**CircularRNA-****0003420 protects viability of the established colorectal neuroendocrine carcinoma cells acting as mediator of miR-1278/SHP-1 signaling pathway**

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**Abstract.** Circular RNAs (circRNAs), aberrantly expressed, can regulate tumorigenesis and subsequent tumor progression in a wide spectrum of cancers. This study aims to investigate the function and effect of hsa\_circ\_0003420 in colorectal neuroendocrine carcinoma (CNEC). We established two CNEC cell lines (SS-2 and HROC57) based on previous reports. The circRNA microarray and RT-qPCR were used for detecting the expression of hsa\_circ\_0003420 in CNEC tissues and cell lines. The cell proliferation was examined using Cell Counting Kit 8 (CCK8) assay, colony formation assay and Xenograft tumor in nude mice. Wound-healing assay were used for cell viability. Flow cytometry was used to measure cell apoptosis. The protein detection was detected by western blot assay. The expression of hsa\_circ\_0003420 was down-regulated in CNEC tissue and cell lines. Upregulated hsa\_circ\_0003420 restored cell proliferation, viability, and repressed apoptosis, as tested in SS-2 and HROC57 cells. Bio-informatics and Dual-luciferase reporter assay confirmed that circ\_0003420 acted as a sponge of miR-1278, while miR-1278 could target the 3’UTR of SHP-1 gene. Circ\_0003420 could interact with miR-1278, and thereby upregulated the expression of SHP-1, which subsequently deactivated STAT3 sensor. Simultaneously, miR-1278 was found to be elevated, and SHP-1 was down-regulated in CNEC specimens and cell lines. Rehabilitation of miR-1278 counterbalanced the impact of circ\_0003420 on proliferation, viability, apoptosis in SS-2 and HROC57 cells. Moreover, the effect of SHP-1 overexpression in the SS-2 and HROC57 cells presented a similar phenotype with circ-0003420. The present study suggested that hsa\_circ\_0003420 was able to protect CNEC cell lines against apoptosis via mediating the miR-1278/SHP-1 signal pathway.

**Keywords**: Colorectal neuroendocrine carcinoma; CNEC; hsa\_circ\_0003420; miR-1278; SHP-1

**Introduction**

Neuroendocrine cell carcinomas (NEC) are uncommonly identified in colorectal, accounting for only roughly 0.1% of all colorectal cancers (1). However, compared to colon and rectal adenocarcinomas, their prognosis is much worse, with about half of these patients died within one year (2-4). Neuroendocrine tumors are staged according to the World Health Organization (WHO) classification (5).

Circular RNAs (circRNAs) are a relatively new category of single-stranded, non-coding RNAs, which form a covalently bonded continuous loop (6). They have been detected in many different organisms and are abundantly expressed in the eukaryote life tree, especially between humans and mice (7). Their unique structure provides them with a stable and longer half-life than linear RNAs, and their expression also indicates developmental and tissue stage-specific characteristics, very similar to linear mRNAs (8). They have multiple functions, such as serving as protein scaffolds or miR sponges, and have important biological roles in a wide spectrum of diseases. Recent experimental studies suggest that circRNAs control the levels of encoded transcripts by interacting on a limited library of microRNAs (miRNAs) (9, 10).

MiRNAs serve as critical regulators of both normal physiologic as well as pathologic processes of the body. These include regulating a series of cellular processes through post-transcriptional inhibition of their target genes. Although previous studies have indicated a potential role of circRNAs and miRNAs in neuroendocrine tumors, a detailed analysis of that mechanism remains obscure (11, 12).

Shinji et al. established an NEC SS-2 cell line stemed from colon tumor and verified with typical immunohistochemical markers of neuroendocrine tumors. Moreover, it retained an elevated Ki-67 labeling index in original tumor when implanted into nude mice. The SS-2 cell line formed a spherical shape and expressed high-level a specific cancer stem cell (CSC) marker (13). Gock et al. also established an HROC57 cell line derived from a colonic large cell neuroendocrine carcinoma (14). These two newly established and verified neuroendocrine midgut cell lines were utilized in the present study. Initially, the expressions of circ\_0003420 in CNEC tissue and CNEC cell lines vs. normal healthy colorectal samples and colorectal adenocarcinoma cells were examined. Then, CCK-8 assay, colony formation assay, Xenograft tumor in nude mice, wound-healing assay and flow cytometry were utilized to determine the effect of circ\_0003420 on CNEC cell proliferation, viability, apoptosis. The results from the bioinformatics prediction and Dual-luciferase reporter assay confirmed that circ\_0003420 served as an important ceRNA for miR-1278, and the miR-1278 targeted SHP-1 gene. However, the relationship of circ\_0003420/miR-1278/SHP-1 is still unknown. Therefore, we pay attention to regulatory mechanisms of circ\_0003420, miR-1278 and SHP-1 in CNEC treatment.

**Methods**

***Specimen acquisition***

Surgical tumor specimens from 32 patients with colonic neuroendocrine carcinomas operated at Harbin Medical University Cancer Hospital from Aug 2010 to Sep 2019 provided the basis for this study. The diagnostic criteria for all study neuroendocrine carcinomas were consistent with the World Health Organization guidelines (WHO, 2019) (15). All enrolled participants gave their informed consent, and this study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital.

