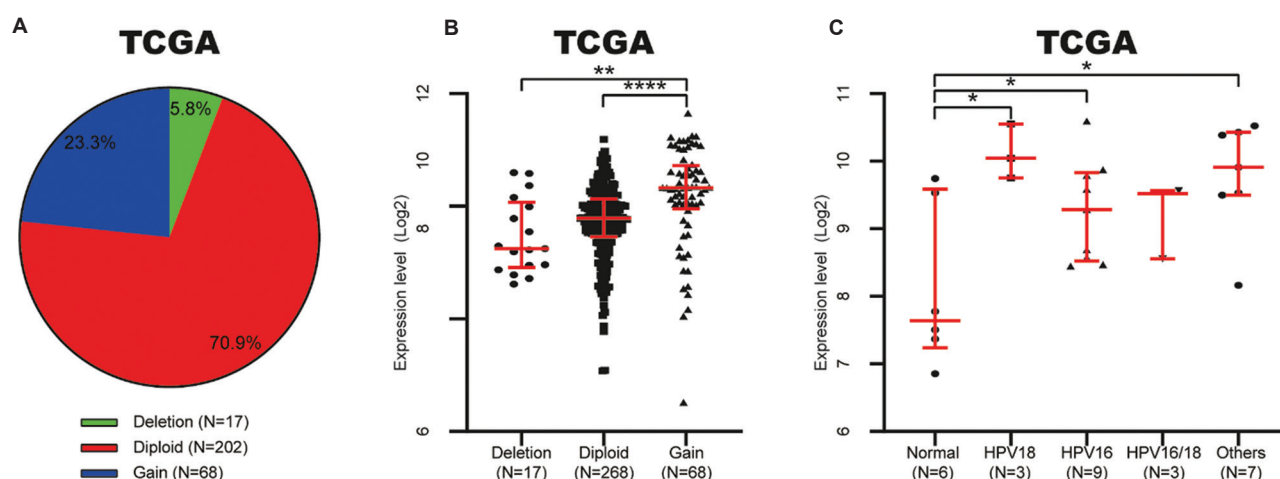


**Figure 2.** Increased TET3 expression in CC. TET3 expression was analyzed in CC tissue samples versus normal tissue samples from the (A) TCGA, (B) GSE7803, and (C) HPA datasets using the GEPIA database, GEO2R software, and the HPA database, respectively. Differences in expression levels were determined using a one-way analysis of variance and Mann-Whitney t-test. \* $P < 0.05$  and \*\*\*\* $P < 0.0001$ . (D) Identification of mutations in TET3 protein from CC tissue samples from the TCGA dataset using the cBioPortal database. A green circle with black line and red letters: Missense mutations. Green box: Oxygenase domain. X-axis: Numbers of aa. Y-axis: Frequency of mutations. (E) TET3 expression in CC tissue samples according to FIGO stage from TCGA dataset using cBioPortal database. The statistical differences were calculated using Mann-Whitney t-tests. \* $P < 0.05$ . (F) TET3 expression was analyzed in CC tissue samples with lymph node metastasis and compared to CC tissue samples without metastasis from GSE26511 using GEO2R software. Statistical differences were determined using Mann-Whitney t-tests. \* $P < 0.05$ . Analysis of (G) receiver operating characteristic and (H) Kaplan-Meier curves of TET3 expression in normal and CC tissue samples from GSE7803 and TCGA datasets using GraphPad Prism software and Kaplan-Meier database.

Abbreviations: aa: Amino acid; CC: Cervical cancer; FIGO: International federation of gynecology and obstetrics; GEPIA: Gene expression profiling interactive analysis; HPA: Human Protein Atlas; TCGA: The Cancer Genomes Atlas; TET3: Ten-eleven translocation 3.

and cg21855109) and one genomic region in the gene body (cg25299214)—that exhibit hypomethylation correlated with shorter OS in patients with CC from the TCGA dataset using the MethSurv database (Figure 4D and Table 1).

