High Expression of Circular RNA–Mitochondrial tRNA Translation Optimization 1 Assists the Diagnosis of High-Risk Human Papillomavirus Infection in Cervical Cancer

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Objective: Persistence of high-risk human papillomavirus (HR-HPV) infection is a paramount determinant in cervical cancer (CC) development. Circular RNAs have the potential to be promising biomarkers for various cancers. This study explored circular RNA–mitochondrial tRNA translation optimization 1 (circMTO1) expression in the serum of CC patients and its clinical value in diagnosing CC and predicting HR-HPV infection.

Materials and Methods: In total, 125 CC patients (including 78 cases with HR-HPV) were enrolled, with another 76 healthy people as controls. Serum circMTO1 and miR-199a expressions were detected by reverse transcription–quantitative polymerase chain reaction, and the diagnostic efficacy of circMTO1 for CC and HR-HPV infection was analyzed by the receiver operating characteristic curve. According to the median of serum circMTO1 expression, CC patients were assigned into circMTO1 low/high expression groups to analyze the correlation between circMTO1 and clinical parameters using the Fisher and χ^2 tests. Independent association of circMTO1 with HR-HPV infection in CC was evaluated via logistics multivariate regression analysis. Targeted relationship between miR-199a and circMTO1 was predicted by Starbase Web site and validated via dual-luciferase assay, with their correlation further assessed by Pearson analysis.

Results: Serum circMTO1 was increased in CC patients and prominently elevated in HR-HPV–positive CC patients, with a level greater than 1.485 assisting CC diagnosis and a level greater than 2.480 assisting HR-HPV–positive diagnosis. The circMTO1 was interrelated to clinical stage, tumor differentiation, lymph node metastasis, invasion depth, and independently linked with HR-HPV infection in CC. Serum miR-199a was downregulated in HR-HPV–positive CC patients and inversely correlated with circMTO1.

Conclusions: Serum circMTO1 is upregulated in HR-HPV–positive CC patients and has a diagnostic value for HR-HPV infection in CC.

Key Words: cervical cancer, high-risk human papillomavirus, serum, circMTO1, miR-199a, receiver operating characteristic, person analysis, logistic regression

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Cervical cancer (CC) ranks the fourth most frequent cancer and is a major cause of cancer-related mortality in women, with appropriately 604,000 women diagnosed and 342,000 people dying worldwide in 2020.[1] Human papillomavirus (HPV) infection is a dominant cause of CC.[2] Most cases of CC develop from HPV acquisition, HPV maintenance, evolution to cervical precancer, and advancement of invasive cancer.[3] Based on the oncogenic ability, HPV can be divided into low- and high-risk HPV (HR-HPV) types, among which HR-HPV 16/18 contribute to 70% of infections in invasive CC and are the most frequent carcinogenic HPV types.[4,5] Most HPV infections are ephemeral, for the immunocompetent individual's immune system presumably eliminates the infection.[6] However, the impaired immune property reduces the capability to clear HPV infection, and the persistent HR-HPV infection in the cervix increases the peril of developing CC.[7,8] Over the past decades, the morbidity and mortality of invasive CC have lowered in numerous countries, primarily because of the screening program implementation in high-resource countries.[9] The HPV test is an efficient screening strategy to prevent CC.[10] Nonetheless, some CC patients have false-negative HR-HPV test results, indicating the limitation of HPV screening.[11,12] Hence, finding novel and valid biomarkers of HR-HPV infection is of great magnitude for the initial diagnosis and treatment of CC.

Circular RNA (circRNA) is a particular type of covalently closed circular structure noncoding RNA, mainly existing in cytoplasm and exosome, which is generated from pre-mRNA by backsplice, including 2 prominent means of formation: intron cyclization and exon cyclization.[13,14] Circular RNAs are imperative in epigenetically regulating transcriptional and posttranscriptional gene expression and possess tissue specificity, high stability, and conservation.[15,16] Intriguingly, circRNAs can serve as oncogenic genes or tumor suppressors to modulate cancer cell chemosensitivity, metastases, differentiation, and proliferation.[17] Diverse circRNAs are aberrantly expressed in CC and promise to be new markers for diagnosis and prediction of tumor episode and development.[18] Circular RNA–mitochondrial tRNA translation optimization 1 (circMTO1) plays an oncogenic function in CC cell (CCC) lines and is documented to be upregulated in the tissues of CC patients.[19] However, circMTO1 expression in the serum of CC patients, its clinical diagnostic value, and its correlation with HR-HPV infection in CC have rarely been reported.

