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Hsa_circ_0007534 knockdown represses the development of colorectal cancer cells through regulating miR-613/SLC25A22 axis

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Abstract. – OBJECTIVE: Colorectal cancer (CRC) is a common tumor around the world. Circular RNAs (circRNAs) have been reported to be related to the development of CRC. However, the detailed mechanism is complicated. This study aimed to reveal the functional mechanism of circ_0007534 in CRC.

PATIENTS AND METHODS: Quantitative real time polymerase chain reation (qRT-PCR) and Western blot assay were performed to analyze gene expression. 3-(4,5-dimeth azol-2-yl)-2,5-diphenyltetrazolium bromide assay and colony formation assay were c ed out to determine cell proliferation ability. thermore, cell migratory and invasive abili were assessed by transwell a y. Glycol ic metabolism was examined measure ments of extracellular acidi ion (ECAR), glucose consumption, a ctate duction. Also, the interaction be circ or solute carrier family 2 me) and miR-613 was icted onfirmed by starBase v2.0 an e Dual-Luc e reporter assay, respect use xenog as performed to inv gate effect of ci 0007534 on tumor g wth *in vivo*.

RESU Circ_000753 SLC25A22 levels were u gulated, and miR- evel was down-regu d in C tissues/cells. Circ_0007534 kn wn essed CRC cell proliferation, colon on, mi on, invasion, and gly-lysis. restin Circ_0007534 target-iR-6 d -613 targeted SLC25A22. Ci 00753 ed its function by repressing mi 3 expre ion, and miR-613 exerted its f hibiting SLC25A22 expression. Al- Circ_ 534 repressed miR-613 expression upregulate SLC25A22 level. Circ_0007534 de- ion repressed tumor growth *in vivo*.

NCLUSIONS: We demonstrated that circ_0007534 knockdown suppressed the growth of CRC cells by regulating miR-613/SLC25A22 axis, providing potential target for the treatment of CRC.

Key Words:
Circ_00 4, Mi Cell develop ent, SLC25A22, Colorectal cancer.

Introduction

orectal ca (CRC), one of the most com- mo r ty s a cancer that is related to age, smoki ct[1]. CRC brings a great threaten to people' live and health. According to the statistics 5 there were over 376,000 new cases and pp nately 191,000 deaths of CRC patients in China[2]. Despite some methods, including surgery and chemotherapy, were applied for the therapy of CRC, the overall survival rate was not remarkably improved[3].

Circular RNAs (circRNAs) were initially considered as a type of products of splicing errors[4]. Nowadays, it is widely accepted that circRNAs acted as non-coding RNAs to regulate cancer cell progression, such as proliferation, migration/invasion, apoptosis, and autophagy[5]. Chen et al[6] suggested that circRNA cRAPGEF5 repressed cell proliferation and mobility by modulating miR-27a-3p expression in renal cell carcinoma. Chi et al[7] detected that circRNA-104075 was related to matrine-induced cell apoptosis, as well as autophagy in glioma. Furthermore, circ_0007534, as an endogenous circRNA, was highly expressed in CRC tissues and promoted the growth of CRC cells[8]. However, the role of circ_0007534 in CRC *in vivo* and the detailed mechanism remains unclear.

MicroRNAs (miRNAs), with 19-25 nucleotides in length, were identified as a family of small non-coding RNAs that modulated the level of target gene by inhibiting translation or inducing degradation of message RNA (mRNA) in human

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cancers[9,11]. Wang et al[12] revealed that miR-1296-5p inhibited the proliferation, as well as the mobility of osteosarcoma cells. Zhang et al[13] indicated that miR-337-3p repressed cell proliferation in ovarian cancer. MiR-613 was shown to inhibit a series of cell progression in CRC, including proliferation, migration/invasion, and cycle, meaning that miR-613 played a pivotal role in CRC development[14]. Therefore, it is essential to explore the detailed molecular mechanism of miR-613 in CRC.

Solute carrier family 25 member 22 (SLC25A22), also known as a member of mitochondrial carrier system (MCS) family, encodes a mitochondrial glutamate carrier that transports glutamate from inner mitochondrial membrane to mitochondrial matrix[15,16]. SLC25A22 was related to the development of various human cancers, such as gallbladder cancer[17], osteosarcoma[18], and CRC[19]. Furthermore, SLC25A22 was reported to suppress CRC cell proliferation, mobility, and tumor growth in mice[20]. These data revealed that SLC25A22 exerted an important role in CRC development. Therefore, it is necessary to further explore the role of SLC25A22 in CRC.

Here, the levels of circ_0007534, miR-613 and SLC25A22 in CRC tissues and cells were ed. Furthermore, we first explored the effe of circ_0007534 on proliferation, migration, sion, and glycolysis in CRC *in vitro* and eluci ed a new regulatory mechanism . Beside we proved the function of cir n tumo growth *in vivo*. The prese udy de nstrated that circ_0007534 regula CRC *via* miR-613/SLC25A axi might p- vide a new light fo therapy C.

Pa nts Method

Tissues Cell Cultu

CR d adja ent normal ues (N=60) were obt from nts at China-Japan Union Hospita U rsity. T s research was approved by the Review mmittees of China-Japan H U University. Informed consen as pro y each patient.

mal hum colon mucosal epithelial cell l y and two CRC cell lines (LoVo and V620) were provided by Shanghai Institute for logical Sciences (Shanghai, China). All cells kept in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) as well as 1% penicillin/streptomycin at 37°C with 5% CO_2.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was obtained using TRIzol reagents (Invitrogen, Carlsbad, CA, USA on the user's manual. Next, the first ad c NA synthesis kit (TaKaRa, Komat apan) was employed to generate complemen DNA (cDNA), and then qRT-PCR was carried ith the use of the SYBR Green dy KaRa, tsu Japan). The level of gene s normalized or glyceraldehyde 3- sphate ydrogen (GAPDH) using the 2 eth he primer sequences were: cir 007 ward -GTGACGGAAAT AATTG -3' reverse (R) 5'-ATGG GCTGGC -3'; miR-613, F 5' TCCTTCT GC-3' and R 5'-GAACATGTCTC ATCTC-3'; SLC25A22, F 5' ACGAGG CTACTCTG-3' and R GAAGTAGACCA TGTGCGAT-3'; U6, F TGCGGGTGCTCGCTTCGGCAGC-3' and R 5'-CCAGTG GGGTCCGAGGT-3'; GAPDH F 5'-GCAC TCAAGCTGAGAAC-3' and R GTGA ACGCCAGTGGA-3', 18S rRNA, F CCTGGTTGATCCTGCCAGT-3' and R 5'-GGCACCAGACTTGCCCTC-3'.

ular Fractionation Location

The PARIS Kit (Life Technologies, Carlsbad, CA, USA) was employed for cytoplasmic fraction in line with the recommended instruction. Briefly, the cells were incubated with lysis buffer for 10 min, and then, centrifuged at 4°C. Then, the supernatant and pellets were used to extract cytoplasmic RNA and nuclear RNA, respectively. Finally, the levels of RNAs in nuclear and cytoplasm were analyzed by qRT-PCR.

RNase R Treatment

For the measurement of circ_0007534 stabilization, the total RNA (5 μg) was incubated with RNase R (3 U/μg; Epicentre Biotechnologies, Madison, WI, USA) for 15 min at 37°C for 2 times. Subsequently, qRT-PCR was performed to analyze the levels of circ_0007534 and linear mRNA (GAPDH).

Cell Transfection

The small interfering RNA against circ_0007534 (si-circ_0007534; GenePharma Co., Ltd., Shanghai, China) and miR-613 inhibitor (anti-miR-613; GenePharma Co., Ltd., Shanghai, China) were applied to deplete circ_0007534 and miR-613, respectively. For overexpression of SLC25A22, its

sequence was inserted into the pcDNA3.1 vector (GenePharma Co., Ltd., Shanghai, China). The cells were transfected using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

Cell Proliferation Assay

Cell proliferation ability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) kit (Promega, Madison, WI, USA) in line with the recommended protocol. In brief, the cells were transfected, and then, cultured for 0 h, 12 h, 24 h, or 48 h. Then, the cells were incubated with MTT solution, and then dissolved using dimethyl sulfoxide (DMSO). Finally, optical density was examined by ELx800 absorbance reader (BioTek Instruments, Winooski, VT, USA) at 490 nm.

Colony Formation Assay

Briefly, the cells were transfected, and kept in 6-well plates for 14 d. Then, the cells were washed using PBS, fixed by paraformaldehyde, and then, stained with 0.5% crystal violet. Finally, a microscope (Olympus, Tokyo, Japan) was used to take a picture, and then, the number of the clone[illegible] counted.

Cell Migration and Invasion Assay

In briefly, transfected cells in 100 μL serum-f[illegible] medium were introduced into th[illegible] transwe[illegible] chamber (BD Biosciences, [illegible]klin[illegible]kes, NJ, USA), and the bottom of t[illegible]hamber[illegible]s introduced with 500 μL mediu[illegible]tain[illegible] After 24 h, migratory [illegible]s o[illegible]om chamber were counted by a[illegible]roscope ([illegible]pus, Tokyo, Japan). For cell i[illegible] Matrigel ([illegible]Biosciences, Franklin L[illegible]s, [illegible]SA) was co[illegible]ed on the insert, and the steps we[illegible]agreement with that in cell m[illegible]tory assay.

