

ORIGINAL RESEARCH ARTICLE

Integrated bioinformatic analysis of *RNH1* role in cervical cancer: Methylation, expression, prognostic value, diagnostic value, and association with immune infiltration

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Abstract

Cervical cancer (CC) is the fourth leading cause of cancer incidence and mortality among women worldwide. Currently, the survival rate in patients with advanced CC remains low. Thus, it is necessary to investigate the molecular mechanisms involved in CC. *RNH1* has been reported as a tumor suppressor gene in several cancer types; its role in CC remains unclear. In this study, we analyzed the role of *RNH1* in CC using a bioinformatics approach. Methylation of the *RNH1* promoter was investigated using data from the Cancer Genome Atlas (TCGA) and GSE30760 datasets in the DiseaseMeth and Gene Expression Omnibus (GEO) databases. *RNH1* expression was evaluated in samples from TCGA, GSE9750, GSE7803, GSE67522, and the Human Protein Atlas (HPA) datasets in the University of Alabama at Birmingham Cancer Data Analysis Portal, GEO, and HPA platforms. The prognostic and diagnostic value of *RNH1* expression was assessed using Kaplan–Meier and receiver operating characteristic curves. Genes correlated with *RNH1* expression in CC were identified using the cBioPortal database. Pathway and Gene Ontology (GO) enrichment analyses were performed using Enrichr. Finally, immune infiltration analysis was conducted using the Tumor Immune Estimation Resource database. Our results showed that the *RNH1* promoter is aberrantly methylated in CC. Decreased *RNH1* expression could be used as a prognostic and diagnostic biomarker in CC. Pathway and GO analyses identified terms associated with CC pathways and biological processes related to immune response. Furthermore, immune infiltration analysis demonstrated that *RNH1* expression correlates with the presence of various immune cell types. In conclusion, our findings indicate that *RNH1* plays a key role in CC.

Keywords: *RNH1*; Methylation; Biomarker; Immune infiltration; Cervical cancer; Bioinformatic analysis

1. Introduction

Cervical cancer (CC) ranks in fourth position in both incidence and mortality among women globally, accounting for 6.8% of new cancer cases and 8.1% of cancer-related deaths. 661,021 new cases and 348,189 deaths from CC were reported globally in 2022.¹ The American Cancer Society (ACS) estimated 13,360 new cases and 4,320 deaths in the USA in 2025.² Persistent infection with high-risk (HR) human papillomavirus (HPV), such as HPV16 and HPV18 genotypes, is the primary etiological factor in cervical carcinogenesis.³ Currently, diagnosis relies on screening and clinical procedures, including Papanicolaou testing, cervical cytology, HPV testing, and colposcopy.⁴ Treatment for patients with CC is based on disease stage and includes surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy.^{5,6} Unfortunately, the 5-year relative survival rate remains steady at 91% for early-stage CC; however, this percentage decreases to 60% for late-stage diagnoses.⁷ Thus, it is essential to understand the molecular mechanisms underlying this disease's biology.

Ribonuclease inhibitor 1 (RI1) is a 50-kDa cytosolic protein identified in 1952.⁸ This protein belongs to the cytosolic RNase inhibitor family and inhibits the ribonucleolytic activity of the pancreatic ribonuclease superfamily (RNase A), including angiogenin, by direct interaction. Moreover, RI1 binds to 40S ribosomes, thereby regulating protein translation.⁹ Several studies have reported that RI1 acts as a tumor suppressor in various cancers, including bladder,¹⁰ lung,¹¹ melanoma,¹² colorectal,¹³ and breast cancer.¹⁴ RI1 participates in the regulation of cellular processes, including proliferation, migration, invasion, apoptosis, cell cycle, autophagy, and tumor growth.^{11–13} Mechanistically, the *RNH1* gene, which encodes RI1, overexpression inhibits the protein kinase B (AKT)/mechanistic target of rapamycin (mTOR)/Unc-51-like autophagy activating kinase 1 signaling pathway, promoting autophagy-related gene-dependent autophagy in HT29 cells.¹³ In contrast, another study found that *RNH1* is overexpressed in gastric cancer cells resistant to histone deacetylase inhibitors, where it contributes to reduced reactive oxygen species levels. Despite these findings, the role of *RNH1* in CC remains to be elucidated.

In this work, the role of *RNH1* in CC was analyzed utilizing a systematic bioinformatic approach based on multiple public databases, including The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO), and the Human Protein Atlas (HPA). Our analysis included promoter methylation, gene expression, the diagnostic and prognostic value of *RNH1*, and the signaling pathways and biological processes (BPs) associated with this gene in CC.

2. Materials and methods

2.1. Island cytosine-phosphate-guanine prediction

The data of the *RNH1* promoter was downloaded from the Eukaryotic Promoter Database¹⁵ hosted on the ExPasy server.¹⁶ Subsequently, we analyzed the *RNH1* promoter ($\pm 2,000$ bp relative to the transcription start site) with Methprimer software¹⁷ (Version 2.0, Ractigen Therapeutics, USA) using the default parameters, except for the window size, which was set to 200.

2.2. Methylation analysis

The TCGA and GSE30760¹⁸ datasets from DiseaseMeth V3.0¹⁹ and GEO²⁰ databases were used for the analysis of methylation levels in the *RNH1* promoter (cytosine-phosphate-guanine [CpG] island). The TCGA dataset (450K [Illumina Infinium Human Methylation 450 BeadChip; HM450]) contains data from three normal and 307 CC tissue samples. The GSE30760 dataset (Platform: GPL8490, Illumina Human Methylation 27 Bead Chip [Human Methylation 27_270596_v.1.2]) comprises data of 152 normal and 63 CC tissue samples. For the TCGA analysis, the parameters selected are shown in Table 1. For the GSE30760 analysis, the dataset was analyzed in GEO2R²¹ using the default parameters.