***circRNA microarray***

TRIzol reagent was applied to extract total RNA from CNEC tissue and adjacent normal samples (n=3). The rRNA Ratio (28s/18s) of all enrolled samples surpassed 1.8, with an RNA Integrity Number (RIN) over 8.0. The data was analyzed by miRNA QC tool software (Affymetrix). The microRNA differences in CNEC tissues were P<0.05 and 1.5 times, which were considered dysregulated circRNAs.

***Cell lines establishment and culture***

SS-2 and HROC57 CNEC cell lines were established according to the previous reports (13, 14). Cells were cultured with 5% CO2 at 37 ℃ in a humidified incubator. The dishes were maintained in RPMI-1640 medium 10 ml containing 10% FBS and 1% penicillin / streptomycin. The medium for cell lines was changed every three days, and new cell lines were cultured for over 60 generations.

***Cell transfection***

The hsa\_circ\_0003420 (pCD-ciR-circ\_0003420) and SHP-1 (pcDNA3.1-SHP-1) overexpression vectors was synthesized by Genscript Co., Ltd. (Beijing, China). MiR-1278 mimic sense as follows: 5’-UAGUACUGUGCAUAUCAUCUAU-3’; and NC mimic sense as follows: 5’-UUGUACUACACAAAAGUACUG-3’) were synthesized by Genscript Co., Ltd.

Lipofectamine 3000 was applied to transfect SS-2 and HROC57 cells with the synthesized vector noted above on the basis of the manufacturer’s instruction book. Following forty hours of transfection, the SS-2 and HROC57 cells were harvested after transfection forty hours for subsequent experiments.

***Real-time quantitative PCR (RT-qPCR)***

In the light of the manufacturer’s instructions, total RNA was segregated with an RNeasy Mini Kit (Qiagen, Hilden, Germany). After RNA isolation, reverse transcription and polymerase chain reaction (PCR) was determined via RNase R, PrimeScript RT Master Mix, 2 × PCR Master Mix (Thermo Fisher Scientific). The ΔΔCt method was applied to estimate the relative level of various genes, and GAPDH was used for a control gene. Primers for RT-qPCR were as below: hsa\_circ\_0003420 Forward, 5’-UAGAGAAGCGCGUUCUAGC-3’; hsa\_circ\_0003420 Reverse, 5’-AGUGCUCACGUCCCGUAGT-3’; GAPDH Forward, 5’-TCAAGGCTGAGAACGGGAAG-3’; GAPDH Reverse, 5’-TCGCCCCACTTGATTTTG GA-3’.

***Cell Counting Kit 8 (CCK8) assay***

The cells were incubated at 2 × 103 cells/well into the 96-well plates for 0, 24, 48, and 72 h. Then, 10 µl CCK8 reagent and 90 μl fresh medium were added to the 96 well plate for 1 hour. The optical densities (OD) at 450 nm were detected by the microplate reader. The OD values were analyzed and a line chart was created by the GraphPad 8.0.

***Colony formation assay***

500 cells for a single cell suspension were planted into a 6-well plate with 3 ml culture medium. After 4 days of cultivation, cell samples in 1×PBS were harvested and stained with crystal violet. Clones with a cell count greater than 50 were determined by microscopy (Olympus Corp., Tokyo, Japan).

***Flow cytometry***

In accordance with the manufacturer's instruction, the cells were stained with 5 μl Annexin V-FITC and Propidium Iodide following the Apoptosis Assay Kit. Then, the Annexin V+/PI- and Annexin V+/PI+ stained cells were served as the apoptotic cells according to FACS Calibur flow cytometer.

***Western blot (WB) analysis***

The total proteins of cells and specimen were extracted via RIPA buffer. Then, the protein concentration was assessed by BCA Protein Assay Kit. The primary antibodies were as followed: SHP-1 (CST, #26516, 1:1000), GAPDH (CST, #2118, 3:1000). The secondary antibodies were as followed: HRP-conjugated goat anti-rabbit (Beyotime Biotechnology, A0208, 1:5000), HRP-conjugated goat anti-mouse (Beyotime Biotechnology, A0216, 1:5000). Chemiluminescence was evaluated using the Millipore chromogenic solution.

***Wound-healing assay***

The confluent cells were bruised by a 200 μl pipette tip in 6-well plates. The cells were conceded to migrate into the wound. After fixation, the scratched area was detected by microscopy. Migration ratio (%) was calculated by dividing the width at 48 hours by the width at 0 hours.

***Bioinformatic prediction***

The prediction algorithm MiRanda (https://sourceforge.net/projects/miranda/), starBase (<http://starbase.sysu.edu.cn/>), PicTar (https://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org) were used to distinguish targets of circ\_0003420 and miR-1278. The listed divination were based on targeted efficacy predictions. The predictions were ranked based on their probability of conservative targeting (16).