To identify potential transcription factors involved in TET3 overexpression in CC, we searched for binding sites in the *TET3* promoter using the ALGGEN-PROMO and Alibaba programs. We found 39 potential binding sites in



**Figure 3.** TET3 expression according to putative copy number alterations and HPV genotype in CC. (A) Percentage of putative copy number alterations (deletion and gain in copy number) in CC tissue samples from the TCGA dataset using the cBioPortal database. (B) TET3 expression according to putative copy number alterations (deletion and gain in copy number) compared with diploid. (C) TET3 expression in CC tissue samples with different HPV genotypes versus normal tissue samples from the TCGA dataset using the cBioPortal database. Differences in (B) and (C) were determined using Mann-Whitney t-tests. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

Abbreviations: CC: Cervical cancer; TCGA: The Cancer Genomes Atlas; TET3: Ten-eleven translocation 3.

ALGGEN-PROMO and 14 in Alibaba. Among these, we identified three transcription factors in both programs: AP-2 $\alpha$ , c-Ets-1, and NF- $\kappa$ B (Figure 4E). Interestingly, the AP-2 $\alpha$  expression was found to be increased (Figure 4F) and positively correlated with TET3 expression (Figure 4G) in CC tissue samples from the TCGA dataset, as analyzed using the GEPIA and cBioPortal databases, respectively.

These results indicate that aberrant methylation in the *TET3* promoter, combined with increased expression of the AP-2 $\alpha$  transcription factor, may induce TET3 overexpression in CC.

### 3.5. Alteration of key pathways by TET3 overexpression in CC

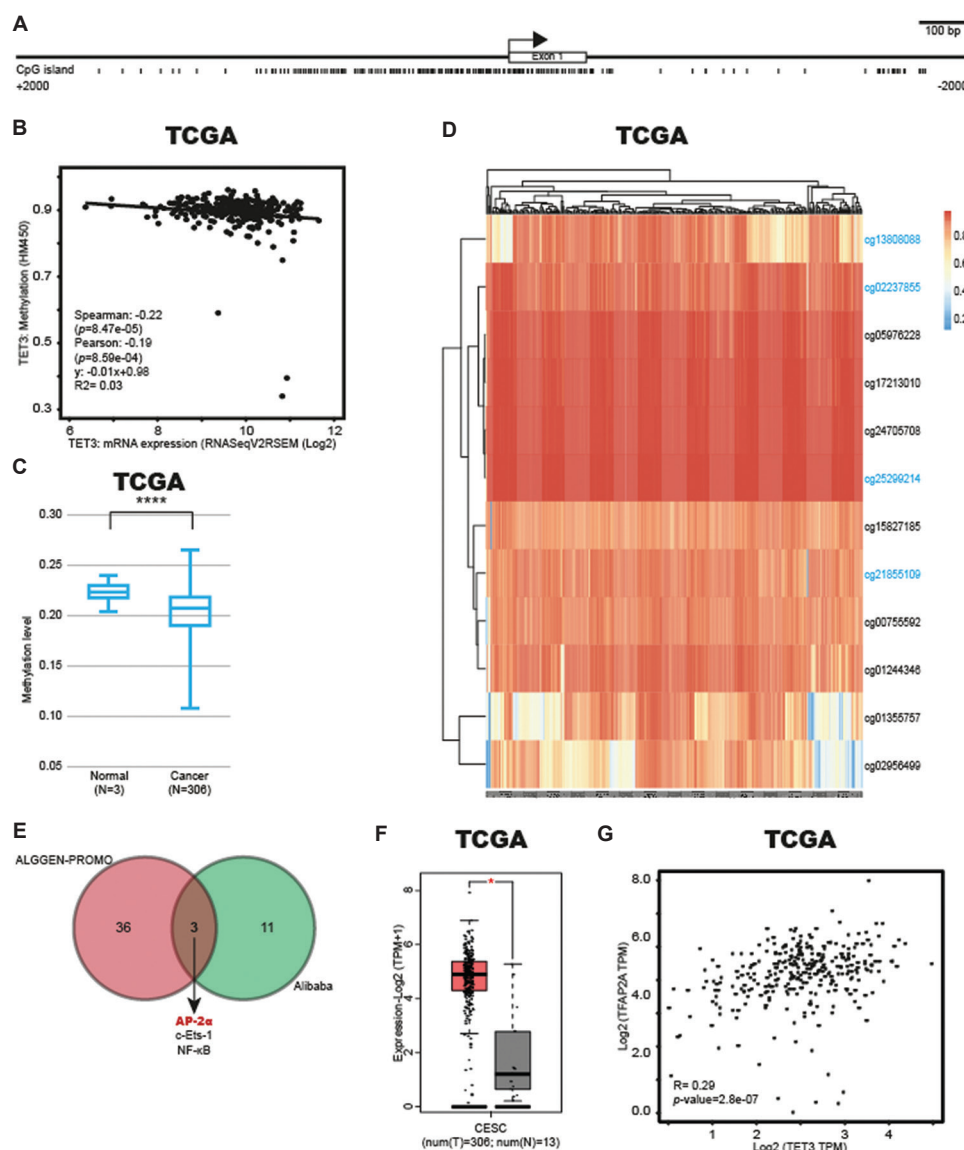
To explore the potential role of TET3 in CC, we employed two approaches: (i) identifying genes that positively (Spearman's correlation  $>0.44$  and  $P < 0.05$ ) and negatively (Spearman's correlation  $<0.4$  and  $P < 0.05$ ) correlate with TET3 expression in CC tissue samples from the TCGA dataset using the cBioPortal database; and (ii) identifying DEGs in CC tissue samples from GSE7803 dataset using GEO2R software. In the first approach, we identified a total of 787 genes that positively correlated with TET3 expression and 499 genes that negatively correlated. In the second approach, we identified 1846 overexpressed and 1,602 underexpressed genes. Interestingly, we found 112 genes that were both positively correlated with TET3 expression and overexpressed in CC (Figure 5A), as well as 33 genes that were both negatively correlated with TET3 expression and underexpressed in CC (Figure 5B).