Western Blot Assay

T[illegible]ro[illegible]s in CR[illegible] tissues and cells were isolate[illegible] lysis b[illegible]r (Beyotime, Shanghai, [illegible]). T[illegible]We[illegible] blot assay was performed in [illegible] with [illegible]revious steps[21]. Briefly, the pr[illegible]s were s[illegible]parated, transfected onto polyvi[illegible]fluoride membranes (PVDF; Millipore, Billerica, MA, USA), and blocked by 5% non-fat milk. After that, the membranes were incubated with the primary antibodies against matrix metallopeptidase 2 (MMP-2), MMP-9, hexokinase 2 (HK2), Glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA), SLC25A22, and GAPDH (1:1,000; Abcam, Cambridge, MA, USA). After incubation by the corresponding secondary antibodies (1:2,000; Abcam, Cambridge, MA, USA), the protein bands were measured using enhanced chemiluminescent (ECL) agents (BD Biosciences, [illegible] Lakes, NJ, USA).

The Measurement of ECAR

Firstly, the cells were trans[illegible]fected, [illegible]kept in 6-well plates. Next, ECAR [illegible]determi[illegible]ing the Seahorse XF analyze[illegible]gilent Techno[illegible] Santa Clara, CA, USA) [illegible]previou[illegible]describe[illegible]

The Measurem[illegible]of [illegible]se Co[illegible]mption and Lac[illegible] Produ[illegible]

Glucose [illegible]ption and [illegible]production were anal[illegible]by [illegible]cose Assay [illegible]t (Sigma-Aldrich, St. Louis, MO, [illegible]A) and Lactate Assay kit (Sig[illegible]rich, St. Lo[illegible]O, USA), respectively. [illegible] experiments were [illegible]ried out as previous[illegible]scribed[23].

T[illegible]Dual-Luci[illegible]ase Reporter Assay

[illegible]ide-t[illegible]r mutant-type of circ_0007534 or SL[illegible]-untranslated region (UTR) was cloned into the pGL3 vector (Promega, Madison, [illegible]SA). Then, the cells were transfected with [illegible]or and miR-613 or miR-NC. After 48 h, luciferase density was examined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Mouse Xenografts

4-week-old BALB/c nude mice were used in this research. This assay was performed in line with the guidance of the National Animal Care and Ethics Institution. Briefly, LoVo cells transfected with small hairpin RNA against circ_0007534 (sh-circ_0007534) or sh-NC (GenePharma Co., Ltd) were subcutaneously injected into the mice. Then, tumor volume (length × width^2/2) was calculated every 7 d. After 35 d, the tumor was removed, and tumor weight was analyzed. This study was approved by the Animal Research Committee of China-Japan Union Hospital, Jilin University.

Statistical Analysis

The results were obtained from at least three independent experiments, analyzed by Student's *t*-test or Analysis of Variance (ANOVA) with Tukey's honestly significant difference (HSD) post-hoc test, and expressed as the means ± standard deviation (SD). The relationship between

the levels of the two genes was explored through the analysis of Pearson's correlation coefficient. $p<0.05$ was considered significant.

Results

Circ_0007534 Level was Upregulated in CRC Tissues and Cells

We first analyzed the level of circ_0007534 in CRC tissues and adjacent normal tissues from CRC patients (Table I) and found that circ_0007534 level was significantly upregulated in CRC tissues (Figure 1A). Then, circ_0007534 level in CRC cells was investigated. As shown in Figure 1B, its level was higher in CRC cells than that in normal cells. Next, we analyzed the distribution of circ_0007534 in LoVo and SW620 cells. The results suggested that circ_0007534 was mainly located in cytoplasm (Figure 1C and D). On the other hand, RNase R was used to detect the stabilization of circ_0007534. Compared with linear RNA, circ_0007534 was resistant against RNase R (Figure 1E and F). Besides, we found that circ_0007534 level in CRC patients with high survival rate was lower tha in CRC patients with low survival rate (Figu These data indicated that circ_0007534 expr was positively correlated with CRC developm

Circ_0007534 Knockdown ssed Ce Proliferation, Colony For on, ration, Invasion, and Glycoly n CRC lls

To investigate the effec rc_0 growth of CRC cells. and 0 cells were transfected with s _00075 knockdown circ_0007534. Si-NC was used as negative control. Knockdown efficiency was confirmed by qRT-PCR (Figure 2A and B). Next, cell proliferation ability was determined by MTT assay. As demonstrated in Figure 2C and D, circ_0007534 kn dramatically inhibited cell proliferation colony formation assay suggested the number of colonies was significantly d ed due to circ_0007534 knockdown (Fig e 2E). quently, transwell assay was used sess cel lity. The results revealed that 0007534 knoc remarkably suppressed migrati nd inva (Figure 2F and G; 10 im , the l els of two proteins (MM and 9) relat o cell migration and sion we wn ated by circ_0007534 down (Figu d I).

On the el d, we ana zed whether circ_0007534 affec glycolytic metabolism by ng extrac r acidification rate (E). The results indic d that ECAR was re- d by circ_0007534 knockdown in LoVo and 20 cells (Fig 2J and K). Furthermore, our r showed t circ_0007534 knockdown decre ucos nsumption and lactate production (F and M). In addition, circ_0007534 knockdown significantly downregulated the levels of glycolysis-associated GULT1, HK2, and gure 2N and O). Therefore, circ_0007534 knockdown inhibited the growth of CRC cells.

Circ_0007534 Targeted MiR-613

Through bioinformatics tool starBase v2.0, we found that miR-613 was a potential target of circ_0007534 (Figure 3A). Then, the Dual-Luciferase reporter assay was performed to veri-

Table I. Co tion betwee irc_0007534 level and pathological indexes of colorectal cancer patients (n=60).

		circ_0007534 expression		
P eter	Case	Low (n=27)	High (n=33)	*p*-value[a]
Age (y				0.4846
	35	16	29	
	25	11	14	
G er				0.6717
	24	10	14	
	36	17	19	
Tumor size				0.0030*
≤3 cm	39	23	16	
cm	21	4	17	
ph node metastasis				0.0020*
Absent	29	19	10	
Present	31	8	23	

*$p < 0.05$; [a]Chi-square test.

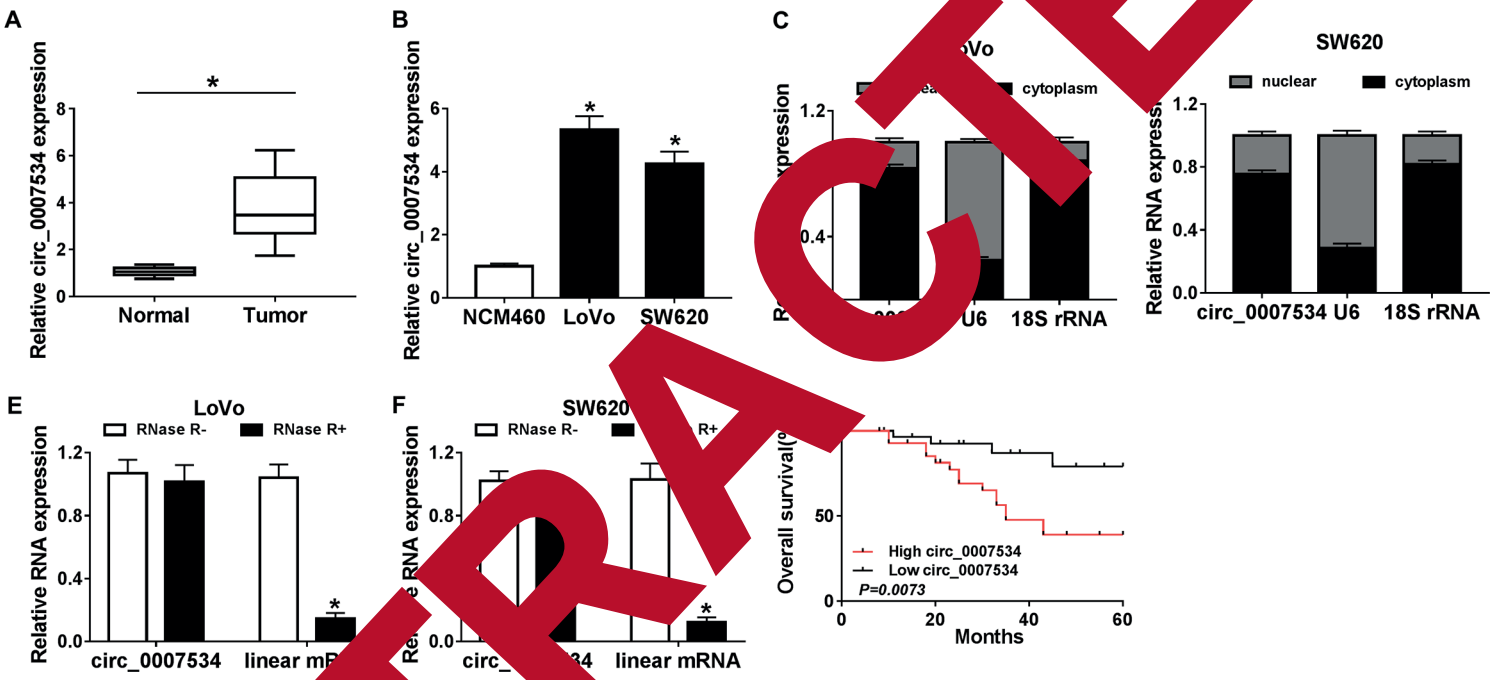

Figure 1. Circ_0007534 was u[illegible]ulate[illegible]RC tissues/c[illegible]s and related to overall survival rate. **A,** Circ_0007534 level was detected by qRT-PCR in CRC tissues and adjacent normal t[illegible]ues. **B,** Circ[illegible]534 level was determined in CRC cells and normal cells. **C,** and **D,** The levels of circ_0007534, U6, and 18 S rRNA were measured in[illegible]ear and cytopla[illegible]LoVo and SW620 cells. **E,** and **F,** The levels of circ_0007534 and linear mRNA were examined after the treatment of RNase R. **G**[illegible]e relationship betwe[illegible]c_0007534 and overall survival rate of CRC patients was analyzed. *p<0.05.