***Dual-luciferase reporter assay (DLRA)***

Regulatory interrelations among circ\_0003420, miR-1278 and SHP-1 were evaluated using DLRA. Additionally, the WT, mutant 3'-UTR of circ\_0003420 and SHP-1 were performed by the assay. After incubation for 36 hours, cells were transfected with miR-1278 mimic and NC mimic.

***Xenograft tumor in nude mice***

A total of 24 clean grade 6-week-old female Balb/c nude mice with weighing (20 ± 2) g were purchased from Charles River Laboratories. The SS-2 cells, following 35 passages transfected with adenovirus with NC or circ\_0003420 (1 × 107 cells/ml). Then, The 2 × 106 cells were injected subcutaneously under the left axilla of every mouse, with eight mice in every group. On the 28th day, the mice were euthanized, then, the tumor weights were determined. All experiments were conducted in accordance with the relevant guidelines formulated by the Animal Ethics Committee of Harbin Medical University Cancer Hospital.

***Statistical analysis***

All data in graphs were represented the mean ± SD from three independent experiments. ANOVA test and t tests were performed to evaluate the p values of multiple groups and two groups. All statistical data were analyzed by using SPSS 22.0 software. Statistical significance was considered at a P-value less than 0.05.

**Results**

***Expression of circ\_0003420 in CNEC specimens and SS-2 and HROC57 cell lines***

To discriminate the differential expression of circRNA between CNEC specimens and normal colon tissue, microarray was performed. Notably,\_circ\_0003420 was shown to be downregulated in three CNEC specimens (Fig. 1A). To identify the ectopic expression of circ\_0003420 during CNEC development, we analyzed its expression by qRT-PCR in 32 CNEC samples and 11 healthy normal colon tissues; circ\_0003420 expression was dramatically reduced in CNEC specimens compared with the control group (Fig. 1B). Moreover, circ\_0003420 was as well downregulated in the established cell lines (SS-2 and HROC57), compared with colon adenocarcinoma cells (HCT116) (Fig. 1C), suggesting that circ\_0003420 played a role in CNEC development.

***Ectopic overexpression of circ\_0003420 suppressed cell proliferation of SS-2 and HROC57 cells***

The role of circ\_0003420 on proliferation and survival of SS-2 and HROC57 cells was then investigated. Initially, both cell lines were transfected with either circ\_0003420 overexpression plasmid or empty plasmid. A definite promotion of circ\_0003420 level was demonstrated following transfection (Fig. 2A, 2B). CCK-8 method was used to detect growth of SS-2 and HROC57 cells at different time points under circ\_0003420 overexpression. We found that compared with the NC group, the proliferation rates of SS-2 and HROC57 cells were clearly reduced at 24 hours, 48 hours and 72 hours after transfection (Fig. 2C, 2D).The colony formation experiment confirmed that circ\_0003420 overexpression prominently reduced the quantity of colonies formed by SS-2 and HROC57 cells (Fig. 2E, 2F). These data provided strong evidence that circ\_0003420 played a crucial role in proliferation and viability of CNEC cells.

***Ectopic overexpression of circ\_0003420 induced apoptosis of SS-2 and HROC57 cells***

To explore the effect of circ\_0003420 on the viability of SS-2 and HROC57 cells, we used flow cytometry to detect cell apoptosis after transfection. Upregulation of circ\_0003420 triggered apoptosis of both cell lines as evidenced by an increased number of Annexin V+/PI- and Annexin V+/PI+ stained cells (Fig. 3A, 3B).

***Ectopic overexpression of circ\_0003420 enhanced cell migration of SS-2 and HROC57 cells***

To determine the influence of circ\_0003420 on migration, CNEC cell lines with dysregulation of circ\_0003420 come in for wound-healing assay. According to the wound-healing assay, circ\_0003420 promotion significantly enhanced migration of SS-2 and HROC57 cells (Fig. 4A, 4B). These data suggested circ\_0003420 might impact the capacity of CNEC cells to metastasize.

***Circ\_0003420 targets miR-1278***

Previous studies noted that miR-1278 modulated cell proliferation in glioma and nasopharyngeal cancer (17, 18), but its effect on carcinomas of the colon is still unclear. However, bio-informatics analysis indicated that the circ\_0003420 gene covered a hypothetical binding site complementary to the miR-1278 seed region (Fig. 5A). Then, DLRA was used to study the direct relation between miR-1278 and circ\_0003420 (Fig. 5B). Luciferase activity was restrained by approximately 60% in HEK293T cells transfected with the miR-1278 mimic bound to WT circ\_0003420. Then, miR-1278 expression in 32 CNEC specimens, SS-2 and HROC57 cell lines was investigated. The qRT-PCR data indicated that miR-1278 was dramatically promoted in CNEC samples, SS-2 and HROC57 cell lines, compared to controls (Fig. 5C, 5D). Moreover, overexpression of circ\_0003420 caused a notable reduction of miR-1278 in both SS-2 and HROC57 cells compared with NC groups (Fig. 5E, 5F). These data confirmed that circ\_0003420 acts as an endogenous sponge for miR-1278.