2.3. Correlation analysis

The correlation analysis between *RNH1* methylation (HM450) and expression levels (RNA sequencing version 2 data quantified using RNA-Seq by expectation-maximization, Log₂ values) was analyzed in 310 CC tissue samples from the TCGA dataset using the cBioportal database^{22,23} with default parameters.

2.4. Expression analysis

RNH1 levels were evaluated in samples from the TCGA, GSE9750²⁴, GSE7803²⁵, GSE67522^{26,27} and HPA²⁸ datasets using The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN)^{29,30}, GEO²⁰ and HPA databases. The TCGA dataset included three non-tumorigenic and 305 cancer tissue samples. The GSE9750 dataset (GPL96 [HG-U133A] Affymetrix Human Genome U133A Array) contains 21 normal and 33 cancer tissue samples. The GSE7803 dataset (GPL96 [HG-U133A] Affymetrix Human Genome U133A Array) includes 10 controls and 21 tumorigenic tissue samples. The GSE67522 dataset (GPL10558 Illumina HumanHT-12 V4.0 expression beadchip) comprised 22 non-tumorigenic and 20 tumorigenic tissue samples. Two representative images of CC and normal tissue samples from the HPA database are shown. The *RNH1* expression was detected using HPA039223 and HPA040781 antibodies. The GSE9750,

GSE7803, and GSE67522 datasets were analyzed using GEO2R.²¹

Table 1. Parameters selected in the Cancer Genome Atlas analysis

Parameters	Value
Disease	CESC
Gene symbol	<i>RNH1</i>
Array-based technology	450K
Control type	Controls from the same tissue/ cell line
Method of differential analysis	<i>t</i> -test
Significant <i>p</i> -value	<0.05
Absolute methylation difference	>0.2
Multimatched	NM_002939
Select region of interest	Promoter
Definition of promoter	2 kb upstream of the TSS to 0.5 kb downstream relative to the TSS, and a CpG island in the promoter

Abbreviations: CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CpG: Cytosine-phosphate-guanine; TSS: Transcription start site.

2.5. Survival analysis

Overall survival (OS) and disease-free survival (DFS) analyses were performed with the TCGA dataset in the Gene Expression Profiling Interactive Analysis (GEPIA) database.³¹ Patients with CC were classified according to *RNH1* levels in high ($n = 146$) or low ($n = 146$) based on the median *RNH1* expression level as the cut-off value. Glyceraldehyde-3-phosphate dehydrogenase expression was used to normalize *RNH1* expression.

2.6. Analysis of receiver operating characteristic curves

The *RNH1* expression levels in cancer and non-tumorigenic tissue samples from the GSE9750,²⁴ GSE7803,²⁵ and GSE67522^{26,27} datasets were used to perform the receiver operating characteristic (ROC) curve analysis in GraphPad Prism (V8.0.0, GraphPad Software, Inc., USA). The area under the curve (AUC) was calculated with a 95% confidence interval (CI). AUC values were interpreted as follows: $AUC \geq 0.9$, excellent; $0.8 \leq AUC < 0.9$, considerable; and $0.7 \leq AUC < 0.8$, fair.³² Sensitivity and specificity were determined using a cut-off specific to each dataset, based on the Youden index.³³

2.7. Biological processes and pathways analysis

The Enrichr database³⁴ was used for BP and pathways analyses. Kyoto Encyclopedia of Genes and Genomes

(KEGG) and Molecular Signatures Database (MSigDB) libraries were selected for pathway analysis, while Gene Ontology (GO) BP was selected for process analysis. The significant terms found were selected, and their *p*-values were transformed to $-\text{Log}_{10}$ for visualization.

2.8. Immune infiltration analysis

Immune infiltrates and *RNH1* expression were used to evaluate their correlation in the cervical squamous cell carcinoma and endocervical adenocarcinoma dataset in the Tumor Immune Estimation Resource (TIMER) database.^{35,36} The immune infiltrates analyzed included B cells, CD8⁺ T cells, CD4⁺ T cells, macrophages, neutrophils, and dendritic cells.

2.9. Statistical analysis

The differences were determined using the Student's *t*-test for methylation and expression analysis. The Spearman and Pearson correlation coefficients were determined for the correlation analysis. The Log-rank test and hazard ratio were calculated to assess differences in survival. For analysis of immune infiltration, purity-corrected partial Spearman's rho values were calculated, and *p*-values < 0.05 were considered statistically significant. For all analyses, a *p*-value < 0.05 was considered statistically significant.

3. Results

3.1. Methylation in the *RNH1* promoter in cervical cancer

We identified a CpG island in the *RNH1* promoter using MethPrimer software (Figure 1A). Subsequently, we quantified the methylation levels in this CpG island in CC and control samples from the TCGA dataset using the DiseaseMeth database. We found that the *RNH1* promoter was hypermethylated in CC tissue samples (Figure 1B). These results were validated in CC samples from the GSE30760 dataset (two regions) (Figure 1C and 1D). Finally, the correlation between *RNH1* methylation and expression was analyzed in CC tissue samples from the TCGA dataset through the cBioPortal database. Interestingly, the results revealed a negative correlation between methylation and expression of *RNH1* in CC tissue samples (Spearman coefficient: -0.25 , *p*-value: 1.414×10^{-5} ; Pearson coefficient: -0.23 , *p*-value: 6.980×10^{-5}) (Figure 1E). These results strongly suggest that the *RNH1* promoter is hypermethylated in CC, potentially negatively regulating its expression.