These results suggest that TET3 promotes CC by positively regulating the expression of the 112 genes and negatively regulating the expression of the 33 genes.

To investigate the role of these 145 potential target genes of TET3 in CC, we performed GO and pathway analyses. As shown in Figure 5C, we found that the 112 genes participate in key pathways and biological processes related to CC, such as cell cycle, DNA replication, MAPK signaling pathway, and basal transcription factors (Figure 5C), as well as metabolic process, DNA-templated DNA replication, positive regulation of cell cycle process, and DNA damage response (Figure 5D). On the other hand, the 33 negatively regulated genes were found to be involved in pathways associated with other diseases, such as coronavirus, Parkinson's, and Huntington's diseases (Figure 5E). Moreover, the GO analysis suggested that these 33 genes are involved in biological processes, such as cytoplasmic translation, translation, ribosome biogenesis, and peptide biosynthetic process (Figure 5F).

To confirm these results, we performed a Gene Set Enrichment Analysis with the 3,448 DEGs using WebGestalt software. The results revealed enrichment in pathways related to the cell cycle (Figure 6A) and DNA replication (Figure 6B), as well as in biological processes such as telomere organization (Figure 6C), DNA recombination (Figure 6D), and double-strand break repair (Figure 6E).

Overall, these results suggest that TET3 overexpression deregulates gene expression and alters key pathways and biological processes to promote CC.



**Figure 4.** Increased AP-2α expression in CC induces TET3 expression. (A) Identification of a CpG island in the TET3 promoter using the MethPrimer software. (B) Correlation between methylation level in the TET3 promoter (Y-axis) and its expression (X-axis) in CC tissue samples from the TCGA dataset using the cBioPortal database. Correlation was analyzed using Pearson, Spearman, and R2 coefficients, and a  $P < 0.05$  was considered significant. (C) The methylation level in the TET3 promoter was analyzed in CC tissue samples and normal samples from the TCGA dataset using the DiseaseMeth v3.0 database. Differences were determined using a Student's t-test. \*\*\*\* $P < 0.0001$ . (D) Heatmap of methylation levels in TET3 using 12 probes of the 450k microarray in CC tissue samples from the TCGA dataset using the MethSurv database. X-axis: CC tissue samples. Y-axis: Methylation probes. Blue probes: Probes associated with OS. Blue scale: Low methylation. Red scale: High methylation. (E) Identification of binding sites to transcription factors in the TET3 promoter using the ALGGEN-PROMO and Alibaba v2.1 programs. (F) AP-2α expression was analyzed in CC tissue samples versus normal tissue samples from the TCGA dataset using the GEPIA database. Differences were determined using a one-way analysis of variance. \* $P < 0.05$ . (G) Correlation between TET3 expression (X-axis) and AP-2α (or TFAP2A, Y-axis) expression in CC tissue samples from the TCGA dataset using the GEPIA database. Abbreviations: CC: Cervical cancer; GEPIA: Gene expression profiling interactive analysis; OS: Overall survival; TCGA: The Cancer Genomes Atlas; TET3: Ten-eleven translocation 3.