***miR-1278 restoration obviated the effect of circ\_0003420 on proliferation, apoptosis, migration of SS-2 and HROC57 cells***

To determine whether miR-1278 was involved in circ\_0003420 regulated cell proliferation, migration, apoptosis of SS-2 and HROC57 cells, these cells were co-transfected with circ\_0003420 overexpressing vector and miR-1278 mimic (or NC mimic) to upregulate the level of miR-1278 in cells with circ\_0003420. We found that miR-1278 expression was dramatically elevated in the circ\_0003420 overexpressing cells, compared with circ\_0003420 + NC mimic groups (Fig. 6A, 6B). However, circ\_0003420 level was not changed with miR-1278 upregulation (Fig. 6A, 6B). Next, the role of miR-1278 upregulation on proliferation of CNEC cells was evaluated. CCK-8 assay performed that transfection of miR-1278 mimic caused a significant restoration in the proliferation of SS-2 and HROC57 cells with the circ\_0003420 overexpression, as compared with circ\_0003420 + NC mimic group (Fig. 6C, 6D). Colony formation assay also indicated that miR-1278 restoration increased the number of colonies (Fig. 6E, 6F). Additionally, data from flow cytometry demonstrated that miR-1278 upregulation eliminated the apoptotic cell percentage of SS-2 and HROC-57 cells which was induced by circ\_0003420 (Fig. 6G, 6H). These data confirmed that miR-1278 participated in the circ\_0003420-mediated cell proliferation via repressing apoptosis. Moreover, miR-1278 upregulation remarkably recovered the amount of migrated SS-2 and HROC57 cells with circ\_0003420 overexpression, as determined by Wound healing assay (Fig. 7A, 7B). In short, these data shown that circ\_0003420 influenced migration of SS-2 and HROC57 cells via targeting miR-1278.

***MiR-1278 targets 3’-UTR of SHP-1***

Bio-informatic prediction was then undertaken to identify a prospective target of miR-1278, and SHP-1 gene was identified (Fig 8A), a key tumor suppressor in the development of multiple cancers (19). Data from DLRA suggested that SHP-1 was an important target gene of miR-1278, and transfection of miR-1278 mimic led to downregulation of luciferase activity through interacting with WT SHP-1 (Fig. 8B). This led to studying the expression level of SHP-1 mRNA in CNEC specimens and cell lines. The qRT-PCR shown that SHP-1 mRNA was significantly lower in CNEC samples and cell lines than those in normal healthy tissues and HCT-116 cells (Fig. 8C, 8D). Further clarify this relationship between circ\_0003420, miR-1278 and SHP-1 expression, cells were also transfected with various plasmids. SS-2 and HROC57 cells were transfected with miR-1278 inhibitor (or NC inhibitor), knowing that the expression level of miR-1278 was upregulated in the CNEC cells, as expected, miR-1278 was downregulated as assessed by qRT-PCR. Then qRT-PCR and western blot indicated that transfection of miR-1278 inhibitor resulted in an upregulation of SHP-1 expression level in SS-2 and HROC57 cells (Fig. 8E, 8F, 8I, 8J). To investigate the reverse actions, cells were also transfected with circ\_0003420 overexpressing vector and NC vector, then circ\_0003420 overexpression resulted in increased expression of SHP-1 (Fig. 8G, 8H, 8K, 8L). These data strongly indicated that SHP-1 expression was regulated negatively by miR-1278, and its expression was positively associated to circ\_0003420 in CNEC cells.

***Effect of SHP-1 overexpression on proliferation, migration apoptosis of CNEC cells***

To evaluate the role of SHP-1 on CNEC cell phenotypes, the SHP-1 was overexpressed in SS-2 and HROC57 cells. After transfection with pcDNA3.1-SHP-1 vector, SHP-1 levels in SS-2 and HROC57 cells were upregulated at both protein and mRNA levels (Fig. 9A-9D). As a result of SHP-1 overexpression, cell proliferation and viability were impaired, as evidenced (Fig. 9E-9H). The proportion of apoptotic cells was significantly increased after SHP-1 overexpression as determined by flow cytometry (Fig. 9I, 9J). Moreover, the migration capacity of SS-2 and HROC57 cells were prominently reduced in cells with SHP-1 overexpression, compared with NC groups (Fig. 10A, 10B). These results indicated that SHP-1 acted as a tumor suppressor for CNEC via inhibiting tumor phenotypes such as proliferation, migration.

***Circ\_0003420 repressed tumorigenesis of CNEC in nude mice***

The final step in determining the role of circ\_0003420 in CNEC tumor formation was performing xenograft studies. Nude mice were injected subcutaneously with SS-2, SS-2-NC and SS-2-circ\_0003420 cells, euthanized at 28 days post-injection, and the tumors were excised and weighed. Comparing tumors in the Model and NC groups, tumors in the circ\_0003420 overexpression group showed a slower rate of growth with a lower mean tumor weight and volume (Fig. 11A, 11B). Expression of circ\_0003420, miR-1278 and SHP-1 mRNA in the tumor tissues (n = 8) in each group were quantitated, and circ\_0003420 and SHP-1 mRNA were upregulated whereas the expression of miR-1278 was diminished in the circ\_0003420 group, compared with mice in the Model and NC groups (Fig. 11C, 11D).