3.2. Expression of *RNH1* in cervical cancer

To evaluate whether *RNH1* expression was altered in CC, its expression was quantified in CC and normal

tissue samples from the TCGA dataset in the UALCAN database. Surprisingly, the expression of *RNH1* decreased in CC (Figure 2A). Consistently, we found similar results in CC samples from GSE9750, GSE7803, and GSE67522 datasets using the GEO database (Figure 2B, 2C, and 2D), supporting the results in Figure 2A. In addition, *RNH1* expression was evaluated at the protein level in the HPA database, yielding similar results (Figure 2E). These results, obtained from five independent databases of mRNA and protein expression levels, strongly suggest that *RNH1* expression is decreased in CC compared to non-cancerous cells.

3.3. Expression of *RNH1* in patients with cervical cancer and human papillomavirus infection

To explore the role of HPV infection in *RNH1* expression in CC, the GSE9750 samples were classified into normal, HPV16⁺ CC, and HPV18⁺ CC groups. Interestingly, we found that patients with CC and HPV16 or HPV18 infection presented a low *RNH1* expression (Figure 3A). On the other hand, samples from the GSE67522 dataset were classified into normal, HPV16⁺ normal, and HPV16⁺ CC groups. As shown in Figure 3B, *RNH1* levels decreased only in HPV16⁺ CC patients. These results suggest that HPV infection may regulate *RNH1* expression in the CC context.

3.4. *RNH1* expression as a potential prognostic and diagnostic biomarker in cervical cancer

To explore the clinical significance of *RNH1* expression in CC patients, OS and DFS were evaluated using Kaplan–Meier curves in the GEPIA database for CC tissue samples from the TCGA dataset. Interestingly, CC patients with low *RNH1* expression exhibited poorer OS (Figure 4A). However, no significant association was observed between *RNH1* expression and DFS (Figure 4B).

On the other hand, the diagnostic value of *RNH1* expression was evaluated in CC and normal tissue samples from the GSE9750, GSE7803, and GSE67522 datasets by ROC curves using GraphPad Prism. Surprisingly, we found an AUC of 0.9293 with a *p*-value: < 0.0001, a sensibility of 87.88% and a specificity of 85.71% for GSE9750 dataset (Figure 4C), an AUC of 0.9286 with a *p*-value: 0.0001, a sensibility of 90.48% and a specificity of 90.00% for GSE7803 dataset (Figure 4D), and an AUC of 0.8205 with a *p*-value: 0.0004, a sensibility of 80.00% and a specificity of 68.18% for GSE7803 dataset (Figure 4E).

These findings suggest that *RNH1* expression may be clinically relevant in CC.

3.5. Association of *RNH1* expression with key signaling pathways to cervical cancer

To investigate the role of *RNH1* in CC, a correlation analysis was performed to identify genes that correlate with *RNH1* expression in CC samples from the TCGA dataset using the cBioPortal database. *RNH1* expression correlates with 1,521 genes in total. Specifically, 994 genes showed negative correlation with *RNH1* expression, while 527 showed positive correlation (Table S1). Pathway enrichment analysis using these 1,521 genes with KEGG and MSigDB libraries in the enrichment database revealed that these genes participated in signaling pathways associated to synthesis or metabolism of proteins, such as ribosome and proteasome, to processes associated to cancer, including apoptosis and viral carcinogenesis, bladder cancer and acute myeloid leukemia, as well as infections of agent pathogens, as pathogenic *Escherichia coli* infection, bacterial invasion of epithelia cells, and shigellosis (Figure 5A). Consistently, the pathways analysis using a second MSigDB library revealed that genes that correlated with *RNH1* participate in key pathways to cervical carcinogenesis, such as the p53 pathway, phosphoinositide 3-kinase/AKT/mTOR signaling, and mTOR complex 1 (mTORC1) signaling, as well as pathways associated with immune response, including complement and interferon gamma response (Figure 5B). Finally, GO BP analysis (Figure 5C) identified BPs associated with positive regulation of the canonical Wnt signaling pathway. In addition, several BP associated with the immune response were observed, including the interleukin-1-mediated signaling pathway, the cytokine-mediated signaling pathway, the tumor necrosis factor-mediated signaling pathway, and the cell surface receptor signaling pathway that activated the innate immune response. These results suggest that in patients with CC, *RNH1* may participate in BP associated with the immune response.

3.6. Correlation of *RNH1* expression with immune infiltration in cervical cancer

The tumor niche is key to tumorigenesis, progression, and metastasis, as well as to treatment response and patients' prognosis.^{37,38} Immune infiltration plays a fundamental role in cancer development, including CC.^{39,40} Therefore, we analyzed the association between *RNH1* expression and immune infiltration levels in the TIMER database. We identified that *RNH1* expression levels positively correlated with CD4⁺ T lymphocytes, neutrophils, and dendritic cells, while a negative correlation was observed with macrophages (Figure 6). Our findings suggest that *RNH1* may participate in the recruitment of these cells or be expressed by them, promoting an inflammatory microenvironment.