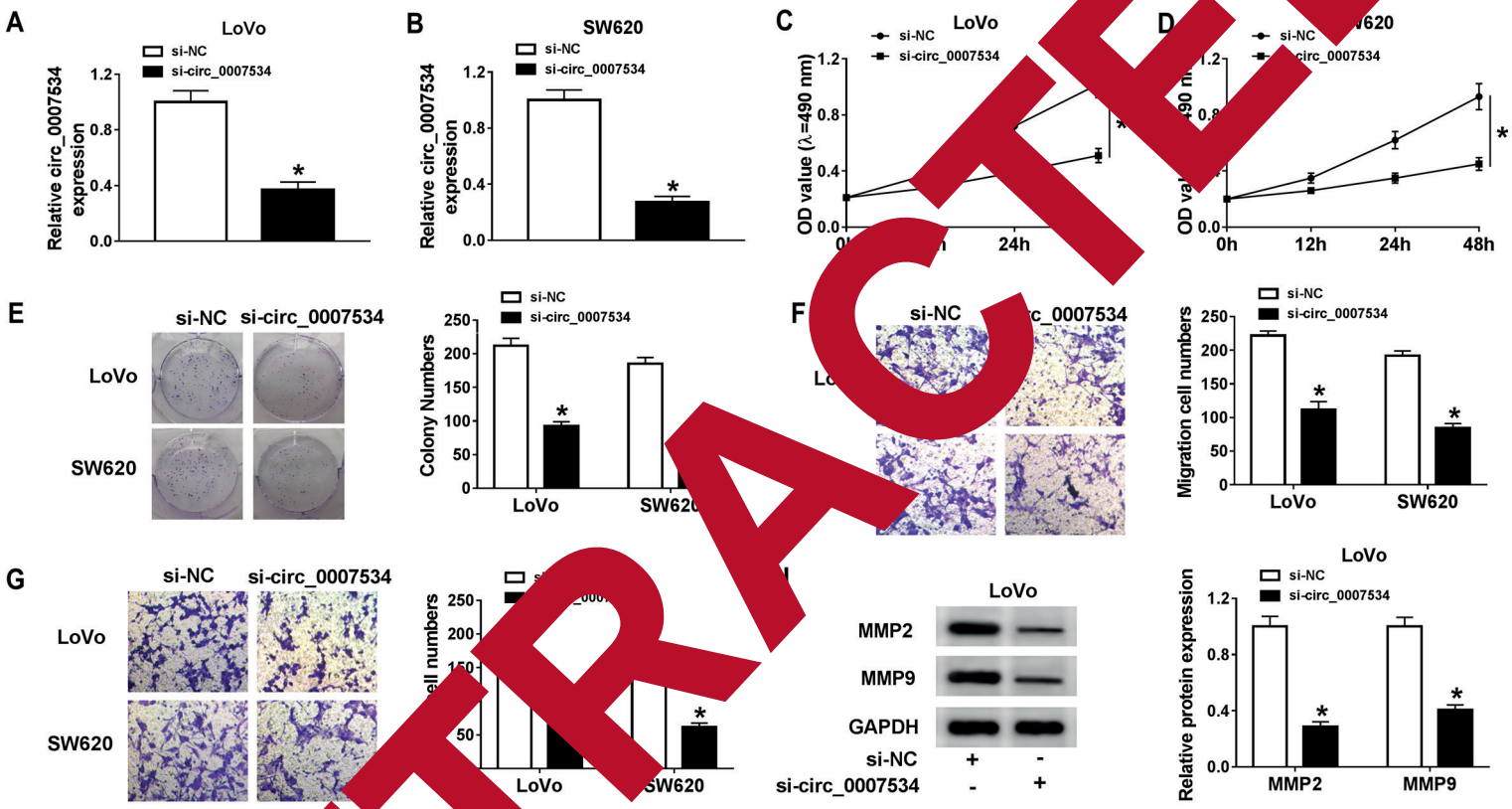

Figure 2. Circ_0007534 was ...ucogene in CRC ... **A,** and **B,** The expression of circ_0007534 was detected in LoVo and SW620 cells transfected with si-NC or si-circ_0007534. **C,** and ... was performe... to assess cell proliferation ability. **E,** Colony formation assay was carried out. **F,** and **G,** Cell migratory and invasive abilities were de...mined usi...answell assay (magnification 100X). **H,** and **I,** The levels of MMP2 and MMP9 were examined by Western blot assay. **J,** and **K,** XF 96 was used t... mi...AR. **L,** ...d **M,** Glucose consumption and lactate production were investigated by related kits. **N,** and **O,** Western blot assay was performed to detect the ...HK2, G...1, and LDHA. *p<0.05.

Figure continued

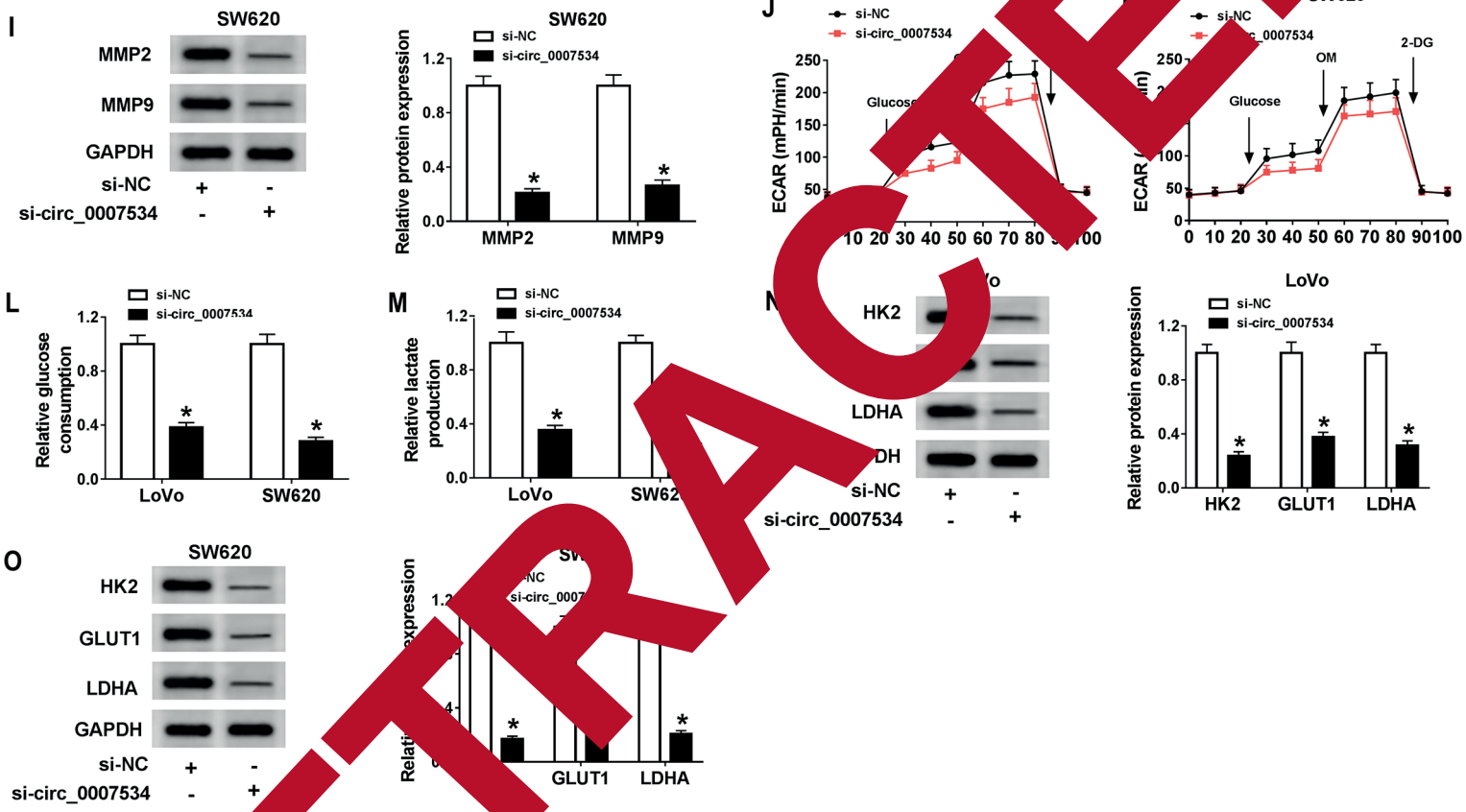

Figure 2 (Continued) ...and **I,** T...vels of MMP2 and MMP9 were examined by Western blot assay. **J,** and **K,** XF 96 was used to determine ECAR. **L,** and **M,** Glucose consumption... lactate production were investigated by related kits. **N,** and **O,** Western blot assay was performed to detect the levels of HK2, GLUT1, and LDHA. *$p<0.05$.

