Dysregulated SNORD93 promotes colorectal cancer progression

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Abstract

Background Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. Recently, mounting evidence has identified dysregulated snoRNAs active in cancer by influencing cell transformation and carcinogenesis, and meanwhile, SNORD93 is abnormally expressed and associated with poor prognosis in many cancers. Thus, the present study aimed to investigate the function of SNORD93 in CRC. Methods The expression levels of SNORD93 were measured in CRC tissues and cells by qRT-PCR. Then, the biological significance of SNORD93 on proliferation, migration, cell cycle, and cell apoptosis with gain function analyses was explored by CCK8, colony formation, wound healing, and flow cytometry. Additionally, The expression of related proteins was quantified through Western blotting. Result A significant downregulation of SNORD93 was identified in CRC tissues and cells. Simultaneously, downregulated SNORD93 was considerably associated with poor survival of CRC patients. Furthermore, the overexpression of SNORD93 suppressed CRC cell proliferation via inducing G0/G1 cell cycle arrest and apoptosis, while it didn't affect CRC cell migration. Conclusion SNORD93 functions as a significant tumor suppressor snoRNA in CRC.

Introduction

Colorectal cancer (CRC) ranked third in the most common and was the second leading cause of cancer-related deaths, accounting for approximately 10% of all cancers [1]. Progress against CRC has accelerated by advances in early screening, surveillance, and high-quality treatment, leading to a decline in CRC incidence and mortality to some extent [2][3]. Nevertheless, the recent overall prognosis of CRC patients has remained largely discouraging. About 25% of patients with incurable CRC and 35–45% of the patients with CRC stages II and III succumb to relapse within five years after surgery [4]. Additionally, the surge of Early-onset colorectal cancer is particularly alarming. The CRC death rate increased by 1.2% per year in individuals younger than 50 years between 2005 and 2019 [5]. Therefore, the high morbidity necessitates new biomolecular indicators and therapeutic targets for CRC.

Small nucleolar RNAs (snoRNAs), a subclass of non-coding RNAs, are typically distributed in the nucleolus [6]. Previously, snoRNAs were identified as housekeeping genes because of their unique function in post-transcriptional modification of RNA, including ribosomal RNA (rRNA) and small nuclear RNA (snRNA) by base-pairing with target RNAs [7][8]. Until 2002, Chang and colleagues discovered that h5sn2, H/ACA box snoRNA, exhibited lower expression in meningioma and contributed to the poor prognosis, establishing a groundbreaking connection between snoRNA and cancer development [9]. Since then, numerous investigations revealed that snoRNAs acted in dual roles of promoting and restraining malignancy [10][11]. For instance, SNORA42, as an oncogene, enhanced migration and invasion ability in prostate cancer cells via reprogramming EMT [12]. While SNORA21 functioned as a tumor inhibitor, inducing cells to stagnate in the G0/G1 phase [12]. Consequently, exploring the regulatory role of snoRNAs in the pathogenesis and progression of cancer is beneficial in identifying new markers for the diagnosis, treatment, and prognosis of cancer patients.

SNORD93, a subclass of C/D box snoRNA, has been found to be essential in tumor development. In particular, SNORD93 is aberrantly expressed in renal clear cell carcinoma, reflecting the poor prognosis of patients [13]. Moreover, upregulated SNORD93 acts as a catalyst for the invasion of breast cancer, which can be converted into the brief fragment sdRNA93 resembling miRNA to inhibit the production of the Pipox [14]. However, the expression of SNORD93 and its biological significance in CRC is still unknown. Here, we revealed that SNORD93 was downregulated in CRC and functioned as a CRC suppressor. This study investigated the biological behavior of SNORD93 and its potential molecular mechanism in CRC, which was anticipated to provide new insights into the molecular mechanisms underlying the development and progression of CRC.