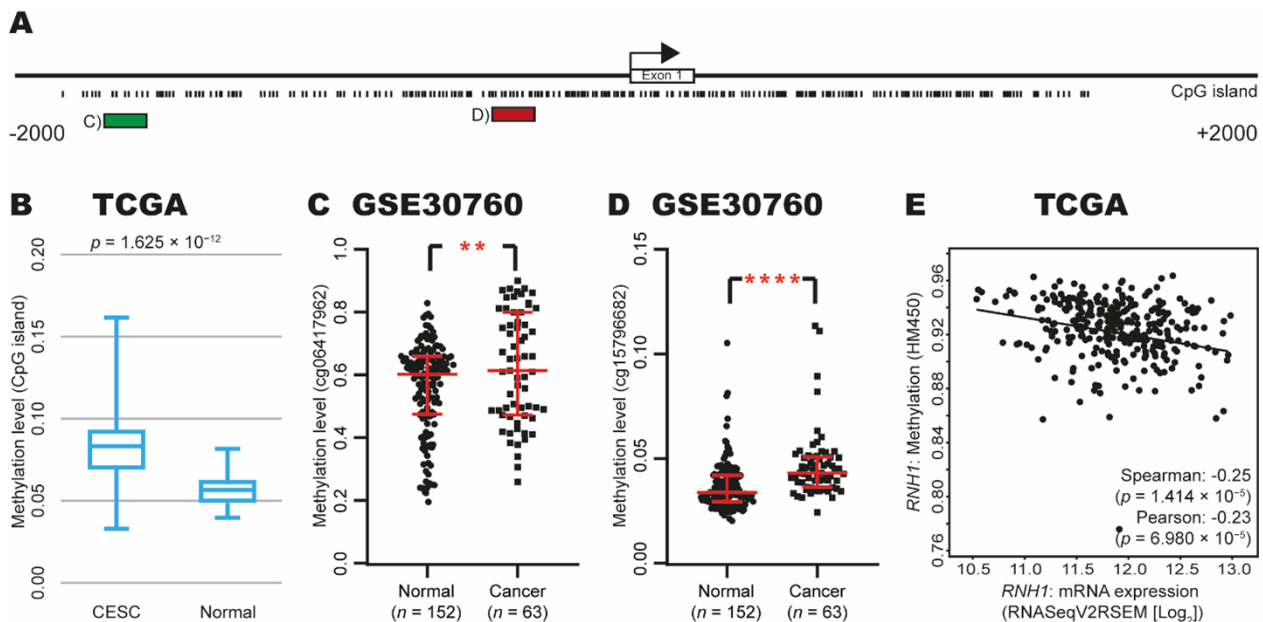


Figure 1. Identification of methylation in *RNH1* promoter in cervical cancer. (A) Identification of a CpG island in the *RNH1* promoter. Green box: Probe used in C incise. White box: Exon 1. Arrow: Transcription start site. Black lines: CpGs. Red box: Probe used in D incise. (B) Analysis of methylation level in the complete CpG island on the *RNH1* promoter in the TCGA dataset using the DiseaseMeth database. (C and D) Methylation analysis in specific regions located on the CpG island in the *RNH1* promoter for (C) C region from A incise and (D) D region from A incise. (E) Correlation analysis between *RNH1* expression and methylation in the TCGA dataset using the cBioportal database.

Notes: ***p*-value < 0.01; *****p*-value < 0.0001.

Abbreviations: CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; CpG: Cytosine-phosphate-guanine; TCGA: The Cancer Genome Atlas.

4. Discussion

Cervical cancer is one of the most common female cancers worldwide.¹ Treatment outcomes depend on the disease stage; for example, surgery or chemoradiotherapy can cure up to 95% of patients with CC in the early stage.⁴¹ In addition, according to ACS, the 5-year relative survival rate drops dramatically to 19 % in women diagnosed with distant CC.⁷ Thus, it is necessary to investigate the molecular mechanisms underlying the origin and development of CC.

In this regard, several studies have reported that hypermethylation is key in CC, including DNA hypermethylation of tumor suppressor gene promoters, such as *PAX1* and *SOX1*.⁴² In this study, hypermethylation of the *RNH1* promoter in CC was observed. Consistently, a previous study reported similar results in patients with CC, but only in a dataset using the UALCAN database.¹⁰ It will be interesting to identify the transcription factors associated with the *RNH1* promoter that could be affected by this aberrant methylation. For example, a study found that the E2F transcription factor 1 (E2F1) inhibits *RNH1* expression, promoting the development of lung cancer.¹¹ Interestingly, E2F1 expression has been shown to increase in CC;⁴³ therefore, it is possible that this transcription

factor inhibits the expression of *RNH1* in this type of cancer. More studies are needed to identify other factors or mechanisms that regulate *RNH1* expression in CC, such as oncogenic miRNAs, long non-coding RNAs, or circular RNAs.

In addition, we observed a negative correlation between *RNH1* methylation and expression in CC, suggesting that aberrant *RNH1* promoter methylation may downregulate *RNH1* expression. Consistently, low *RNH1* expression was observed in patients with this cancer. We hypothesize that *RNH1* may act as a tumor suppressor. Several studies have found low *RNH1* levels and demonstrated its role as a tumor suppressor in multiple cancers, including bladder,¹⁰ lung,¹¹ melanoma,¹² colorectal,¹³ and breast,¹⁴ supporting our results. Further studies are needed to confirm the potential role of *RNH1* in CC through functional assays to evaluate key processes, such as migration, invasion, and proliferation. Furthermore, HR-HPV (HPV16 or HPV18) is known to promote the CC by downregulating tumor suppressor genes.⁴⁴ Therefore, it is possible that HR-HPV contributes to the downregulation of *RNH1* expression in CC. Thus, we found that patients with CC and HPV infection have low *RNH1* levels; experimental evidence is necessary to support our findings.