#### 4. Discussion

CC ranks fourth in global cancer incidence and mortality among women. Projections over the next 25 years suggest that CC incidence will increase by 43.2% and mortality by 55.6% worldwide.<sup>1</sup> Therefore, it is necessary to investigate

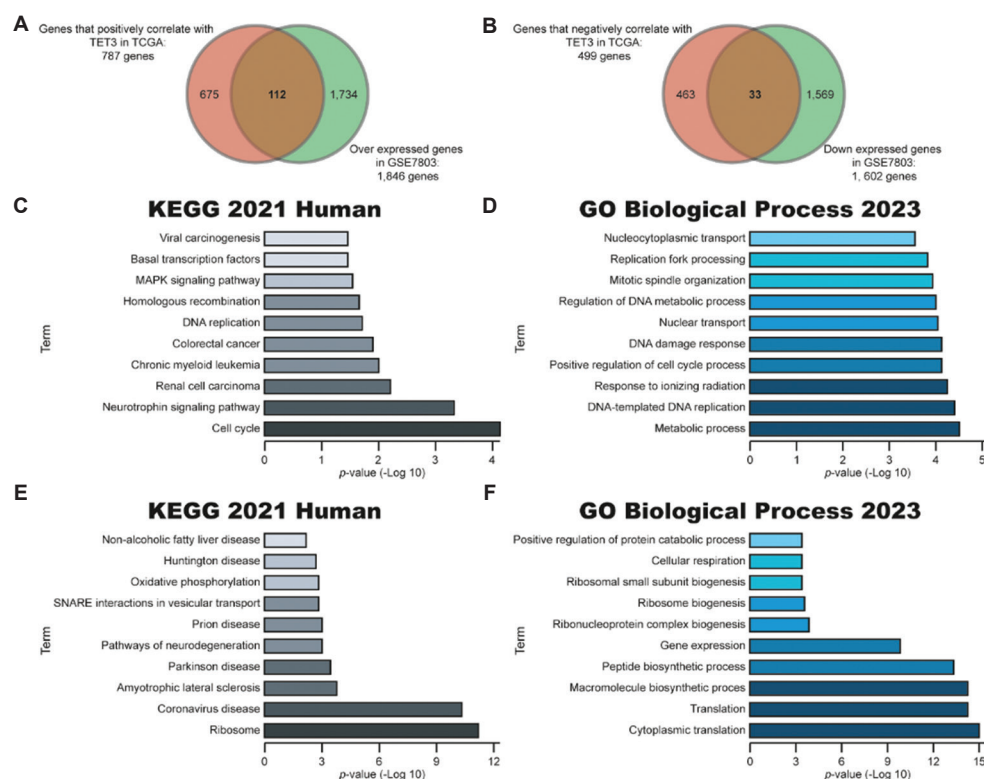
and understand the molecular mechanisms during the carcinogenesis, progression, and metastasis of CC, with the aim of proposing novel, more effective diagnostic and prognostic biomarkers, as well as identifying potential therapeutic targets for this cancer.



Table 1. Association between methylation level in *TET3* gene with overall survival in cervical cancer patients

Gene-CpG	Hazard ratio	Likelihood ratio test <i>P</i> -value	Methylation status
TET3-3'UTR-Open_Sea-cg02237855	0.591	0.034192138	Hypomethylation
TET3-3'UTR-Open_Sea-cg13808088	0.552	0.046156262	Hypomethylation
TET3-3'UTR-Open_Sea-cg21855109	0.601	0.036085864	Hypomethylation
TET3-Body-Open_Sea-cg25299214	0.571	0.028813106	Hypomethylation

Abbreviations: TET3: Ten-eleven translocation 3; CpG: Cytosines that precede guanine.



**Figure 5.** Identification of target genes of TET3 and their pathways and biological processes in CC. Identification of (A) positively and (B) negatively regulated target genes by TET3 in CC from the TCGA and GSE7803 datasets. (C) KEGG pathway and (D) GO biological process analysis using the KEGG 2021 Human and GO Biological Process 2023 libraries in the Enrichr database, taking into account the 112 genes positively regulated by TET3. The *P*-values were  $-\log_{10}$ -transformed. (E) KEGG pathway and (F) GO biological process analysis using the KEGG 2021 Human and GO Biological Process 2023 libraries in the Enrichr database, taking into account the 33 genes negatively regulated by TET3. The *P*-values were  $-\log_{10}$ -transformed.