**Discussion**

Although CNEC constitute less than 1% of all colorectal malignancies, their typically highly aggressive growth pattern and strong propensity for metastasis portends a lethal outcome (20). As has been said before, the distant metastasis, frequency of lymph node metastasis, and one-year survival rates are 38-73%, 60-87% and 15-46%, respectively, indicating that an early diagnostic indicator of CNEC is urgently needed.

Accumulating investigations have performed that circRNAs play significant roles in the growth and metastasis of malignant cells. Dysregulated circRNAs have been found in a wide spectrum of tumors, including colon, gastric, breast, bladder, and lung(21-26). As an example, in colorectal cancer (CRC), knockdown of circ-BANP with siRNA significantly reduced CRC proliferation and the expression of phosphorylated Akt (27). Compared with matched normal mucosa, ciRS-7 was prominently upregulated in colorectal cancer tissues, and its overexpression was related to lower patient survival rate. Overexpression of ciRS-7 in HT29 and HCT116 cells resulted in the prohibiting of miR-7 and led to a more aggressive carcinogenic phenotype, and overexpression of ciRS-7 allowed for the suppression of miR-7 and soon afterwards activation of EGFR and RAF1 oncogenes (28). Li et al. performed high-throughput RNA sequencing in 4 paired colorectal cancer tissues, and circVAPA was confirmed as a potential functional circRNA. In addition, circVAPA level was related to adverse clinical and pathological features in colorectal cancer. Studies on functional acquisition and loss of CRC cell lines showed that circVAPA enhanced CRC cell proliferation, inhibited apoptosis, migration and invasion (29). In our study, the microarray was utilized to differentiate expression of circRNA in CNEC and normal colon tissue. The results shown that circ\_0003420 was downregulated, which was confirmed by qRT-PCR results in CNEC samples and established cell lines. Gain-of function study showed that circ\_0003420 overexpression reduced CNEC cell proliferation, promoted apoptosis, migration; these results provided strong evidence of an anti-tumor effect in vitro.

Increasingly, reports have demonstrated that malignant tissues have exhibited distinctive miRNA expression profiles in cancer patients (30), and miRNA signatures may be distinctive among various cancer types (31). Potentially carcinogenic and inhibitory miRNAs have been identified, such as specific miRNA signals to predict clinical outcome of lung neuroendocrine tumors (32-34) as well as in small intestine (35, 36). Nevertheless, people have limited understanding of the differential expression of miRNAs in human colonic neuroendocrine tumor. In the present study, miR-1278, a not well-documented miRNA, was found to be increased in CNEC tissue and cell lines. Its restoration expunged the inhibition effect of circ\_0003420 on oncogenic phenotypes of SS-2 and HROC cells. Furthermore, miR-1278 was elucidated as an upstream miRNA of SHP-1, which has been reported as a tumor suppressor (37), suggesting a potential effect of miR-1278 on CNEC development.

The SHP-1 and SHP-2 are comprised of a C-terminal protein tyrosine phosphatase domain and two SH2 NH2-terminal domains (38, 39). SHP-1 and SHP-2 regulate several cellular functions with a similar signal axis (40). Liang et al. shown that RNF6 expression was dramatically upregulated in colorectal tumors. Downregulation of RNF6 inhibited invasion and proliferation of colorectal cancer cells through activation of pSTAT3 that regulated the degradation and ubiquitination of SHP-1 (41). SC-43 and SC-78, two novel SHP-1 agonists, showed suppression of human colorectal cancer cells in vitro (42), suggesting that SHP-1 functions as a tumor suppressor of colorectal cancer. This suggested that SHP-1 could serve as a suppressor of CNEC. As a target of oncogene miR-1278, single overexpression of SHP-1 in SS-2 and HROC57 cells suppressed cell proliferation, induced apoptosis, migration of CNEC cells, all consistent with previous reports (41, 42).

**Conclusions**

The overall conclusion was that circ\_0003420 inhibited cell proliferation, migration and expedited cellular apoptosis by targeting the miR-1278/SHP-1 axis, which provides a new therapeutic strategy for CNEC treatment. Therefore, circ\_0003420/SHP-1 agonists and miR-1278 inhibitors may logically be investigated in human studies as potential treatments of CNEC.

**Declarations**

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No.

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**Availability of data and materials**

All original data proposed in the study are included in the published article. For further inquiries, please contact the corresponding author.

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No.

**Conflict of interest**

The authors declare that they have no conflicts of interest.

**Ethics approval and consent to participate**

The study was approved by the Ethics and Animal Research Committee of Harbin Medical University Cancer Hospital (Approved No.82271845).

**Consent for publication**

Not applicable.