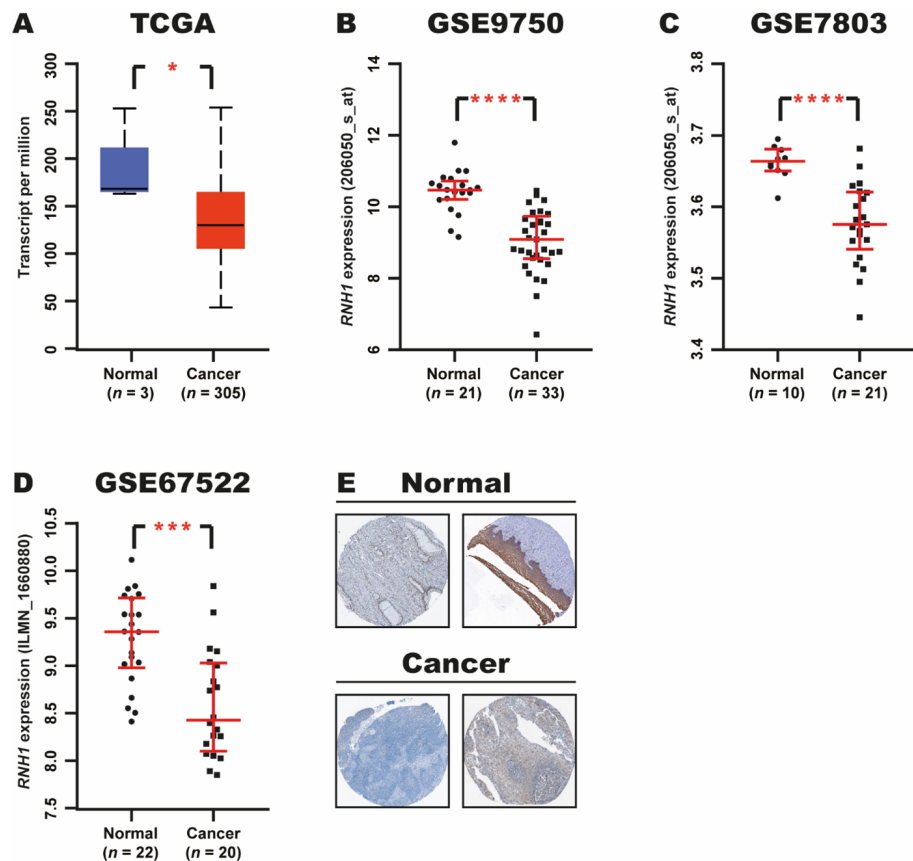


Figure 2. Analysis of *RNH1* expression in cervical cancer. *RNH1* expression was analyzed in samples from the (A) TCGA, (B) GSE9750, (C) GSE7803, (D) GSE67522, and (E) HPA datasets using UALCAN, GEO, and HPA databases.

Notes: **p*-value < 0.05; ****p*-value < 0.001; **** *p*-value < 0.0001.

Abbreviations: GEO: Gene Expression Omnibus; HPA: Human Protein Atlas; TCGA: The Cancer Genome Atlas; UALCAN: The University of Alabama at Birmingham Cancer Data Analysis Portal.

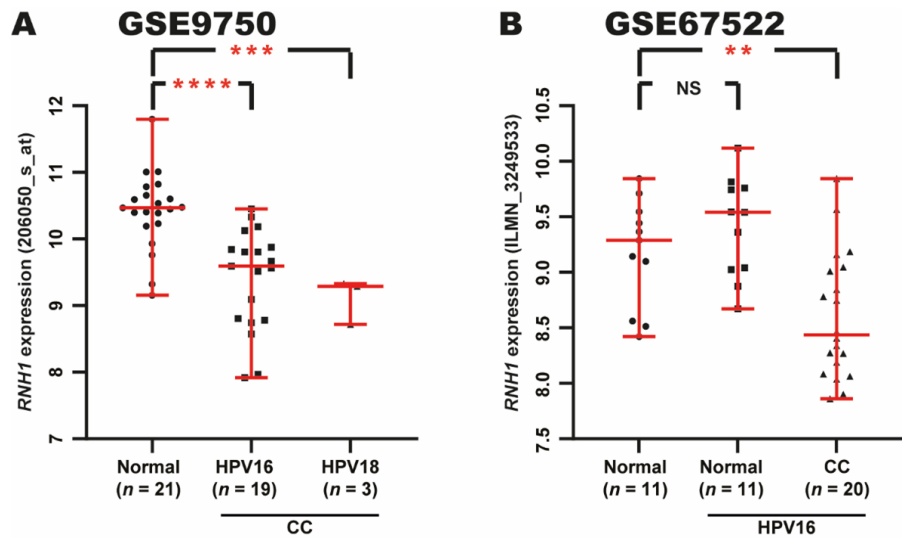


Figure 3. Evaluation of *RNH1* expression according to the HPV genotype in CC. (A) *RNH1* levels were quantified in normal, HPV16+ CC, and HPV18+ CC tissue samples from the GSE9750 dataset. (B) *RNH1* levels were determined in normal, HPV16+ CC, and HPV18+ CC tissue samples from the GSE67522 dataset.

Notes: ***p*-value < 0.01; ****p*-value < 0.001; **** *p*-value < 0.0001.

Abbreviations: CC: Cervical cancer; HPV: Human papillomavirus; NS: Non-significant.