Materials and Methods

Cell culture

The human CRC cell lines HT-29(HTB-38), SW480(CCL-228), HCT-15 (CCL-225), and the human normal intestinal epithelial cell line HIEC(CRL-3266) were obtained from the American Type Culture Collection(ATCC). Then all cell lines were cultured in RPMI-1640 complete medium(Gibco) containing 10% fetal bovine serum (FBS, Gibco), and 1% penicillin -streptomycin(Invitrogen) in a 5% CO_2 humidified atmosphere incubator at 37°C.

Plasmids and transfection

The full-length SNORD93 sequence was cloned into the pGPU6-GEP-Neo vector(Invitrogen) for overexpression SNORD93 purposes. Cells were transfected with the pGPU6-SNORD93 plasmid using lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. The expression of SNORD93 was further confirmed by qRT-PCR.

RNA extraction and real-time quantitative PCR

By the Column Extraction Kit, total RNA was extracted, then using HiScript III RT SuperMix for qPCR following the protocol provided by the manufacturer, RNA underwent reverse transcription into cDNA. Subsequently, the SNORD93 mRNA level was measured by quantitative real-time PCR amplifying SYBR Green Master Mix kit, while the expression was normalized to 18S rRNA, and the relative expression was evaluated by the $2 - \Delta\Delta Ct$ method.

Cell counting kit-8 (CCK-8) assay

Cell Counting Kit 8 assessed the cell viability following the CRC cells transfected with pGPU6-SNORD93 or the control. The cells were seeded into the 96-well plates with 5000 cells in each well, then cultivated for 0, 24, 48, and 72 hours and added the 10ul CCK8-reagent, respectively. Afterward, the plate was incubated for 1.5 hours at 37°C, followed by measurement of the optical density values at 450nm.

Colony formation assay

Colony formation assay evaluated the cell capacity of colony formation after the CRC cells were transfected with pGPU6-SNORD93 or the control. The cells were cultured in 6-well plates with 1000 cells in each well, and the medium was refreshed every two days. Fourteen days later, the cell colonies became visible. Therefore, these were fixed with formaldehyde for 30 minutes and stained with crystal violet for 30 minutes. Cell colonies were then counted manually after the background color was clean.

Cell cycle assay

Cell cycle assay analyzed the cell cycle profile of CRC cells that were respectively treated with pGPU6-SNORD93 or the control. Forty-eight hours went by, and the CRC cells were harvested and then washed twice in cold PBS, resuspended in 70% ethanol, and fixed overnight at 4°C. Later, these were treated with PI for 30 minutes at 37°C. Cell apoptosis assayed the percentages of cells in different phases of the cell cycle and was measured with a flow cytometer, then was analyzed with Flowjo software(FlowJo_v10.6.2).

Cell apoptosis assay

The cell apoptosis assay detected the apoptosis in CRC cells transfected with the control or pGPU6-SNORD93. After 48 hours, the CRC cells were digested with trypsin without EDTA and harvested following treatment with the Annexin-V-FITC binding buffer, Annexin-V-FITC, and PI. Subsequently, the CRC cells were incubated for 20 minutes at room temperature and shielded from light, followed by an ice bath, and Flow cytometric analysis was then performed. PI is red fluorescent, whereas Annexin V-EGFP is green fluorescent.

Wound healing assay

The wound healing assay measured the migration capacity of CRC cells after transfection with the control or pGPU6-SNORD93. A total of 5×105 CRC cells were cultured in 6-well plates, then a vertical wound was marked in the Petri dish using the tip of a 200 μL pipette. The outcomes of scratch migration to the central zone were respectively evaluated at 0, 24, and 48 hours.

Statistical analysis

For statistical analysis, GraphPad Prism8 software was utilized. The t-test was employed to compare statistical differences between the two groups, and one-way ANOVA was applied to compare data from multiple groups. P values less than or equal to 0.05 were considered statistically significant, while values greater than 0.05.