**References**

1. Wu H, Yu Z, Liu Y, et al. Genomic characterization reveals distinct mutation landscapes and therapeutic implications in neuroendocrine carcinomas of the gastrointestinal tract. Cancer Commun (Lond) 2017; 42: 1367-1386.
2. Hrabe J. Neuroendocrine Tumors of the Appendix, Colon, and Rectum. Surg Oncol Clin N Am 2020; 29: 267-279.
3. Volante M, Grillo F, Massa F, et al. Neuroendocrine neoplasms of the appendix, colon and rectum. Pathologica 2021; 113: 19-27.
4. Qiu S, Pellino G, Warren OJ, et al. Mixed adenoneuroendocrine carcinoma of the colon and rectum. Acta. Chir. Belg 2018; 118: 273–277.
5. Assarzadegan N, Montgomery E. What is New in the 2019 World Health Organization (WHO) Classification of Tumors of the Digestive System: Review of Selected Updates on Neuroendocrine Neoplasms, Appendiceal Tumors, and Molecular Testing. Arch Pathol Lab Med 2021;145(6):664-677.
6. Smoniewski CM, Mirzavand Borujeni P, Petersen A, et al. Circular mitochondrial-encoded mRNAs are a distinct subpopulation of mitochondrial mRNA in Trypanosoma brucei. Sci Rep 2023;13(1):7825.
7. Wang PL, Bao Y, Yee MC, et al. Circular RNA is expressed across the eukaryotic tree of life. PLoS One 2014;9(6):e90859.
8. Jeck WR, Sharpless NE. Detecting and characterizing circular RNAs. Nat Biotechnol 2014;32(5):453-461.
9. Johnsson P, Ackley A, Vidarsdottir L, et al. A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. Nat Struct Mol Biol 2013;20(4):440-446.
10. Salmena L, Poliseno L, Tay Y, Kats L, et al. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language?. Cell 2011;146(3):353-358.
11. Li SC, Essaghir A, Martijn C, et al. Global microRNA profiling of well-differentiated small intestinal neuroendocrine tumors. Mod Pathol 2013;26(5):685-696.
12. Roldo C, Missiaglia E, Hagan JP, et al. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. J Clin Oncol 2006;24(29):4677-4684.
13. Shinji S, Sasaki N, Yamada T, et al. Establishment and characterization of a novel neuroendocrine carcinoma cell line derived from a human ascending colon tumor. Cancer Sci 2019;110(12):3708-3717.
14. Gock M, Mullins CS, Harnack C, et al. Establishment, functional and genetic characterization of a colon derived large cell neuroendocrine carcinoma cell line. World J Gastroenterol 2018;24(33):3749-3759.
15. Nagtegaal ID, Odze RD, Klimstra D, et al. The 2019 WHO classification of tumours of the digestive system. Histopathology 2020;76(2):182-188.
16. Friedman RC, Farh KK, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009;19(1):92-105.
17. Zhao Y, Wang P, Wu Q. miR-1278 sensitizes nasopharyngeal carcinoma cells to cisplatin and suppresses autophagy via targeting ATG2B. Mol Cell Probes 2020;53:101597.
18. Zhou X, Lv L, Zhang Z, et al. LINC00294 negatively modulates cell proliferation in glioma through a neurofilament medium-mediated pathway via interacting with miR-1278. J Gene Med 2020;22(10):e3235.
19. Yuan X, Duan Y, Xiao Y, et al. Vitamin E Enhances Cancer Immunotherapy by Reinvigorating Dendritic Cells via Targeting Checkpoint SHP1. Cancer Discov 2022;12(7):1742-1759.
20. Morgan S, Slodkowska E, Parra-Herran C, et al. PD-L1, RB1 and mismatch repair protein immunohistochemical expression in neuroendocrine carcinoma, small cell type, of the uterine cervix. Histopathology 2019;74(7):997-1004.
21. Vo JN, Cieslik M, Zhang Y, et al. The Landscape of Circular RNA in Cancer. Cell 2019;176(4):869-881.e13.
22. Hsiao KY, Lin YC, Gupta SK, et al. Noncoding Effects of Circular RNA CCDC66 Promote Colon Cancer Growth and Metastasis. Cancer Res 2017;77(9):2339-2350.
23. Li P, Chen H, Chen S, et al. Circular RNA 0000096 affects cell growth and migration in gastric cancer. Br J Cancer 2017;116(5):626-633.
24. Liang HF, Zhang XZ, Liu BG, et al. Circular RNA circ-ABCB10 promotes breast cancer proliferation and progression through sponging miR-1271. Am J Cancer Res 2017;7(7):1566-1576.
25. Zhong Z, Huang M, Lv M, et al. Circular RNA MYLK as a competing endogenous RNA promotes bladder cancer progression through modulating VEGFA/VEGFR2 signaling pathway. Cancer Lett 2017;403:305-317.
26. Zhang S, Zeng X, Ding T, et al. Microarray profile of circular RNAs identifies hsa\_circ\_0014130 as a new circular RNA biomarker in non-small cell lung cancer. Sci Rep 2018;8(1):2878.
27. Zhu M, Xu Y, Chen Y, et al. Circular BANP, an upregulated circular RNA that modulates cell proliferation in colorectal cancer. Biomed Pharmacother 2017;88:138-144.
28. Weng W, Wei Q, Toden S, et al. Circular RNA ciRS-7-A Promising Prognostic Biomarker and a Potential Therapeutic Target in Colorectal Cancer. Clin. Clin Cancer Res 2017;23(14):3918-3928.
29. Li XN, Wang ZJ, Ye CX, et al. Circular RNA circVAPA is up-regulated and exerts oncogenic properties by sponging miR-101 in colorectal cancer. Biomed Pharmacother 2019;112:108611.
30. Tang L, Chen HY, Hao NB, et al. microRNA inhibitors: Natural and artificial sequestration of microRNA. Cancer Lett 2017;407:139-147.
31. Ali Syeda Z, Langden SSS, Munkhzul C, et al. Regulatory Mechanism of MicroRNA Expression in Cancer. Int J Mol Sci 2020;21(5):1723.
32. Malczewska A, Frampton AE, Mato Prado M, et al. Circulating MicroRNAs in Small-bowel Neuroendocrine Tumors: A Potential Tool for Diagnosis and Assessment of Effectiveness of Surgical Resection. Ann Surg 2021;274(1):e1-e9.
33. Lee SS, Cheah YK. The Interplay between MicroRNAs and Cellular Components of Tumour Microenvironment (TME) on Non-Small-Cell Lung Cancer (NSCLC) Progression. J. Immunol. Res. 2019, 3046379.
34. Asiedu, M. K., Thomas, C. F., Jr, Dong, J., Schulte, S. C., Khadka, P., Sun, Z., et al. (2018). Pathways Impacted by Genomic Alterations in Pulmonary Carcinoid Tumors. J Immunol Res 2019;2019:3046379.
35. Vicentini C, Fassan M, D'Angelo E, et al. Clinical application of microRNA testing in neuroendocrine tumors of the gastrointestinal tract. Molecules 2014;19(2):2458-2468.
36. Malczewska A, Kidd M, Matar S, et al. A Comprehensive Assessment of the Role of miRNAs as Biomarkers in Gastroenteropancreatic Neuroendocrine Tumors. Neuroendocrinology 2018;107(1):73-90.
37. Huang TT, Su JC, Liu CY, et al. Alteration of SHP-1/p-STAT3 Signaling: A Potential Target for Anticancer Therapy. Int J Mol Sci 2017;18(6):1234.
38. Tai WT, Shiau CW, Chen PJ, et al. Discovery of novel Src homology region 2 domain-containing phosphatase 1 agonists from sorafenib for the treatment of hepatocellular carcinoma. Hepatology 2014;59(1):190-201.
39. Qian H, Deng X, Huang ZW, et al. An HNF1α-regulated feedback circuit modulates hepatic fibrogenesis via the crosstalk between hepatocytes and hepatic stellate cells. Cell Res 2015;25(8):930-945.
40. Garg M, Wahid M, Khan F Dr. Regulation of peripheral and central immunity: Understanding the role of Src homology 2 domain-containing tyrosine phosphatases, SHP-1 & SHP-2. Immunobiology 2020;225(1):151847.
41. Liang Q, Ma D, Zhu X, et al. RING-Finger Protein 6 Amplification Activates JAK/STAT3 Pathway by Modifying SHP-1 Ubiquitylation and Associates with Poor Outcome in Colorectal Cancer. Clin Cancer Res 2018;24(6):1473-1485.
42. Chung SY, Chen YH, Lin PR, et al. Two novel SHP-1 agonists, SC-43 and SC-78, are more potent than regorafenib in suppressing the in vitro stemness of human colorectal cancer cells. Cell Death Discov 2018;4:25.