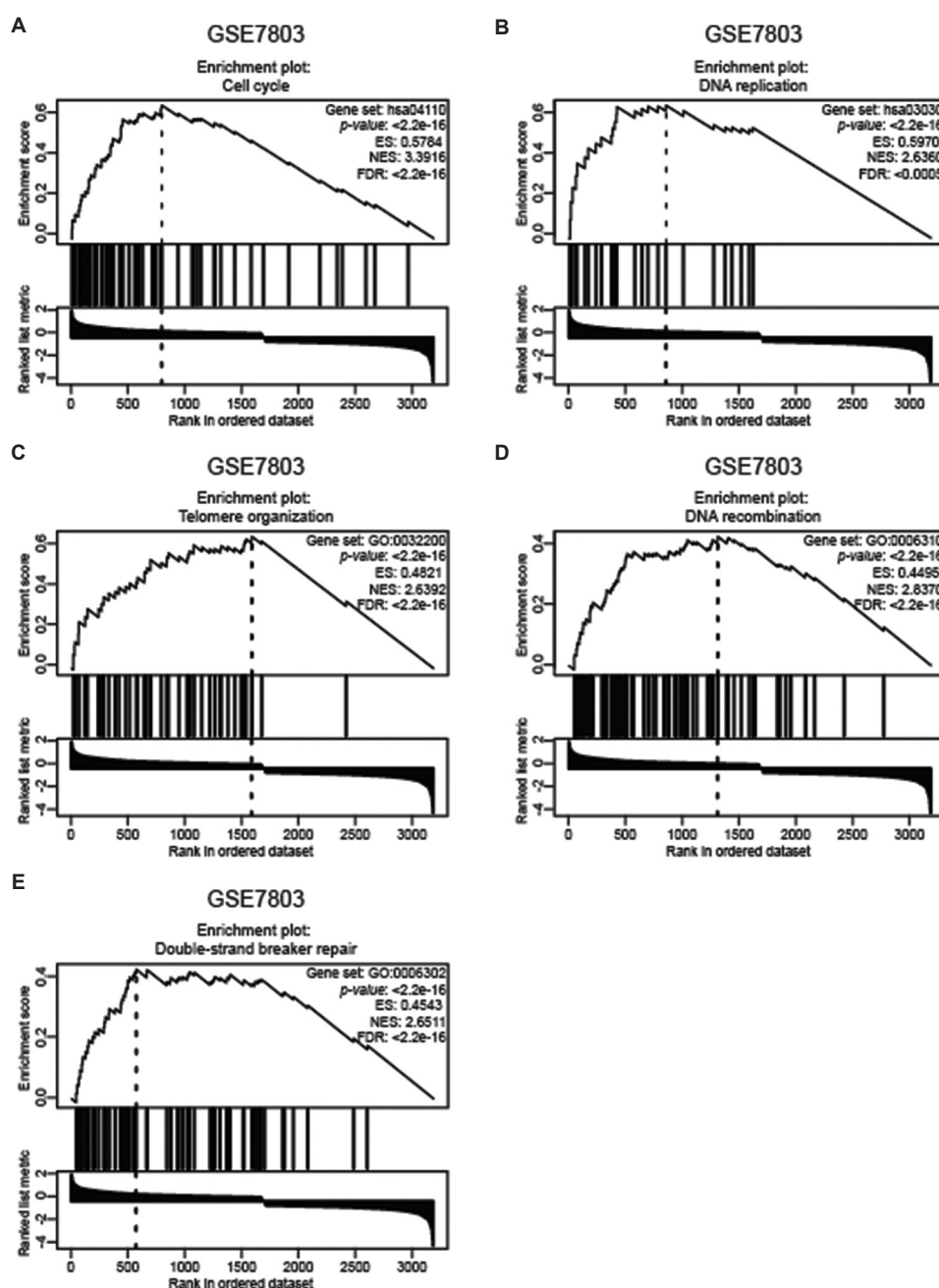
Abbreviations: CC: Cervical cancer; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; TCGA: The Cancer Genomes Atlas; TET3: Ten-eleven translocation 3.