Because of the absence of free ends easily digested by exonuclease, circRNAs are more steady than linear transcripts, which are considered a trait that can augment their potency as microRNA (miRNA) sponges.[20] MicroRNAs extensively mediate diverse biological processes, such as cell proliferation, differentiation, apoptosis, and tumorigenesis.[21] Differentially expressed miRNAs can lead to cancer onset and progression by functioning as oncogenes or tumor repressors.[22] miR-199a is downregulated in HPV-positive CC tissues, and HDAC6 facilitates HPV-positive CC development by elevating Wnt5a and impeding miR-199a transcription.[23] Therefore, we speculated that circMTO1 may play a regulatory action in HR-HPV–positive CC by sponging miR-199a. In the present study, we determined circMTO1 expression in CC and its correlation with HR-HPV infection, expecting to exert valuable reference for early diagnosis and treatment.

METHODS

Ethics Statement

This study was ratified by the ethics committee of our hospital. Patients or their families had voluntarily signed the informed consent form.

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Study Subjects

In total, 125 CC patients (aged 26–57 years, averaged 38.04 ± 6.606 years) who were admitted to our hospital from February 2018 to February 2021 were selected as the CC group. Inclusion criteria included patients: (1) conformed to the International Federation of Gynecology and Obstetrics (FIGO) staging criteria and pathologically diagnosed with CC; (2) with no radiotherapy, chemotherapy, or biological immunotherapy before operation; and (3) cooperating with this study and with complete medical records.

Exclusion criteria were as follows: (1) non-HPV–related cancers that occur in the lower genital track such as melanoma that are not HPV related; (2) with radiotherapy, chemotherapy, or biological immunotherapy before operation; and (3) with acute and chronic infectious diseases, hematological diseases, autoimmune diseases, and other malignant tumors.

The clinical stages of 125 CC patients included stage I (50 cases), stage IIa (41 cases), and stage IIb (34 cases). The pathological types consisted of squamous cell carcinoma (101 cases) and adenocarcinoma (24 cases). Furthermore, the CC patients were assigned into 24 cases with lymph node metastasis and 101 cases without lymph node metastasis; or 86 cases with invasion depth equal or less than 1/2 muscle layer and 39 cases with invasion depth greater than 1/2 muscle layer; or 47 cases with negative HR-HPV infection and 78 cases with positive HR-HPV. Another 76 healthy women who took physical examination during the same period were enrolled as the normal group. There was no significant difference in age between the 2 groups ($p > .05$), so the 2 groups were comparable.

High-Risk HPV Assay

High-risk HPV infection was detected based on the manufacturer's protocol of the primary Roche cobas HPV real-time PCR assay (Cobas 4800; Roche, Basel, Switzerland). This assay can differentiate following 14 HR-HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. High-risk HPV will be judged as positive as long as any subtype of HPV is identified as positive by the machine.

Reverse Transcription–Quantitative Polymerase Chain Reaction

Fasting venous blood was collected from all participants at enrollment. The serum was separated within 2 hours, then transferred to a dedicated cryopreservation tube, and stored in a −80° C freezer for subsequent experiments. Total RNA was extracted from the serum using the TRIzol reagent (Invitrogen, Carlsbad, CA) and then reversely transcribed into the first-strand cDNA of RNA using the RT kit (Invitrogen). Reverse transcription–quantitative polymerase chain reaction was performed using the SYBR Green PCR kit (TaKaRa, Tokyo, Japan). β-Actin and U6 were internal controls. The relative expression was computed using the $2^{-\Delta\Delta CT}$ method based on the expression of β-actin or U6. Primer sequences are presented in Table 1.

Cell Culture

Human CCC C-33A were provided by Procell (Wuhan, Hubei, China) and cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 100 U/mL of penicillin (Thermo Fisher Scientific), and 100 μg/mL of streptomycin (GE Healthcare Life Sciences, Marlborough, MA) in a humidified atmosphere containing 5% CO_2 at 37°C until passages 2–3. The medium was replaced every 2 days and CCCs were passaged weekly, with well-grown CCCs taken for subsequent experiments.