**Figure legends**

Figure 1. circ\_0003420 is downregulated in CNEC patient samples and CNEC cell lines. (A) microarray was performed to identify the differentially expressed RNA between three CNEC specimen and three normal healthy colon tissue. (B) qRT-PCR analysis shown expression of circ\_0003420 in CRC samples (n = 32) and in samples from healthy tissue (n = 11). (C) qRT-PCR analysis displayed the circ\_0003420 expression in CNEC cell lines (SS-2 and HROC57), compared with a colorectal adenocarcinoma cell line (HCT-116). The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01 , \*\*\*P < 0.001 vs. indicated groups.

Figure 2. Effect of circ\_0003420 on proliferation and viability of SS-2 and HROC57 cells. SS-2 and HROC57 cells were transfected with pCD-ciR-Empty or pCD-ciR-circ\_0003420 for 36 h. (A, B) qRT-PCR analysis was applied to detect circ\_0003420 level in SS-2 and HROC57 cells. (C, D) CCK-8 assay displayed the cell proliferation of SS-2 and HROC57 cells at 0, 24, 48, and 72 h after transfection. (E, F) Colony formation assay shown the growth of SS-2 and HROC57 cells post transfection. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 3. Effect of circ\_0003420 on apoptosis of SS-2 and HROC57 cells. SS-2 and HROC57 cells were transfected with pCD-ciR-Empty or pCD-ciR-circ\_0003420 for 36 h. (A, B) Flow cytometric analysis of CNEC cells transfected with pCD-ciR-Empty or pCD-ciR-circ\_0003420. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01 , \*\*\*P < 0.001 vs. indicated group.