fy this interaction by transfecting circ_0007534 WT or circ_0007534 MUT and miR-613 or miR-NC into LoVo and SW620 cells. As shown in Figure 3B and C, miR-613 remarkably reduced the Luciferase activity of circ_0007534 WT but didn't affect the Luciferase activity of circ_0007534 MUT. This result revealed that miR-613 interacted with circ_0007534. Next, the effect of circ_0007534 on miR-613 expression was investigated. The results demonstrated that circ_0007534 knockdown dramatically increased miR-613 expression in LoVo and SW620 cells (Figure 3D). In addition, we determined the level of miR-613 in CRC tissues and cells, founding that miR-613 level was lower in CRC tissues/cells than that in normal tissues/cells (Figure 3E and F). Besides, the relationship between miR-613 expression and circ_0007534 expression was analyzed. As expected, miR-613 expression was negatively correlated with circ_0007534 expression (Figure 3G). These data confirmed that circ_0007534 targeted miR-613 and repressed its expression.

MiR-613 Depletion Weakened the Ef… of Circ_0007534 Knockdown on th… Development of CRC Cells

To investigate whether circ_0007534 exert… function *via* modulating miR-613 expression, L… Vo, and SW620 cells were trans…ith si-NC, si-circ_0007534, si-circ_000… + a…iR-NC, or si-circ_0007534 + anti…-613, r…ectively. QRT-PCR showed that i…13 e… upregulated by circ_75… …down, and then, downregulat…ue to the …fection with anti-miR-613 (Fi… A and B). …MTT assay suggested… c…oliferation …s greatly inhibited … circ_000… knockdown, which was alm…completely re…d by miR-613 depletio…igure 4C and D). …reover, miR-613 dep…n we…ed the inhibitory effect of circ_75… …nockdo…n on colony formation (Figure … next, t… well assay was employed …ess c… obi… As demonstrated in Figure 4F… G, ci…7534 knockdown significantly su…ssed cell …igration and invasion, whereas t… impaired by miR-613 depletion in …RC cells. Similarly, the positive effect of miR-…3 depletion on circ_0007534 knockdown-medi… …ownregulated levels of MMP2 and MMP9 wa… observed in CRC cells (Figure 4H and I).

On the other hand, the effect of miR-613 on circ_0007534-regulated glycolytic metabolism was explored *via* determining ECAR. As shown in Figure 4J and K, ECAR was downregulated by circ_0007534 knockdown, and then partly rescued due to miR-613 depletion. Moreover, the negative effect of circ_0007534 knockdown on glucose consumption and lactate pr… was reversed by miR-613 depletion …gure … and M). Besides, we found that …_0007534 knockdown-mediated downregu… levels of GULT1, HK2, and LDH w…e up…ed by miR-613 depletion (Figure … nd O). …ore… circ_0007534 inhibited …-613 express… regulate CRC cell deve…ment.

MiR-613 was a…ong… SLC25…

Next, we u… bioinfor… t… starBase v2.0 to pred… potential t… miR-613. The resul…gge… that SLC2…22 possessed a complementary s…ce with miR-613 (Figure 5…n, the Dua…iferase reporter assay w… formed to confirm …e interaction between r…-613 and SLC25A22. As shown in Figure 5B, t… Luciferase …vity of SLC25A22 3'-UTR-W…ut not SL…5A22 3'-UTR-MUT, was re-ma… …dimi…ed by miR-613 overexpression, meani…R-613 bound to SLC25A22. Subsequently, the effect of miR-613 on SLC25A22 ex…n was explored. The results indicated that …depletion significantly increased the expression of SLC25A22 in LoVo and SW620 cells (Figure 5C and D). In addition, we analyzed the level of SLC25A22 in CRC tissues and cells and found that SLC25A22 level was higher in CRC tissues/cells than that in normal tissues/cells (Figure 5F-I). Besides, our results demonstrated that SLC25A22 expression was negatively correlated with miR-613 expression in CRC tissues (Figure 5J). These data showed that miR-613 targeted SLC25A22 and repressed SLC25A22 expression.

SLC25A22 Overexpression Reversed the Effect of MiR-613 Upregulation on the Development of CRC Cells

To analyze whether miR-613 exerted function *via* regulating SLC25A22 expression, LoVo and SW620 cells were transfected with miR-NC, miR-613, miR-613 + Vector, or miR-613 + SLC25A22, respectively. QRT-PCR revealed that SLC25A22 expression was downregulated by miR-613 overexpression and upregulated by the transfection with SLC25A22 (Figure 6A-C). Then, MTT assay was carried out to determine cell proliferation ability. As shown in Figure 6D and E, miR-613 overexpression greatly suppressed cell proliferation, whereas this action was weakened due to SLC25A22 up-

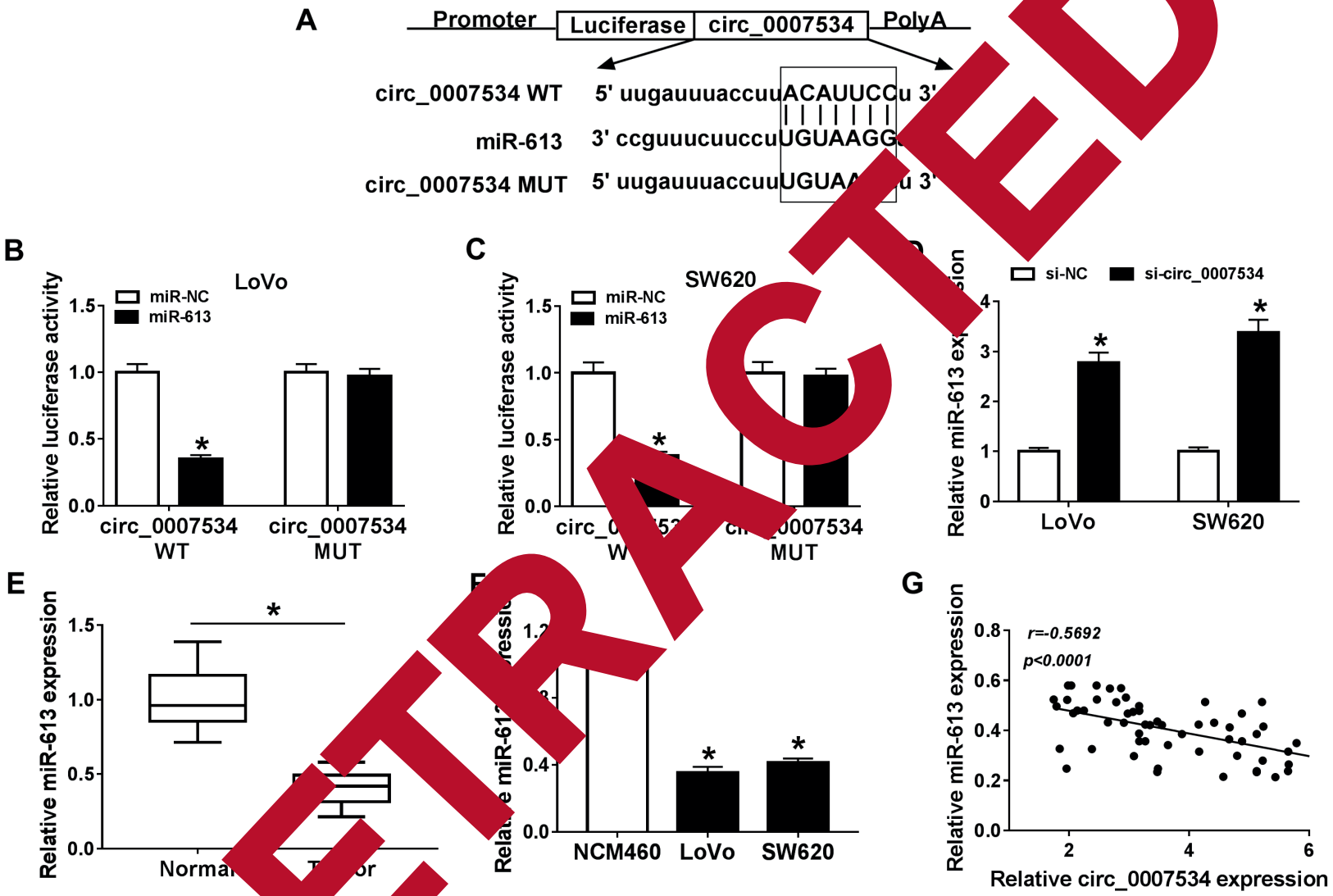

Figure 3. Circ_0007 onge A, The interaction between circ_0007534 and miR-613 was predicted by starBase v2.0. **B,** and **C,** The Luciferase activity of LoVo and SW620 c ansfec with ci 534 WT or circ_0007534 MUT and miR-613 or miR-NC was measured. **D,** MiR-613 expression was determined in LoVo and SW620 cells tran d with s or si-circ_ 007534. **E,** and **F,** The level of miR-613 was detected in CRC and normal tissues (**E**), as well as CRC and normal cells (**F**). **G,** The relationship betw c_00 n and miR-613 expression was analyzed. *p<0.05.