Dual-Luciferase Assay

The binding sites of miR-199a and circMTO1 were determined through the online prediction software Starbase (http://starbase.sysu.edu.cn/). According to the prediction, the mutant (MUT) sequences and wide-type (WT) sequences of the binding sites of miR-199a and circMTO1 were, respectively, designed and cloned into the luciferase vector pGL3 (Promega, Madison, WI) to construct circMTO1-WT and circMTO1-MUT luciferase plasmids. The constructed plasmids were transfected into CCCs with miR-199a mimic or mimic negative control. After 48 hours of transfection, cells were rinsed with PBS and then lysed. Thereafter, the fluorescence activity of cells was detected using a luciferase kit (Yuanpinghao Biotechnology, Beijing, China).

Statistical Analysis

Data analysis and mapping were processed by applying SPSS 21.0 (IBM Corp, Armonk, NY) and GraphPad Prism 8 (GraphPad Software, Inc, San Diego, CA) statistical software. Enumeration data were exhibited as case numbers and percentages. Measurement data were presented as mean ± SD. Fisher test was used for comparisons of enumeration data between 2 groups and χ^2 test was used for multiple group comparisons. The *t* test was performed for comparisons of measurement data between 2 groups. Receiver operating characteristic (ROC) curve was implemented to assess the diagnostic value of circMTO1 for CC patients and HR-HPV–positive CC patients. Binary logistic regression equation was used to evaluate the influencing factors of HR-HPV–positive CC patients, with Enter method for screening independent variables. *P* value was obtained through a 2-sided test, and a *p* value less than .05 indicated statistical significance.

RESULTS

CircMTO1 Was Upregulated in the Serum of CC Patients and Had High Diagnostic Efficacy

A total of 125 CC patients and 76 healthy controls were included in this study. We detected circMTO1 expression in the serum using RT-qPCR, which unveiled an increased circMTO1 expression in CC patients compared with healthy controls ($p < .001$; see Figure 1A). To further investigate the diagnostic value of serum circMTO1 expression for CC, we plotted the ROC curve of

TABLE 1. Primer Sequences

Gene	Forward 5′-3′	Reverse 5′-3′
circMTO1	5′-GCCTGAACACACTGGGAAAT-3′	5′-CACAGATGCGAGAGAACACAGG-3′
miR-199a	5′-ACACTCCAGCTGGGCCCAGTGTTCAGACTAC-3′	5′-TGGTGTCGTGGAGTCG-3′
β-actin	5′-TGGCATCCACGAAACTACCT-3′	5′-TCTCCTTCTGCATCCTGTCG-3′
U6	5′-ATTGGAACGATACAGAGAAGATT-3′	5′-GGAACGCTTCACGATTTG-3′

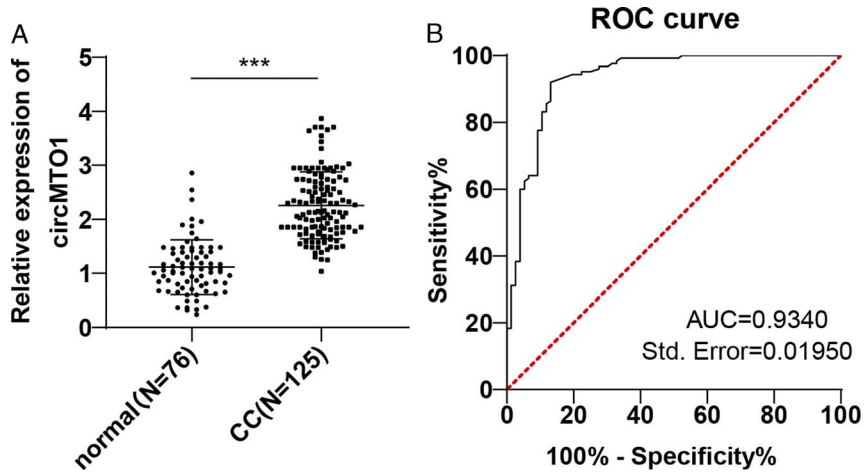

FIGURE 1. CircMTO1 was upregulated in the serum of CC patients and had high diagnostic efficacy. A, RT-qPCR was used to detect the expression of serum circMTO1. B, ROC curve was plotted to analyze the diagnostic efficacy of circMTO1 for CC. The *t* test was adopted for panel A and ROC analysis was used for panel B. ***$p < .001$.