Figure 4. Effect of circ\_0003420 inhibited migration of SS-2 and HROC57 cells. SS-2 and HROC57 cells were transfected with pCD-ciR-Empty or pCD-ciR-circ\_0003420 for 36 h. Migration capacity of SS-2 (A) and HROC57 (B) cells was measured by wound-healing assay. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 5. circ\_0003420 acts as ceRNA of miR-1278. (A) Bioinformatics analysis shown a binding site for miR-1278 in the circ\_0003420 sequence. (B) DLRA was determined, and then co-transfection of a luciferase reporter containing either a WT or MU form of circ\_0003420, and miR-1278 or NC mimic into HEK293T cells. (C) qRT-PCR analysis indicated expression of miR-1278 in CNEC specimens (n = 32) and in samples from healthy tissue (n = 11). (D) qRT-PCR analysis displayed miR-1278 expression in CNEC cell lines (SS-2 and HROC57), compared with HCT116 cells. (E, F) SS-2 and HROC57 cells were transfected with pCD-ciR-Empty or pCD-ciR-circ\_0003420 for 36 h. qRT-PCR analysis was used to detect miR-1278 levels in cells of each group. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 6. MiR-1278 upregulation obviated the influence of circ\_0003420 on proliferation and apoptosis of SS-2 and HROC57 cells. Cells were co-transfected with pCD-ciR-circ\_0003420 and miR-1278 mimic (or NC mimic) for 36 h. (A, B) qRT-PCR analysis was performed to detect circ\_0003420 level in SS-2 and HROC57 cells. (C, D) CCK-8 assay displayed that the cell proliferation of SS-2 and HROC57 cells at 0, 24, 48, and 72 h after transfection. (E, F) Colony formation assay shown the growth of SS-2 and HROC57 cells after transfection. (G, H) Flow cytometric analysis of CNEC cells with different transfection. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 7. miR-1278 upregulation counteracted the effect of circ\_0003420 on metastasis of SS-2 and HROC57 cells. Cells were co-transfected with pCD-ciR-circ\_0003420 and miR-1278 mimic (or NC mimic) for 36 h. Migration capacity of SS-2 (A) and HROC57 (B) were evaluated by wound-healing assay. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 8. miR-1278 targets the 3'-UTR of the SHP-1 mRNA. (A) Bio-informatics analysis shown that miR-1278 possesses a binding site in the 3'-UTR of the SHP-1 gene. (B) DLRA was used to follow by co-transfection of a luciferase reporter containing either a WT or MU form of SHP-1 mRNA, and a miR-1278 mimic into HEK293T cells. (C) The qRT-PCR analysis shown expression of SHP-1 mRNA in CNEC samples (n = 32) and in samples from healthy tissue (n = 11). (D) The qRT-PCR analysis shown expression levels of SHP-1 mRNA in CNEC cell lines (SS-2 and HROC57), compared with HCT116 cells. (E, F) SS-2 and HROC57 cells were transfected with miR-1278 inhibitor (or NC inhibitor) for 36 h. qRT-PCR was utilized to detect the expression of miR-1278 and SHP-1 mRNA in each group. (G, H) Cells were co-transfected with pCD-ciR-circ\_0003420 and miR-1278 mimic (or NC mimic) for 36 h. qRT-PCR were applied to detect SHP-1 expression at both mRNA levels in each group. (I, J, K, L) WB analysis was used to detect SHP-1 protein levels in each group. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 9. Effect of SHP-1 on proliferation and apoptosis of SS-2 and HROC57 cells. Cells were transfected with pCDNA3.1-NC or pCDNA3.1-SHP-1 for 36 h. (A, B) qRT-PCR analysis was used to detect circ\_0003420 level in SS-2 and HROC57 cells. (C, D) WB analysis was used to detect SHP-1 protein levels in each group. (E, F) CCK-8 assay displayed cell proliferation of SS-2 and HROC57 cells at 0, 24, 48, and 72 h after transfection. (G, H) Colony formation assay showed growth of SS-2 and HROC57 cells post transfection. (I, J) Flow cytometric analysis of CNEC cells with different transfection. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 10. Effect of SHP-1 on metastasis of SS-2 and HROC57 cells. Cells were transfected with pCDNA3.1-NC or pCDNA3.1-SHP-1 for 36 h. Migration capacity of SS-2 (A) and HROC57 (B) cells were evaluated by wound-healing assay. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.

Figure 11. Effect of circ\_0003420 overexpression on xenograft CNEC tumorigenesis. The SS-2, SS-2-NC and SS-2-circ\_0003420 cells were subcutaneously injected into the left back flank of 6-week-old female Balb/c nude mice (n = 8). (A) Mice were euthanized, and the tumor were weighed at 28 days post inoculation. Tumors weights were measured from each group. (B, C) Tumor volume at 28 days. (D) Expression of circ\_0003420, miR-1278, and SHP-1 mRNA in the tumor samples of each group was detected by qRT-PCR analysis. The data were displayed as mean ± SD. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001 vs. indicated groups.