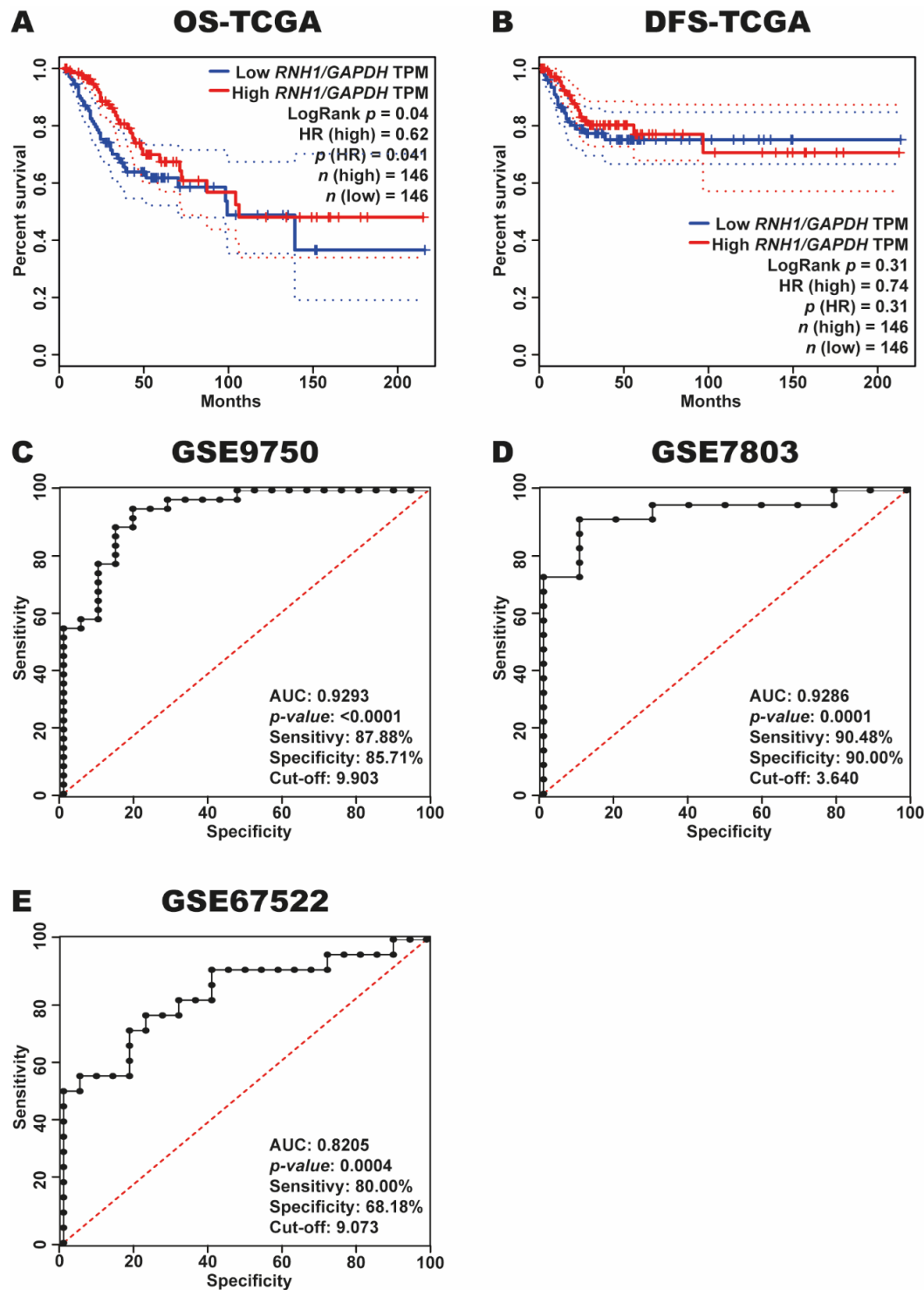


Figure 4. Analysis of prognostic and diagnostic value of *RNH1* expression in CC. (A) OS and (B) DFS were estimated according to *RNH1* expression in patients with CC from the TCGA dataset using the GEPIA database. Red line: High *RNH1* expression. Blue line: Low *RNH1* expression. Receiver operating characteristic curves of *RNH1* in CC and normal tissue samples from the (C) GSE9750, (D) GSE7803, and (E) GSE67522 datasets. Each dot represents a sample. Red line: reference base.

Abbreviations: AUC: Area under the curve; CC: Cervical cancer; DFS: Disease-free survival; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; GEPIA: Gene Expression Profiling Interactive Analysis; HR: Hazard ratio; OS: Overall survival; TCGA: The Cancer Genome Atlas; TPM: Transcript per million.