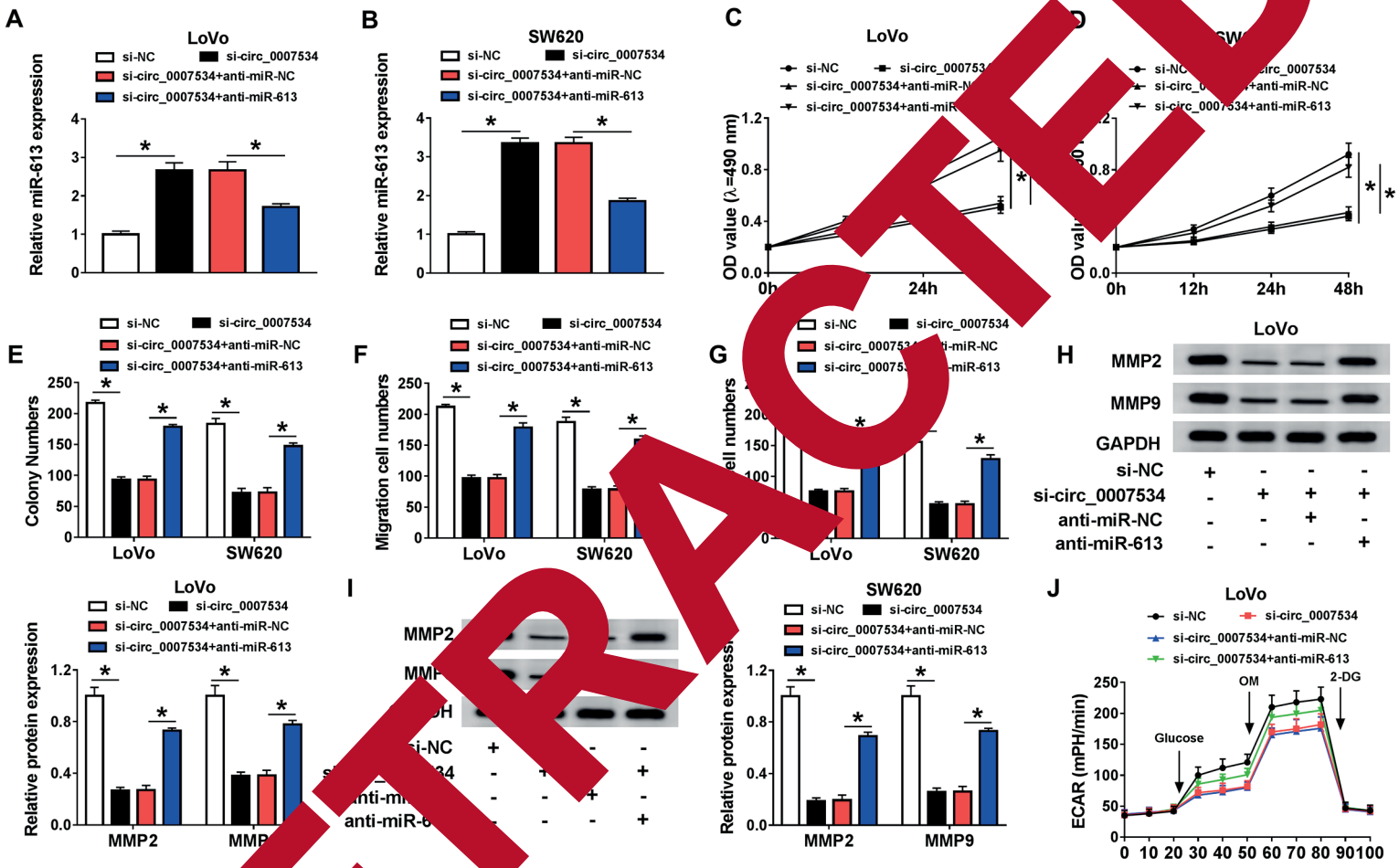

Figure 4. Circ_0007534 [illegible]ated CR[illegible]ll progression by inhibiting miR-613 expression. **A,** and **B,** The expression of miR-613 was detected in LoVo and SW620 cells transfected with si-NC, si-circ_0007534, si-circ_0007534 + anti-miR-NC, or si-circ_0007534 + anti-miR-613, respectively. **C,** and **D,** Cell proliferation ability was examined by MTT assay. [illegible]y form[illegible] assay was performed. **F,** and **G,** Transwell assay was carried out to measure cell migratory and invasive abilities. **H,** and **I,** The lev[illegible]MP2 a[illegible]MP9[illegible] determined by Western blot assay. **J,** and **K,** XF 96 was employed to determine ECAR. **L,** and **M,** Glucose consumption and la[illegible]pr[illegible]ion we[illegible]d by related kits. **N,** and **O,** Western blot assay was used to measure the levels of HK2, GLUT1, and LDHA. *$p<0.05$.

Figure continued

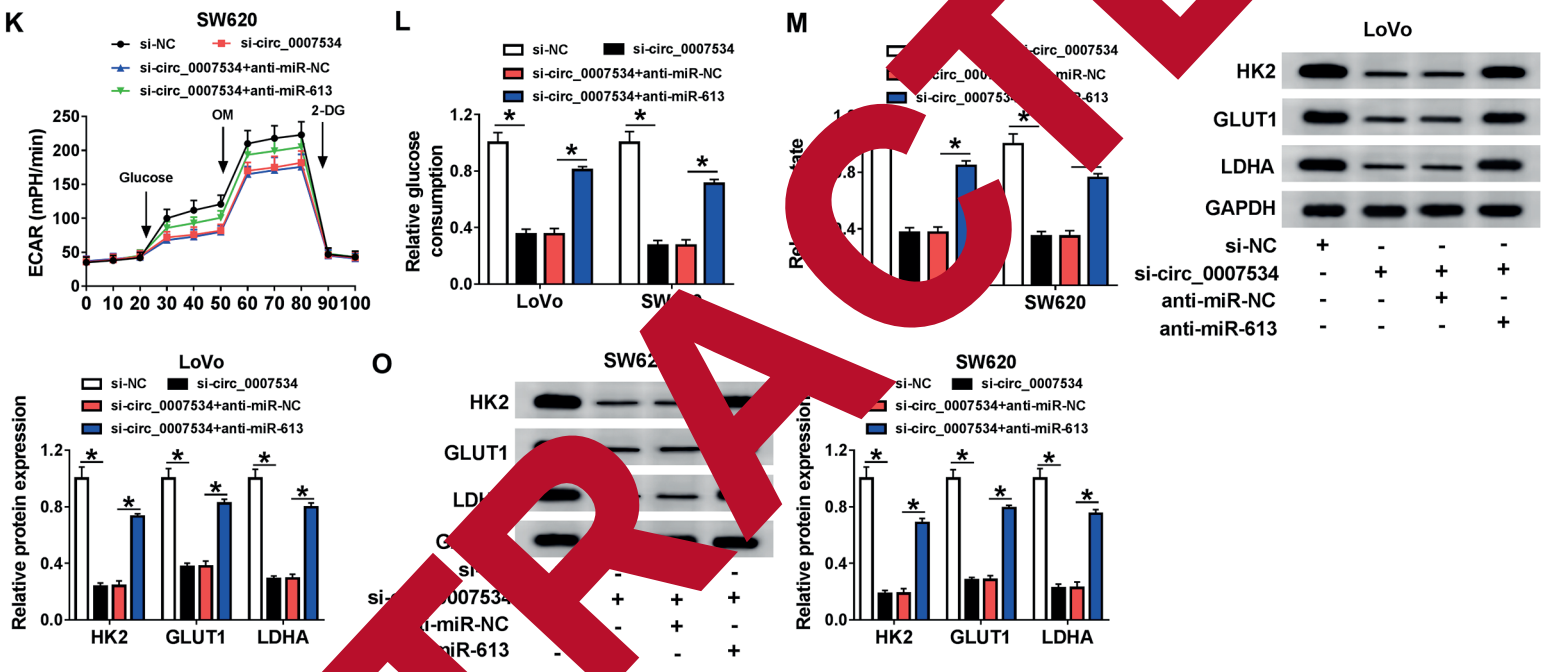

Figure 4 (continued). **J,** and XF 96 was used to determine ECAR. **L,** and **M,** Glucose consumption and lactate production were analyzed by related kits. **N,** and **O,** Western blot assay was used to measure the levels of HK2, GLUT1, and LDHA. *$p<0.05$.