circMTO1 expression to distinguish CC patients from normal healthy people ($p < .0001$; see Figure 1B). The area under the curve was 0.9340 and the cutoff value was 1.485 (92.8% sensitivity and 86.84% specificity). The previously mentioned results demonstrated that a serum circMTO1 level greater than 1.485 could assist CC diagnosis.

Correlation Between Serum circMTO1 and Clinicopathological Features of CC Patients

According to the median of serum circMTO1 level, CC patients were assigned to circMTO1 high expression group and circMTO1 low expression group to further observe and compare the association between circMTO1 levels and clinicopathological characteristics of CC patients. The results revealed that circMTO1 expression was prominently correlated with clinical stage, tumor differentiation degree, lymph node metastasis, and invasion depth ($p < .05$), but not with age, tumor size, and pathological type ($p > .05$; see Table 2).

High CircMOR1 Expression Assisted in Differentiating HR-HPV Infection in CC

To explore the correlation between serum circMTO1 expression and HR-HPV infection, we divided CC patients into HR-HPV–negative group ($n = 47$) and HR-HPV–positive group ($n = 78$) based

TABLE 2. Correlation Between Serum CircMTO1 and Clinical Pathological Features of CC Patients

		CircMTO1		
Clinical pathological features	***n***	**Low expression (*n* = 63)**	**High expression (*n* = 62)**	***p***
Age, y				—
<35	62	32	30	
≥35	63	31	32	
Tumor size, cm				—
≤4	65	31	34	
>4	60	32	28	
Clinical stage				<.0001
Stage I	50	35	15	
Stage IIa	41	21	20	
Stage IIb	34	7	27	
Differentiation degree				.0028
High	36	26	10	
Moderate/low	89	37	52	
Lymph node metastasis				.0002
Yes	24	4	20	
No	101	59	42	
Pathological type				—
Squamous cell carcinoma	101	50	51	
Adenocarcinoma	24	13	11	
Invasion depth				<.0001
≤1/2 muscle layer	86	57	29	
>1/2 muscle layer	39	6	33	

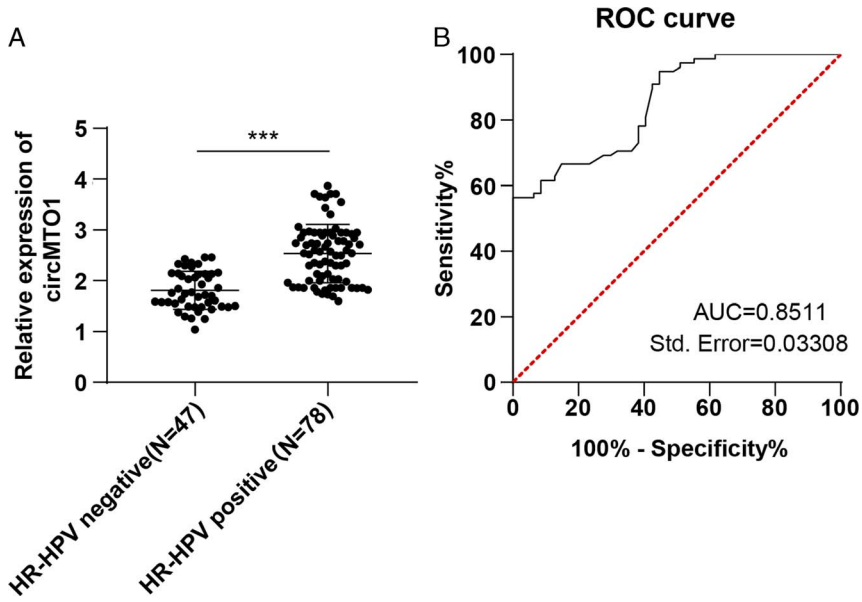

FIGURE 2. High circMOR1 expression assisted in differentiating HR-HPV infection in CC. A, Expression of serum circMTO1 was measured using RT-qPCR. B, Diagnostic efficacy of circMTO1 for HR-HPV–positive CC was assessed by the ROC curve. The *t* test was used for panel A and ROC analysis was performed for panel B. ***$p < .001$.