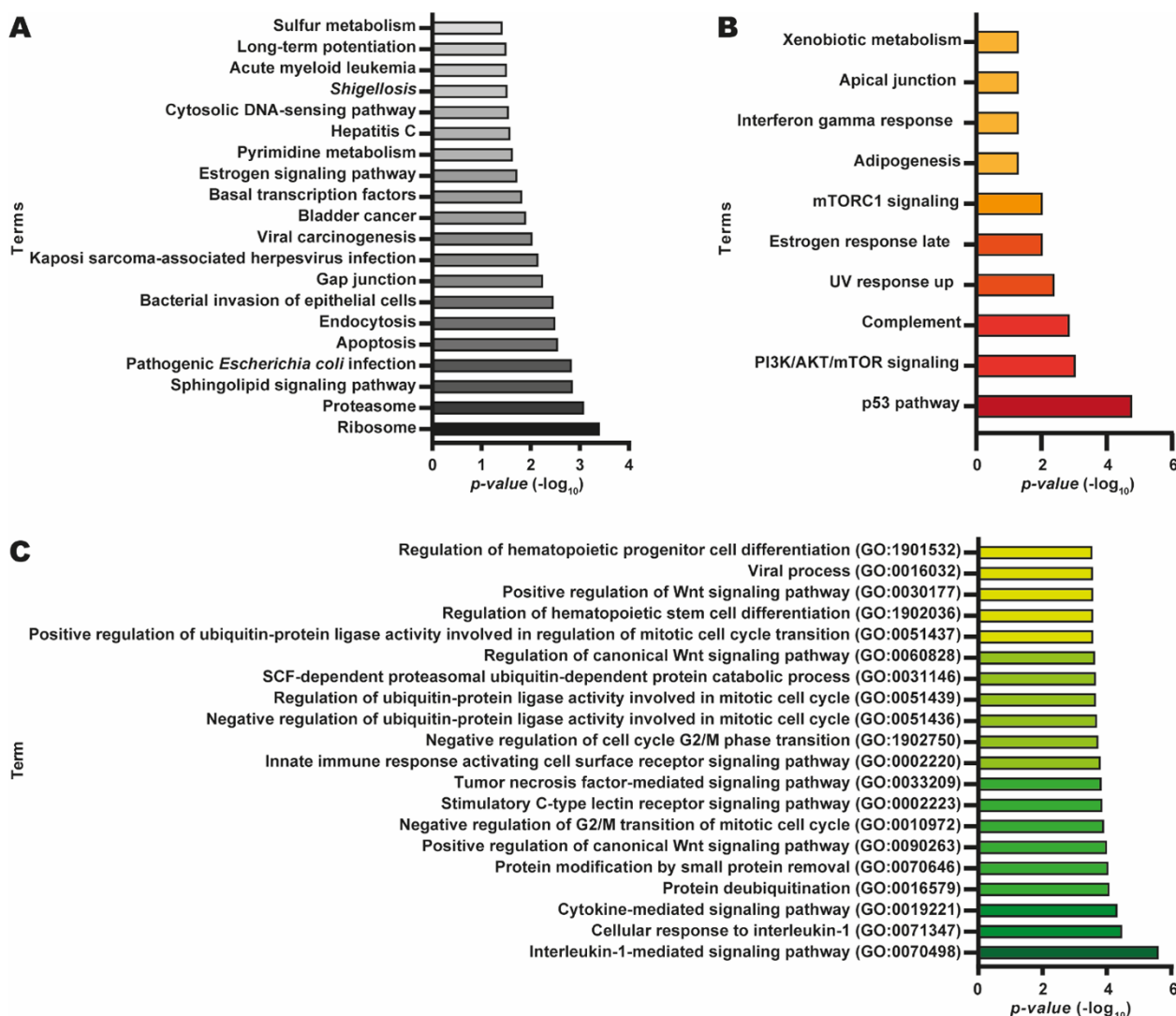


Figure 5. Identification of pathways and BPs associated with *RNH1* expression in CC. Identification of pathways associated with *RNH1* expression using (A) KEGG and (B) MSigDB libraries in CC. (C) BPs associated with *RNH1* expression in CC.

Abbreviations: AKT: Protein kinase B; BPs: Biological processes; CC: Cervical cancer; KEGG: Kyoto Encyclopedia of Genes and Genomes; mTOR: Mechanistic target of rapamycin; mTORC1: Mechanistic target of rapamycin complex 1; PI3K: Phosphoinositide 3-kinase; UV: Ultraviolet.

Given the low *RNH1* expression in CC, we assessed its clinical relevance as a prognostic and diagnostic biomarker using Kaplan–Meier and ROC curves. Interestingly, the results revealed that low *RNH1* expression correlates with a poorer OS, suggesting that *RNH1* expression has potential as a prognostic biomarker in CC. Unexpectedly, we found that low *RNH1* expression is not associated with DFS in CC, a finding that may be explained in part by *RNH1*'s role in this cancer type. For example, *RNH1* does not affect the initial recurrence in CC patients, but it may be involved in progression, metastasis, or response to treatment; thus, low expression of this gene may impact long-term

mortality. In addition, the ROC curves showed an AUC > 0.80, suggesting that *RNH1* expression could be a potential diagnostic biomarker in CC. These results were obtained from CC and normal tissue samples. However, it will be interesting to corroborate the *RNH1* expression in blood samples (serum or plasma) through real-time polymerase chain reaction (RT-PCR). One study has addressed this question by revealing that *RNH1* expression may be valuable in patients with sepsis, suggesting its potential use as a biomarker for renal and hepatic dysfunction and hepatocyte damage⁴⁵, supporting our findings in CC.