In the last two decades, several groups have studied miRNAs in different pathophysiological contexts and shown that these molecules act as tumor suppressor miRNAs or oncomiRNAs.<sup>50-53</sup> Previous studies have reported the role of miR-381-3p expression in several types of cancer, such as oral squamous cell carcinoma, papillary thyroid carcinoma, head-neck squamous cell carcinoma, colorectal cancer, ovarian cancer, non-small cell lung cancer, bladder cancer, breast cancer, and CC.<sup>23,54-61</sup> However, the role of miR-381-3p in CC is still largely unknown.

In this work, we analyzed the miR-381-3p expression in CC through a systematic bioinformatic analysis, and our

results show that miR-381-3p expression is decreased in CC, consistent with a previous study.<sup>23</sup> On the other hand, a study reported that low exosomal miR-377-3p and miR-381-3p levels in serum samples of patients with colorectal cancer improved the diagnostic efficacy.<sup>62</sup> Similarly, we found that low miR-381-3p expression in tissue samples of CC patients may be useful as a diagnostic biomarker. However, this result needs to be validated in a larger cohort of samples.

Several studies have reported the molecular mechanisms of miR-381-3p in cancer.<sup>23,54-61</sup> miR-381-3p decreases the invasion, migration, epithelial-mesenchymal



**Figure 6.** Gene Set Enrichment Analysis of potentially regulated genes by TET3 in cervical cancer. Potential genes regulated by TET3 were enriched in (A) cell cycle and (B) DNA replication pathways, as well as (C) telomere organization, (D) DNA recombination, and (E) double-strand break repair biological processes. Enrichment score and normalized enrichment score were calculated. A  $P < 0.05$  and a false discovery rate of  $<0.05$  were considered significant. Abbreviation: TET3: Ten-eleven translocation 3.

transition, proliferation, and tumor growth *in vivo*, and increases cell cycle arrest and apoptosis by TWIST1, FGFR2, RAB2A, and SOX4 downregulation in colorectal cancer cells, oral squamous cell carcinoma cells, bladder cancer cells, and breast cancer cells.<sup>54,55,60,61</sup> Interestingly, miR-381-3p inhibits the CXCR4 expression, decreasing the resistance to anti-PD-1-based therapy in non-small

cell lung cancer cells.<sup>56</sup> On the other hand, miR-381-3p decreases cell proliferation, migration, and invasion through YY1, LRP6, and NASP downregulation in ovarian cancer, papillary thyroid carcinoma, and head-neck squamous cell carcinoma cells, respectively.<sup>57-59</sup> Recently, it was reported that miR-381-3p decreases FGF7 expression, inhibiting cell proliferation and metastasis,

and inducing cell cycle arrest and apoptosis in CC cells.<sup>23</sup> In this study, we found 1191 potential targets in two databases, including targets previously reported and experimentally validated.<sup>23,54-61</sup> KEGG pathway and GO biological process analysis revealed that these 1191 potential targets participate in key signaling pathways and biological processes in CC, including transcriptional regulation, a process of response to intra and extracellular signals that defines the identity and coordinates the cell activity under specific conditions, such as malignant cellular transformation.<sup>63</sup> Interestingly, the most enriched term in GO Biological Process, identified as positive regulation of transcription by RNA polymerase II (GO: 0045944), contains 124 potential target genes, including two enzymes called TET2 and TET3 that participate in DNA demethylation, a key cellular event in aberrant silencing of tumor suppressor genes in cancer.<sup>15</sup> Previously, a study reported a low TET2 expression but no altered TET3 expression in cervical squamous cell carcinoma tissue samples compared with normal tissue samples.<sup>64</sup> Another study analyzed TET2 and TET3 expression in CC tissue samples compared with normal tissue samples, but their results did not show an aberrant expression of these enzymes. However, statistical analysis showed a low TET2 expression in stage II, as well as a low TET3 expression in stage III, G3 differentiation grade, and squamous type compared with normal tissue samples.<sup>46</sup> These inconsistent results show that TET2 and TET3 expression in CC is unclear, particularly for TET3, which is the focus of this paper. In this work, we found a high TET3 expression in CC tissue samples compared with normal tissue samples based on two independent datasets; moreover, these results were validated to protein level in a third independent dataset, reinforcing the idea that TET3 is a target of miR-381-3p and that low miR-381-3p expression induces TET3 overexpression in CC. Conversely, previous studies did not find an altered TET3 expression, which could be explained by RNA quality, analysis method, and statistical test. Interestingly, we identified four mutations in the TET3 protein, of which two were located in the J-binding protein domain, a key domain to its catalytic activity.<sup>16</sup> In addition, we observed an increase in TET3 expression in FIGO stage II and lymph node metastasis, suggesting that TET3 promotes the progression and metastasis in CC. Finally, high TET3 expression may be useful as a diagnostic and prognostic biomarker in CC patients, consistent with previous reports in ovarian, breast, and esophageal squamous cell carcinoma.<sup>65-67</sup>