on the HPV assay. The RT-qPCR results showed that serum circMOR1 was higher in HR-HPV–positive CC patients than that in HR-HPV–negative CC patients ($p < .001$; see Figure 2A). The ROC curve was further plotted to distinguish HR-HPV–positive CC patients from HR-HPV–negative CC patients based on the circMTO1 expression ($p < .0001$; see Figure 2B), which illustrated that the area under the curve was 0.8511 and the cutoff value was 2.480 (56.41% sensitivity and 100% specificity). Altogether, a serum circMTO1 level greater than 2.480 possessed certain auxiliary diagnostic values for HR-HPV–positive CC.

CircMTO1 Was Independently Associated With HR-HPV Infection in CC

We compared and analyzed the incidence of HR-HPV infection in the circMTO1 high/low expression groups. The results unraveled that the incidence of positive HR-HPV was remarkably elevated in the circMTO1 high expression group (52 cases, 82.54%) relative to the circMTO1 low expression group ($p < .01$; see Table 3), indicating that circMTO1-upregulated CC patients had a higher incidence of HR-HPV infection.

To further investigate whether circMTO1 was independently correlated with positive HR-HPV, we first analyzed the relevance between clinical indexes of CC patients and HR-HPV infection using univariate logistic regression analysis. Based on the results, clinical stage, tumor differentiation degree, lymph node metastasis, invasion depth, and circMTO1 (all $p < .1$) were included as independent variables in multivariate logistic regression analysis, which revealed that serum circMTO1 was independently associated with HR-HPV infection in CC patients after adjusting clinical stage, tumor differentiation degree, and invasion depth ($p = .019$, OR = 2.587, 95% CI = 1.168–5.733; see Table 4). Overall, CC patients with elevated circMTO1 expression had a higher risk of HR-HPV infection than CC patients with low circMTO1 expression.

TABLE 3. Difference of CircMTO1 Expression in HR-HPV–Positive/Negative CC Patients

HR-HPV infection		Low expression ($n = 63$)	High expression ($n = 62$)	p
Positive	47	37	10	<.0001
Negative	78	26	52	

Serum miR-199a Was Downregulated and Inversely Correlated With CircMTO1 in HR-HPV–Positive CC Patients

Circular RNAs can exert regulatory roles via the ceRNA mechanism.[24] To preliminarily investigate the possible regulatory mechanism of circMTO1 in HR-HPV–positive CC, the binding sites between miR-199a and circMTO1 were predicted through the Starbase Web site (https://starbase.sysu.edu.cn/index.php, $p < .001$; see Figure 3A), and the binding relation was validated using dual-luciferase assay ($p < .001$; see Figure 3B). Furthermore, RT-qPCR was implemented to detect miR-199a expression in the serum of HR-HPV–negative/positive CC patients to explore the relationship between miR-199a and HR-HPV infection, which delineated a markedly decreased miR-199a in HR-HPV–positive CC patients compared with HR-HPV–negative ones ($p < .001$; see Figure 3C). Subsequently, a prominent negative relationship between circMTO1 and miR-199a was revealed by Pearson correlation ($p < .001$; see Figure 3D). In a word, serum miR-199a was weakly expressed and negatively linked with circMTO1 in HR-HPV–positive CC patients, and circMTO1 had the potential to function as a ceRNA of miR-199a in regulating HR-HPV–positive CC.

DISCUSSION

Cervical cancer occupies approximately 5% of global cancer, contributing to high morbidity and death rates.[25,26] The persistence of HR-HPV infection remains the primary root of CC and precursor lesion.[27,28] Plentiful circRNAs are reported to engage in CC pathogenesis and as potential biomarkers.[29] This study analyzed the correlation between circMTO1 and HR-HPV–positive CC.