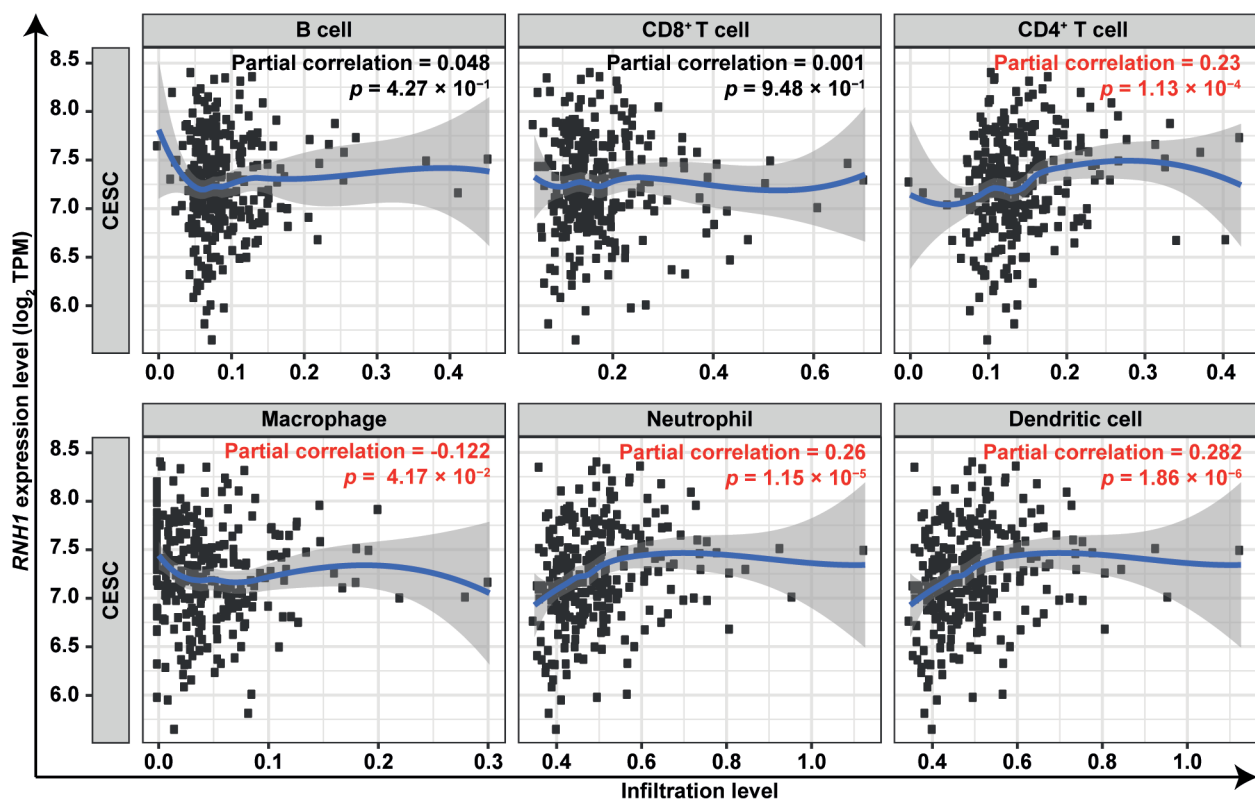


Figure 6. Association of *RNH1* expression (Y-axis) with immune infiltration in CC (X-axis). Association between *RNH1* expression and infiltration level in patients with CC in the TIMER database. Red letters signal only the purity-corrected partial Spearman's rho value with statistical significance. Statistical significance is represented with the *p*-value.

Abbreviations: CC: Cervical cancer; CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; TIMER: Tumor Immune Estimation Resource; TPM: Transcript per million.

The *ANG/RNH1* system is part of the translation machinery and regulates cellular protein synthesis.⁹ Notably, KEGG's analyses suggested that *RNH1* co-expression signature is closely linked to the ribosome and proteasome machinery in the context of CC. A prior work showed that loss of *RNH1* increases myeloid differentiation in acute myeloid leukemia (AML) cell lines, suggesting *RNH1* as a novel regulator of this process.⁴⁶ Consistent with this report, we identified AML terms in our analysis of pathways associated with *RNH1* expression. We also identified other key signaling pathways associated with CC, including viral carcinogenesis, the p53 pathway, and mTORC1 signaling. A study showed that *RNH1* overexpression reduced viral episomes and the expression of E6, E7, and E. Moreover, an enrichment of BP related to the virus was observed in RNA-seq assays.⁴⁷ Another study showed that *RNH1* knockdown promoted DNA damage, p53 activation, and upregulation of type I interferon-stimulated genes in HeLa cells.⁴⁸

In this study, genes that correlate with *RNH1* expression and participate in BP-related immune responses in CC

were identified. *RNH1* expression is also associated with the immune infiltration in CC. Consistent with these findings, *RNH1* has been linked to immunotherapy response in bladder cancer patients.¹⁰ Similarly, another study reported that patients with colorectal cancer and high *RNH1* expression presented a higher immune cell abundance and tumor microenvironment scores. Moreover, a positive correlation between *RNH1* expression and most immune checkpoints was observed in colorectal cancer.⁴⁹ Another study revealed enrichment of BP related to innate immune signaling pathways by *RNH1* overexpression in CIN-612 cells.⁴⁷ Several studies have shown that greater immune cell infiltration into tumors is associated with improved patient survival and predicts better response to immunotherapies, such as checkpoint blockade therapy.⁵⁰ In CC, ongoing efforts aim to enhance immune checkpoint inhibitors and offer new treatments⁵¹; therefore, our findings provide additional support to this field. To our knowledge, our study is the first to explore the role of *RNH1* in CC, including its promoter methylation, expression, prognostic and diagnostic value, and related pathways and BP.

Finally, although our study is based on solid, public, and scientific databases (Table S2), it is only a bioinformatics analysis and lacks experimental support (*in vitro* and *in vivo* assays). Therefore, further experimental validations are required, for example: (i) to quantify the methylation levels in the *RNH1* promoter through methylation-sensitive polymerase chain reaction; (ii) to evaluate the *RNH1* expression using RT-PCR; (iii) to determine the clinical relevance of *RNH1* expression with ROC and Kaplan–Meier curves. Despite the limitation, all our findings are robust ($n = 152$ and $n = 63$ for normal and CC, respectively, from the GSE30760 dataset in the methylation microarray assays), statistically significant (p -value < 0.05 for all our tests), and consistent across multiple datasets (RNA-seq, microarray, and proteomic assays for *RNH1* expression in CC), strongly supporting our results.

5. Conclusion

Our study strongly suggests that the *RNH1* promoter is aberrantly methylated in CC. *RNH1* expression is decreased in this cancer type and may serve as a prognostic and diagnostic biomarker. Moreover, *RNH1* may participate in immune infiltration in CC patients. However, further experimental and clinical validation is required to confirm its functional and clinical significance.

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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

The datasets analyzed in this study are publicly available in the repositories described in Table S2.

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