In this study, we analyzed other molecular mechanisms involved in TET3 overexpression, and we found, for the 1<sup>st</sup> time, a high TET3 expression in patients with a gain in copy number compared with those with diploid in copy number. In addition, we found a high TET3 expression in the presence of HPV, suggesting that HPV infection promotes TET3 overexpression in patients with CC. On the other

hand, a previous study reported a methylation aberrant in *TET3* promoter in CC cell lines.<sup>68</sup> Conversely, we found a decrease in the methylation level of the *TET3* promoter in CC tissue samples; these differences could be explained by the region analyzed, the technique used, and the type of samples.<sup>69,70</sup> Moreover, we found that methylation level positively correlates with TET3 expression level, consistent with our results of TET3 expression. In addition, we found four regions with hypomethylation on the *TET3* gene that correlate with shorter OS in patients with CC. Finally, we found that the AP-2 $\alpha$  transcription factor has binding sites on the *TET3* promoter; its expression is increased and correlates with TET3 expression in CC. Consistent with these results, previous studies have shown that the AP-2 $\alpha$  transcription factor acts as an oncogene in CC.<sup>71,72</sup>

TET3 is a dioxygenase enzyme that catalyzes oxidation of 5mC to 5hmC<sup>15</sup> in several diseases, including cancer.<sup>73</sup> In this study, we found a TET3 overexpression in CC; however, low 5hmC levels in CC tissue samples have been reported,<sup>47</sup> suggesting that TET3 may have an independent catalytic function in CC. In this sense, transcriptional activation or repression independent of TET3 catalytic activity has been previously reported.<sup>74-76</sup> Therefore, we explored the TET3 function in a manner independent of its catalytic activity in CC and identified a total of 112 genes that positively correlate with TET3 expression and are upregulated in CC, suggesting that TET3 induces their expression. Surprisingly, we observed that these genes participate in several critical pathways and processes in CC,<sup>77-79</sup> including cell cycle, DNA replication, MAPK signaling pathway, and basal transcription factors, as well as metabolic process, DNA-templated DNA replication, positive regulation of cell cycle process, and DNA damage response. In addition, we identified 33 genes that negatively correlate with TET3 expression and are downregulated in CC, suggesting that TET3 inhibits their expression. Interestingly, we found that these genes participate in other diseases, such as Coronavirus, Parkinson's, and Huntington's diseases, as well as processes associated with these diseases, including cytoplasmic translation, translation, ribosome biogenesis, and peptide biosynthetic process.

The main limitation of our bioinformatic study is the lack of experimental validation; however, all software, databases, and sets of samples used come from curated, scientific, and reliable sources and are supported by original research papers. Moreover, the statistical analysis was appropriate for each stage. Finally, all our results were consistent across different cohorts, supporting the validity of our findings.

## 5. Conclusion

In this study, we analyzed the role of miR-381-3p in CC through bioinformatic analysis. Our results suggest that

miR-381-3p acts as a potential tumor suppressor by inhibiting the expression of genes involved in pathways and processes that promote CC. Moreover, we identified the miR-381-3p/TET3 axis in CC. Our results show that *TET3* is overexpressed in CC, acting as a potential oncogene by upregulating the expression of genes involved in key pathways and processes in CC. These findings indicate that low miR-381-3p expression and high *TET3* expression may serve as valuable diagnostic and prognostic biomarkers for patients with CC.

## Acknowledgments

None.

## Funding

None.

## Conflict of interest

The authors declare they have no competing interests.

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## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Availability of data

Data used in this work are available from the corresponding author on reasonable request.

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