Aberrant expressions of numerous circRNAs are observed in tumors, and this dysregulation is implicated in the tumorigenesis and metastasis of various cancer.[30] As gene regulators, circRNAs

TABLE 4. CircMTO1 Was Independently Associated With HR-HPV Infection in CC

	Univariate logistic regression analysis			Multivariate logistic regression analysis		
	p	OR	95% CI	*p*	OR	95% CI
Age, y	.744	1.009	0.955–1.067	—	—	—
Tumor size, cm	.345	1.421	0.685–2.946	—	—	—
Clinical stage	.029			.09		
Clinical stage (1)	.319	1.538	0.659–3.589	.211	1.835	0.708–4.751
Clinical stage (2)	.008	3.846	1.424–10.389	.034	3.557	1.102–11.486
Differentiation degree	.003	3.392	1.517–7.586	.004	3.846	1.548–9.556
Lymph node metastasis	.025	3.707	1.181–11.633	.677	1.338	0.341–5.253
Pathological type	.162	0.488	0.178–1.334	—	—	—
Invasion depth	.003	3.975	1.582–9.986	.049	2.821	1.003–7.933
CircMTO1	.001	3.187	1.578–6.437	.019	2.587	1.168–5.733

perform crucial impacts in cancer evolution and are considered prospective markers in diagnosing and treating cancer.[30,31] Hence, we measured the circMTO1 expression and observed an elevated serum circMTO1 level in CC patients. In addition, through the ROC curve, we found that a serum circMTO1 level greater than 1.485 (92.8% sensitivity and 86.84% specificity) could assist the diagnosis of CC. The tumor-promoting effect of circMTO1 is also documented in oral squamous cell carcinoma.[32] The circMTO1 knockdown represses chemoresistance, migration, and invasion of CCCs.[33] Our study initially analyzed the diagnostic value of circMTO1 for CC.

The FIGO stage can provide risk stratification for operable CC patients and predict survival time.[34,35] Lymph node metastasis remains an important contributor to the poor survival in CC patients.[36] Subsequently, we divided CC patients into circMTO1 high/low expression cases to further assess the relevance of circMTO1 and clinicopathological indicators, which unraveled the correlation of circMTO1 with clinical stage, tumor differentiation grade, lymph node metastasis, and invasion depth. The association between circMTO1 and lymph node metastasis is also presented in bladder cancer[37] and colorectal cancer.[38] CircMTO1 acts as an oncogene in CC and circMTO1-upregulated CC patients have short overall survival relative to the circMTO1-downregulated patients.[19] To sum up, our findings could exert certain significance for distinguishing CC individuals based on serum circMTO1 expression.

Interestingly, a similar highly expressed ciRS-7 is interrelated to large tumor size, metastatic lymph node, deep invasion, advanced FIGO stage, and HPV infection in CC patients.[39] Because of the primary pathogenic effect of oncogenic HR-HPV infection in CC,[40] we further explored the correlation between circMTO1 and HR-HPV infection. Based on our results, circMTO1 was much higher in HR-HPV–positive CC patients and its serum level greater than 2.480 (56.41% sensitivity and 100% specificity) exerted a diagnostic value for HR-HPV–positive CC. Endogenous circRNAs are reported to be highly expressed in HPV-positive tumors.[41] In HPV-related malignancies, circRNAs are documented to participate in tumorigenesis, cancer evolution, and drug resistance, among which some are regarded as effective diagnostic