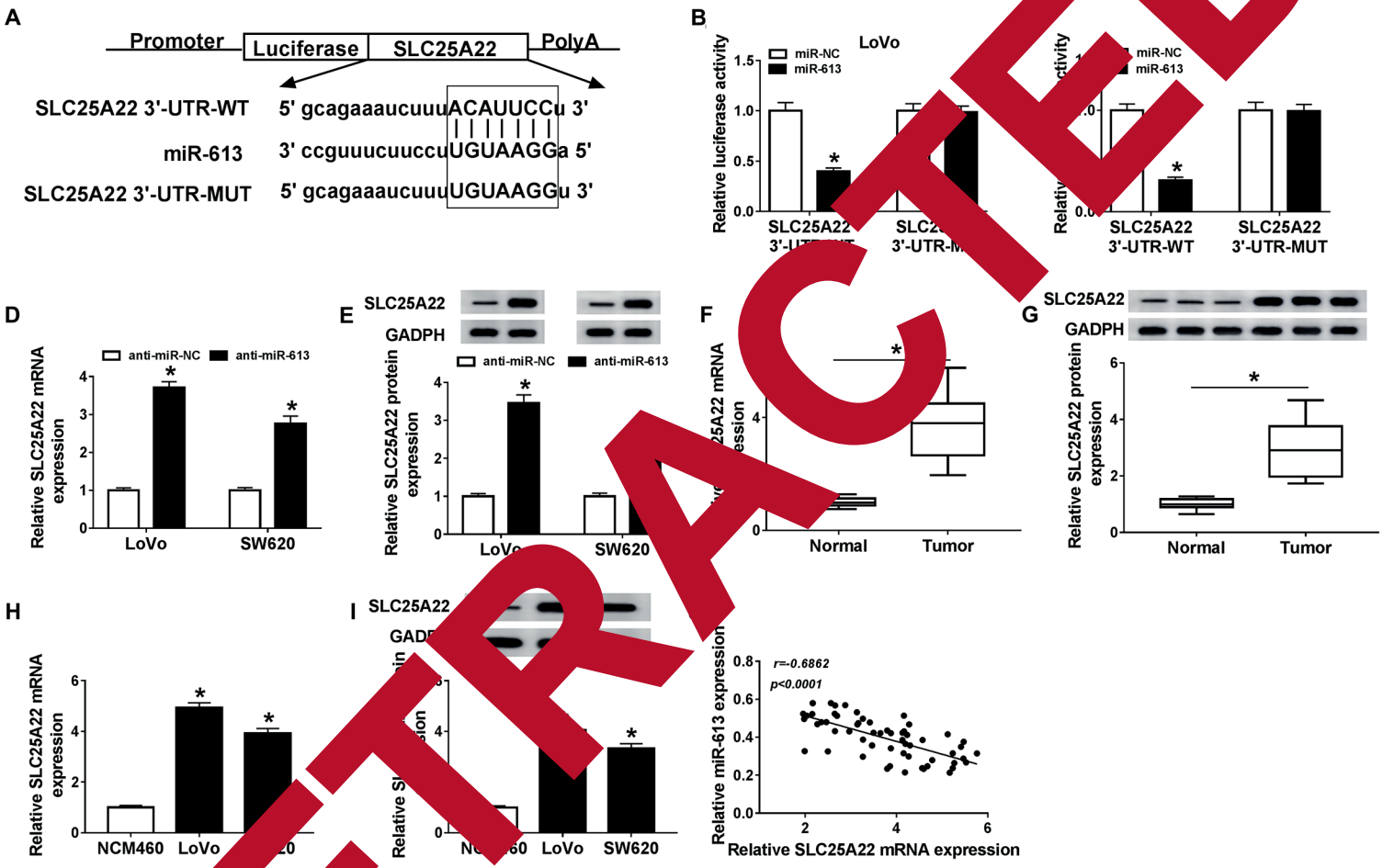

Figure 5. MiR-613 was a[illegible]SLC25A[illegible] **A,** The interaction between miR-613 and SLC25A22 was predicted by starBase v2.0. **B,** and **C,** The Luciferase activity was determined [illegible] and SW[illegible] cells transfected with SLC25A22 3'-UTR-WT or SLC25A22 3'-UTR-MUT and miR-613 or miR-NC. **D,** and **E,** SLC25A22 expr[illegible] detec[illegible] Lo[illegible] nd SW620 cells transfected with anti-miR-NC or anti-miR-613. **F-I,** The level of SLC25A22 was examined in CRC and normal ti[illegible] (**F** a[illegible]), as w[illegible]C and normal cells (**H** and **I**). **J,** The relationship between SLC25A22 expression and miR-613 expression was investigated. *$p<0$[illegible]

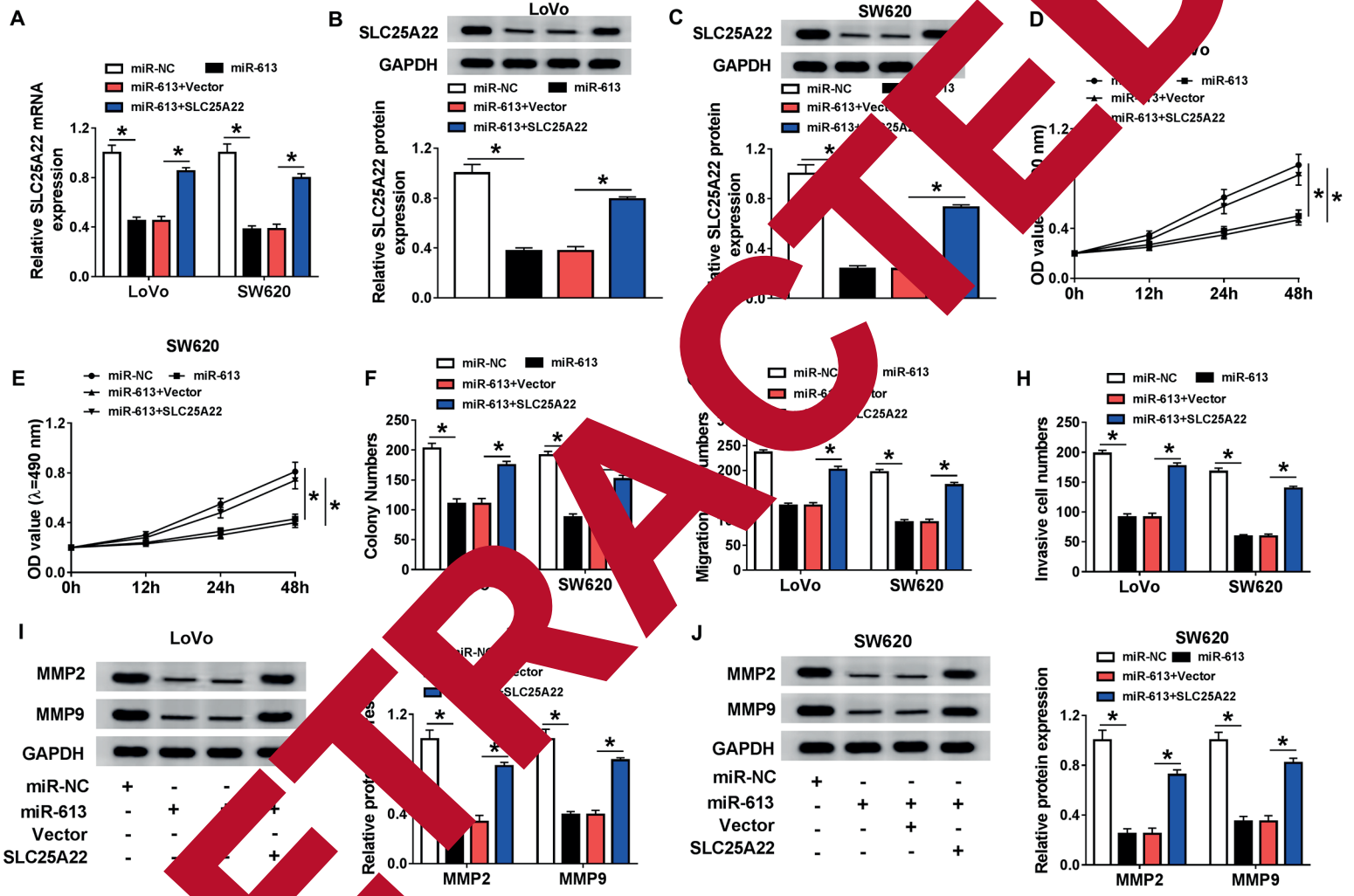

Figure 6. MiR-613 modulates CRC cell progression via repressing SLC25A22 expression. **A-C,** The expression of SLC25A22 was detected by qRT-PCR and western blot assay in LoVo and SW620 cells transfected with miR-NC, miR-613, miR-613 + Vector, or miR-613 + SLC25A22, respectively. **D,** and **E,** MTT assay was carried out to measure cell proliferation ability. **F,** Colony formation assay was performed. **G,** and **H,** Cell migratory and invasive abilities were examined by transwell assay. **I,** and **J,** The levels of MMP2 and MMP9 were detected by Western blot assay.