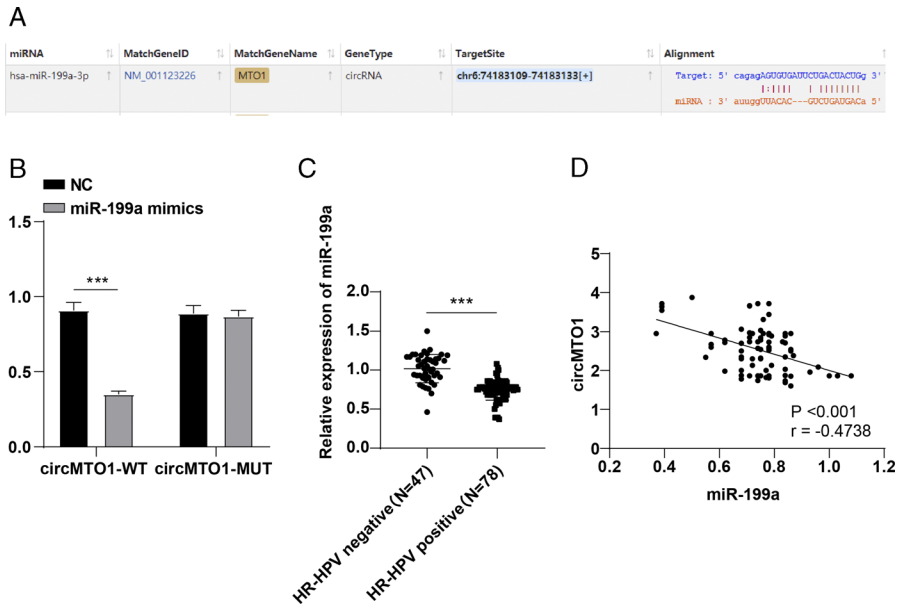

FIGURE 3. Serum miR-199a was downregulated and inversely correlated with circMTO1 in HR-HPV–positive CC patients. A, Starbase Web site was used to predict the targeted binding between miR-199a and circMTO1. B, Dual-luciferase assay was used to validate the targeted binding between miR-199a and circMTO1. C, RT-qPCR was adopted to determine the expression of serum miR-199a. D, Pearson correlation was performed to analyze the relationship between circMTO1 and miR-199a. The *t* test was used for panels B and C. ***$p < .001$.

and prognostic targets.[42] Furthermore, we found that circMTO1-upregulated CC individuals had a higher occurrence of HR-HPV infection and circMTO1 was independently related to HR-HPV infection in CC, pointing to the strong relevance of circMTO1 and HR-HPV infection.

Accumulating reports unveil that circRNAs exert biological functions by functioning as miRNA sponges.[43–46] For instance, circMTO1 can trigger gallbladder cancer progression by sponging miR-219a-5p.[32] CircMTO1 sponges miR-6893 to facilitate CC development and chemoresistance.[33] Consequently, we verified this regulatory regime. The binding sites of circMTO1 and miR-199a were predicted and ascertained. Moreover, miR-199a was observed to have an extreme decrease in HR-HPV–positive CC patients and a negative correlation with circMTO1. The above observations are consistent with the idea that miR-199a-3p is sequestered by circMTO1.[19,47] Consistently, miR-199a, as a tumor suppressor, is weakly expressed in CC, which suppresses CCC growth by targeting B7-H3.[48,49] Furthermore, evident downregulation of miR-199a is revealed in HPV-positive CC individuals relative to HPV-negative patients.[50] Collectively, circMTO1 may regulate HR-HPV–positive CC by sponging miR-199a.

As a species-specific DNA virus, HPV possesses high tissue and host specificity, causing various lesions from mucosal hyperplasia progressing to malignant tumors. After the body is infected with HPV, the immune system will be altered and produce numerous inflammatory cytokines, resulting in inflammatory responses and tissue damages.[51] Most individuals have CC due to long-term HR-HPV infection, so it is pivotal to find new biomarkers associated with HR-HPV infection for the early diagnosis and treatment of CC. Circular RNAs have a closed-loop structure with high stability and can perform regulatory properties through the ceRNA mechanism. The previous studies mostly detected circRNA levels in tissues from CC patients, rarely in the serum. Determining the serum expression of circRNAs in CC patients assists in accurately assessing the disease and adopting reasonable therapeutic measures. This study innovatively elicited the close relevance of circMTO1 with CC onset and HR-HPV infection and verified its diagnostic value, and identified the shunt function of circMTO1 in HR-HPV–positive patients when HPV detection is used as a primary screening, which poses imperative reference significance to ensure more reasonable shunt management for patients in clinical practice. However, this study only looked at patients with existing CC and whether circMTO1 can be used as a biomarker to guide the early diagnosis and treatment of CC warrants more exploration. In addition, the study population was hospital based, so the possibility of potential selection bias may exist. The limited number of samples, mainly the cases with HR-HPV infection, presumably has some impact on the data accuracy. In addition, we only simply demonstrated the targeted binding of miR-199a and circMTO1, lacking in-depth studies. Future studies shall expand the sample size and explore the molecular mechanism of miR-199a and circMTO1 in HR-HPV–positive CC and other mechanisms of regulating circMTO1. Moreover, long-term follow-up of CC patients is important to increase the result credibility.

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