Figure continued

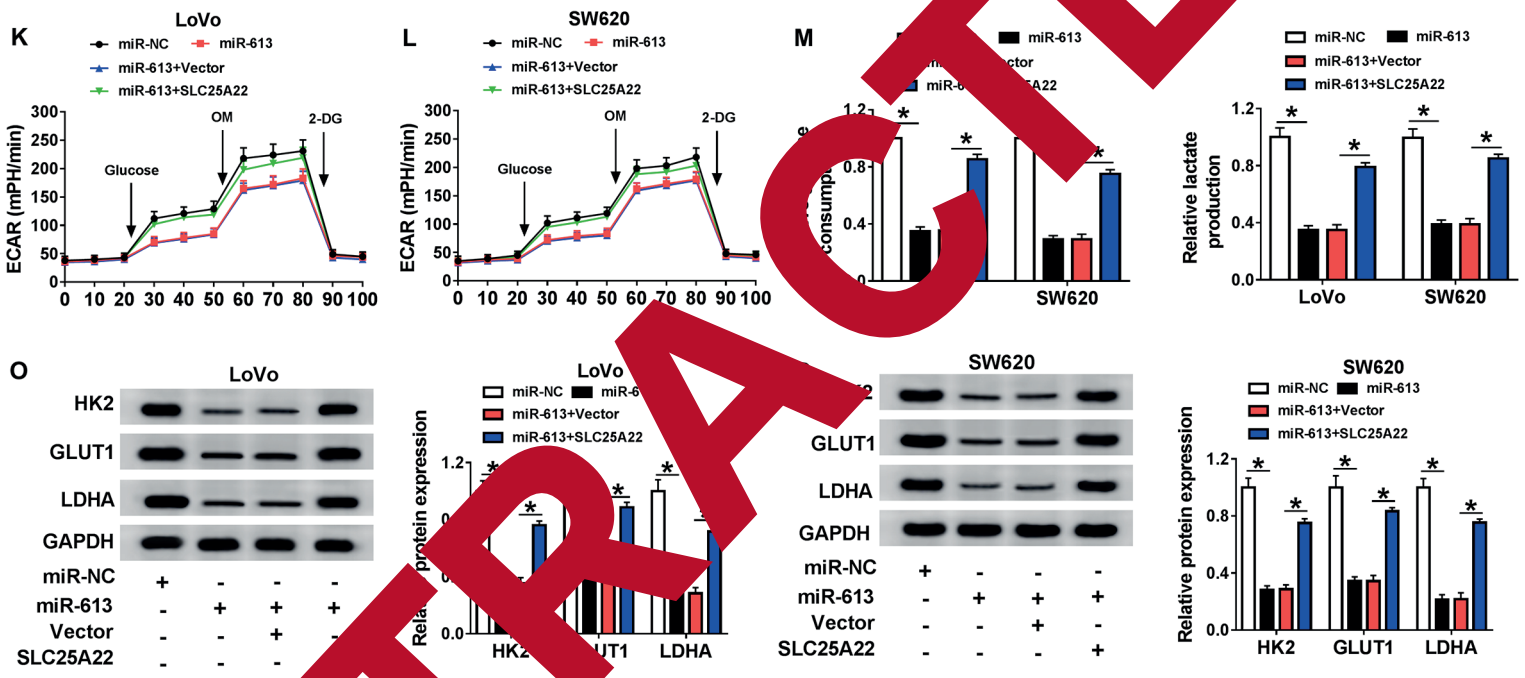

Figure 6. ***(Continued).*** **K** and **L,** XF 96 w[illegible]loyed to analyze ECAR. **M,** and **N,** Glucose consumption and lactate production were explored by related kits. **O,** and **P,** The levels of HK[illegible]LUT1, and LDHA[illegible]determined by Western blot assay. *$p<0.05$.

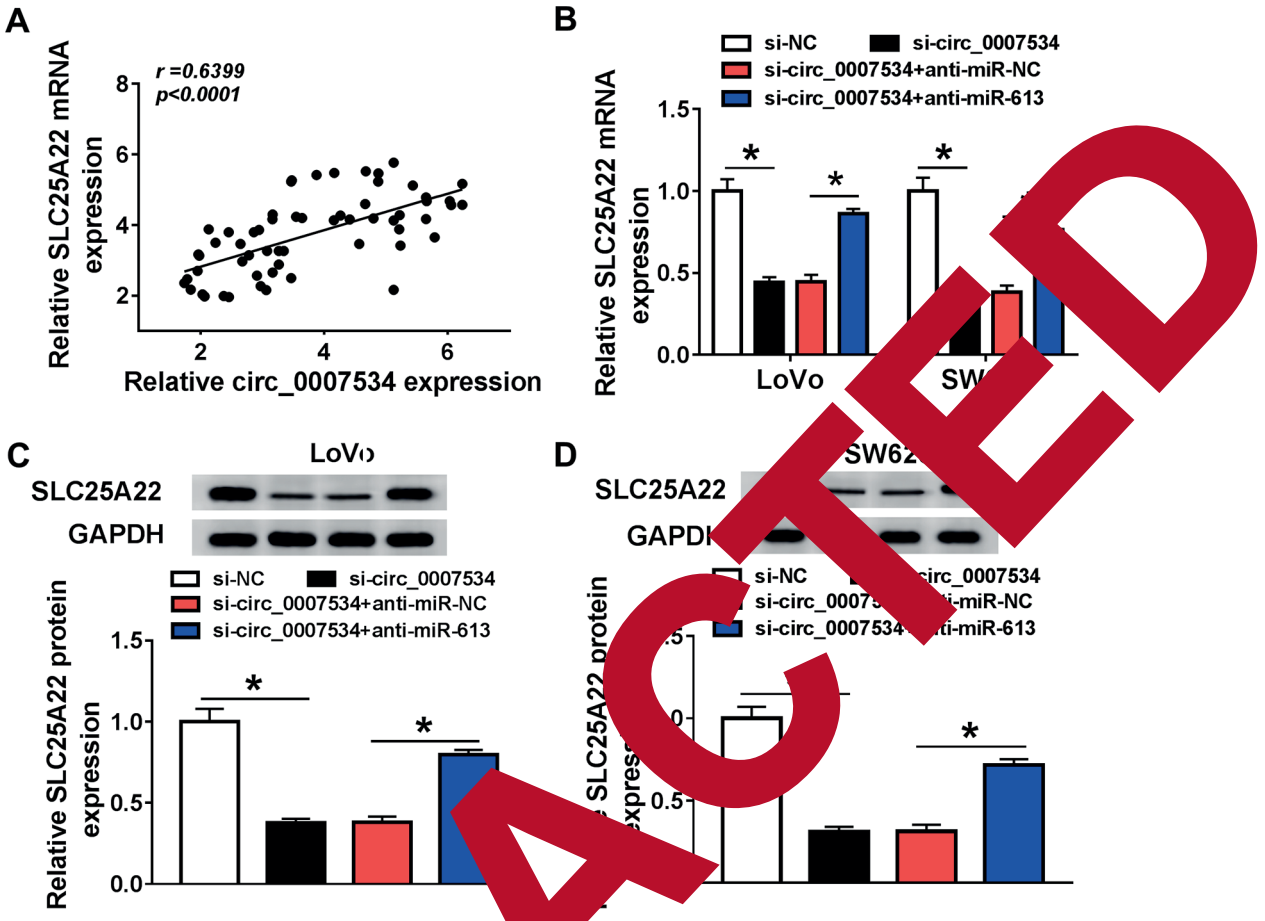

Figure 7. The linear relationship between circ_0007534, miR-613, and SLC25A22 was investigated. **A,** The relationship between SLC25A22 expression and circ_0007534 expression was investigated. **B-D,** The expression of SLC25A22 was detected in LoVo and SW620 cells transfected with si-NC, si-circ_0007534, si-circ_0007534 + anti-miR-NC, or si-circ_0007534 + anti-miR-613, respectively. *p<0.05.

regulation. Further, colony formation assay showed that SLC25A22 upregulation reversed the effect of miR-613 overexpression on colony formation (Figure 6F). Next, cell motility was assessed by transwell assay. The results demonstrated that cell migration and invasion were dramatically suppressed by miR-613 overexpression, and then partially rescued by SLC25A22 upregulation (Figure 6G and H). Similarly, the positive effect of SLC25A22 upregulation on miR-613 overexpression-downregulated levels of MMP2 and MMP9 was observed in CRC cells (Figure 6I and J).

We analyzed glycolytic metabolism in this study and found that miR-613 overexpression decreased ECAR, and this action was impaired due to SLC25A22 upregulation in LoVo and SW620 cells (Figure 6K and L). Moreover, SLC25A22 upregulation reversed the effect of miR-613 overexpression on glucose consumption and lactate production (Figure 6M and N). In addition, we found that miR-613 overexpression-mediated downregulated levels of GULT1, HK2, and LDH were upregulated by SLC25A22 upregulation (Figure 6O and P). These data indicated that miR-613 regulated CRC cell development by inhibiting SLC25A22 expression.

Circ_0007534 Upregulated SLC25A22 Level Through Inhibiting MiR-613 Expression

Next, the relationship between the levels of SLC25A22 and circ_0007534 was investigated. As expected, SLC25A22 level was positively correlated with circ_0007534 level in CRC tissues (Figure 7A). Therefore, we speculated that circ_0007534 promoted SLC25A22 expression

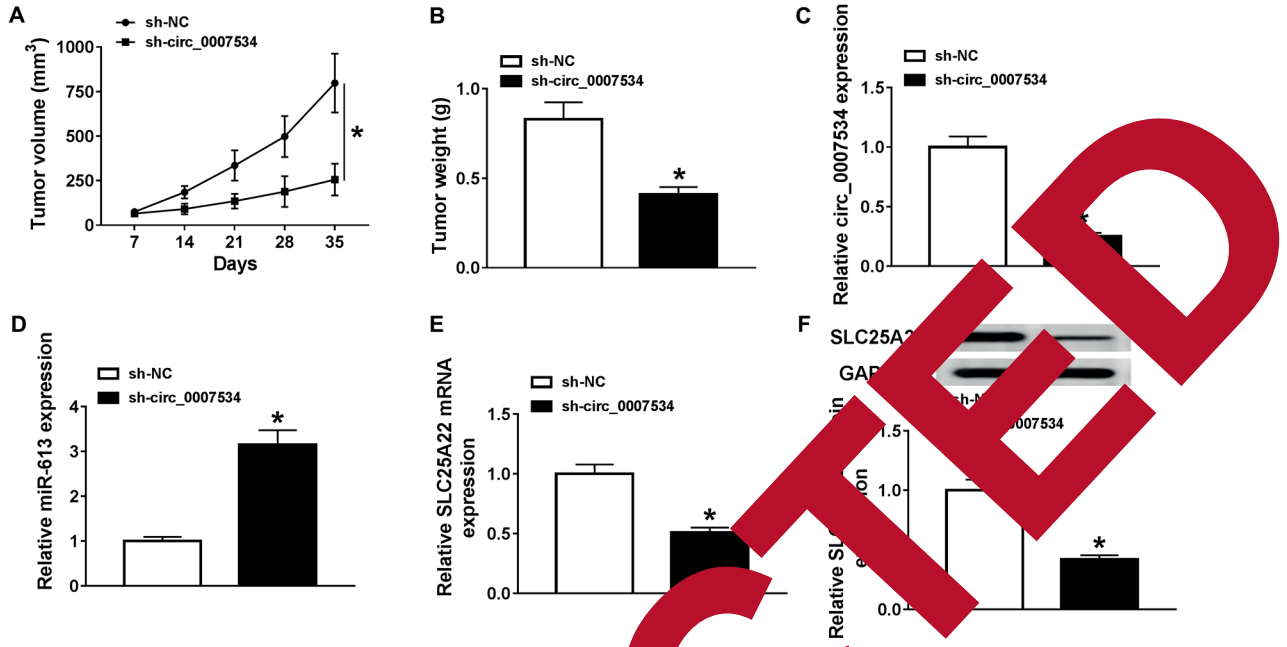

Figure 8. Circ_0007534 knockdown attenuated tumor growth *in* **A,** Tumor vo was calculated in sh-circ_0007534 group and sh-NC group every 7 d. **B,** Tumor weight was analyzed af d of injection **C,** The RNA levels of circ_0007534, miR-613, and SLC25A22 were detected in sh-circ_0007534 group NC group The protein level of SLC25A22 was determined in sh-circ_0007534 group and sh-NC group. *$p<0.05$.

by repressing miR-613 expression. To con this hypothesis, LoVo and SW620 cells w transfected with si-NC, si-c 7534, s circ_0007534 + anti-miR-NC si-c 0007534 + anti-miR-613, respectiv ext, qT CR and Western blot assay reveal t Sl downregulated by _0 knockde n, and then partly re d due t -613 depletion (Figure 7B hese result wed that circ_0007534 res iR-613 e ession to upregulate C25A22 l

Circ 7534 Depletion ppressed Tu Grow n Vivo

T ple whethe circ_0007534 regulated tum wth of *in vivo*, the mice were tane i ed with LoVo cells transfe with s 0007534 or sh-NC. Then, we ca ated tum volume every 7 d, and found t was significantly smaller in sh-c_0007534 group than that in sh-NC group (Figure 8A). Moreover, tumor weight was down-ated by circ_0007534 depletion (Figure 8B). Next, the levels of circ_0007534, miR-613, and SLC25A22 were determined. As expected, the levels of circ_0007534 and SLC25A22 were downregulated, and the level of miR-613 was upregulated in sh-circ_0007534 group (Figure 8C-F). Taken together, circ_0007534 depletion repressed tumor growth *in vivo*.

Discussion

Colorectal cancer is a common cancer with high metastasis rate worldwide[24]. Although some advanced technologies were used for its therapy, the treatment effect of most CRC patients still needs to be improved[25]. Thus, it is of importance to explore the mechanism of CRC development for the treatment of CRC patients. In this research, we investigated the functional mechanism of circ_0007534 in CRC. The results demonstrated that circ_0007534 knockdown suppressed the development of CRC by regulating miR-613/SLC25A22 axis.

Many circRNAs were reported to be involved in the development of CRC. Fang et al[26] confirmed that circ_100290 knockdown repressed cell proliferation and mobility and promoted apoptosis *via* modulating miR-516b/ Frizzled-4 (FZD4) axis in CRC. Li et al[27] demonstrated that circ_102958 promoted CRC development by regulation of miR-585/cell division cycle 25B (CDC25B) axis. Bian et al[28] showed that circ_103809 negative-

ly regulated the growth of CRC cells by targeting miR-532-3p. In this research, we found that circ_0007534 level was increased in CRC tissues and cells. Furthermore, circ_0007534 knockdown suppressed the proliferation, colony formation, and mobility of CRC cells. Previous data indicated that circ_0007534 acted as an oncogene in some cancers, including cervical cancer[29], pancreatic ductal adenocarcinoma[30], breast cancer[31], glioma[32], and CRC[8]. These findings agree with our results. Also, the effect of circ_0007534 knockdown on glycolysis was explored in CRC cells. It is well known that glycolysis is enhanced in cancer cells[33]. It is a process in which glucose is converted into pyruvate accompanied by the production of lactate (fermentation). ECAR is an easily measurable indicator of glycolysis activity[34]. The inhibited ECAR, glucose consumption and lactate production revealed that glycolysis was suppressed. In addition, the levels of glycolysis-related proteins (such as HK2, GLUT1, and LDHA) can also be used to evaluate glycolysis. HK2 is the first rate-limiting enzyme in process of glycolysis[35]. GLUTs take charge of the transportation of glucose, and the overexpression of GLUTs facilitated glycolysis[36]. LDHA is an enzyme converts pyruvate to lactate[37]. Thus, the downregulated HK2, GLUT1, and LDHA resulted by circ_0007534 indicated that circ_0007534 knockdown repressed glycolysis in CRC.

CircDENND4C was highly expressed in breast cancer and promoted glycolysis[38], meaning that circRNA, identified as an oncogene, can positively modulate glycolysis in cancers. However, how circ_0007534 affected glycolysis is unrevealed. Therefore, more experiments are needed to investigate the role of circ_0007534 in CRC.

CircRNA can function as competing endogenous RNAs (ceRNAs) to regulate gene expression in cancers. CircRNA-ACAP2 protected cell development by sponging miR-21-5p to regulate T lymphoblast invasion and metastasis protein 1 (Tiam1) in colon cancer[40]. To investigate the functional mechanism of circ_0007534 in CRC, its target was explored. Using the bioinformatics tool starBase v2.0 and the Dual-Luciferase reporter assay, we found that circ_0007534 targeted miR-613 and negatively regulated miR-613 expression. MiR-613 was reported to repress cell proliferation, migration, as well as invasion in a variety of cancers, including nasopharyngeal carcinoma[41], colon cancer[42], bladder cancer[43], and retinoblastoma[44]. Moreover, Li et al[14] demonstrated that miR-613 was lowly expressed in CRC tissues/cells, and miR-613 suppressed cell development. Also our results showed that miR-613 level was reduced in CRC tissues/cells compared with that in normal tissues/cells. In addition, miR-613 was shown to inhibit glycolysis metabolism in gastric cancer[45]. Taken together, it was speculated that circ_0007534 regulated CRC cell progression *via* inhibiting miR-613 expression. Then, we sustained this hypothesis.

Zahid et al[46] showed that miRNA exerted its function *via* targeting 3'-UTR of downstream mRNA. Here, through bioinformatics tool starBase v2.0 and the Dual-Luciferase reporter assay, we found that SLC25A22 was a target of miR-613, and SLC25A22 level was negatively regulated by miR-613. SLC25A22 was a mitochondrial glutamate carrier[47]. In recent years, its role was analyzed in human cancers. Du et al observed that SLC25A22 level was increased, and SLC25A22 promoted cell growth, as well as repressed apoptosis in gallbladder cancer. In CRC, SLC25A22 was highly expressed, and SLC25A22 accelerated cell proliferation. Our results demonstrated that SLC25A22 level was increased in CRC tissues and cells. The effect of SLC25A22 on glycolysis in human cancers was unknown. Wong et al[20] showed that the silence of SLC25A22 suppressed glycolysis in mice. These data suggested that SLC25A22 acted as a positive regulator in CRC development. Then, whether miR-613 regulates CRC by modulating SLC25A22 level was investigated in this study. As expected, miR-613 repressed the development of CRC cells *via* downregulating SLC25A22 expression.

Finally, our results detected that circ_0007534 inhibited miR-613 expression to upregulate SLC25A22 level in CRC cells, meaning that circ_0007534 exerted its function by regulating miR-613/SLC25A22 axis. Next, the effect of circ_0007534 on tumor growth was explored. As expected, circ_0007534 depletion attenuated tumor growth *in vivo*.

Conclusions

These findings demonstrated that circ_0007534 knockdown suppressed the development of CRC by modulating miR-613/SLC25A22 axis, providing a theoretical basis for the therapy of CRC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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