Results

SNORD93 was downregulated and correlated with poor prognosis in CRC

To investigate the potential role of SNORD93 in CRC, the qRT-PCR was performed to measure the abundance of SNORD93 CRC patients. As shown, the expression of SNORD93 was significantly downregulated in CRC tissue while compared with adjacent normal tissues (Fig. 1A). Furthermore, we analyzed the SNORD93 expression patterns in a subset of CRC lines (SW480, HT-29, and HCT-15) as well as the normal colonic epithelial cell line HIEC. As expected, SNORD93 exhibited lower expression in CRC cell lines (Fig. 1B). These data indicated that aberrant expression of SNORD93 might contribute to the CRC progression. Therefore, we further screened SNORD93 in *SNORic* databases [15]. The patients were divided into two groups based on the median expression level of SNORD93 and then assessed for the association between SNORD93 expression and overall survival. The results demonstrated that a lower SNORD93 expression suffered from a significantly worse prognosis (Fig. 1C). These consistent results above suggested that SNORD93 might be a novel tumor suppressor gene in CRC.

SNORD93 inhibited the proliferation of CRC cells

Tumor cell proliferation is one of the most significant representative indicators of malignant phenotypes. To determine the biological effects of SNORD93 on CRC tumor progression, we upregulated the expression of SNORD93 in the HCT-15 and HT-29 cells by transfecting pGPU6-SNORD93. Without a doubt, the transfection of pGPU6-SNORD93 appeared to enhance the expression of SNORD93 in both HCT-15 and HT-29 cell lines (Fig. 2A). As expected, CCK8 assay showcased that the proliferation was inhibited when the SNORD93 in CRC cell lines was strengthened (Fig. 2B). Consistently, the colony-formation assay presented that overexpressed SNORD93 resulted in fewer and smaller colonies of CRC cells (Fig. 2C). Altogether, these investigations indicated that overexpression of SNORD93 significantly restrained CRC cells proliferation ability.

SNORD93 limited CRC growth by regulating the cell cycle and promoting apoptosis.

The receipt of appropriate cell cycle signals is of great importance for the proliferation of normal cells, which restricts the unfettered proliferative potential [16]. To determine whether the function of SNORD93 in cell proliferation is associated with the cell cycle. The SNORD93 overexpressed and negative control CRC cells were stained with PI. Data from flow cytometry revealed that SNORD93 enhanced the proportion of CRC cells in the G0/G1 phase (Fig. 3A&B). Subsequently, the expression of cell cycle-related proteins was detected. As shown in Fig. 3D, SNORD93 enhanced G1 block-related protein p21 while simultaneously decreasing the levels of cyclinD3, cyclinE2 and CDK9.

Notably, evasion from apoptosis is one of the hallmarks of cancer, and de-regulated apoptotic signaling induces continuous proliferation, ultimately leading to the formation of cancer [17]. To further elucidate the mechanism by which SNORD93 affects CRC cell proliferation, we performed Annexin V/PI staining on CRC cells after transfected with pGPU6-NC or pGPU6-SNORD93 to analyze cell death and apoptosis. Our lab's observations showed that upregulating SNORD93 raised the proportion of apoptotic cells (Fig. 3C). Subsequently, we measured the expression of apoptotic proteins, the data then showed that SNORD93 stimulated the expression of caspase3 caspase7 caspase8, and bim (Fig. 3E). In addition, the

phosphorylation of both RIPK3 and MLKL, which have emerged as essential cellular elements during necroptosis, increased in pGPU6-SNORD93 treated CRC cells (Fig. 3F). Our in vitro findings strongly suggested that overexpressed SNORD93 influenced the proliferation of colorectal cancer by arresting the cell cycle and inducing apoptosis.

SNORD93 failed to influence CRC cell migration

Tumor-metastasis is an ominous property of most aggressive tumors, leading to cancer-related deaths. EMT is an essential cellular progress in which epithelial cells exhibit mesenchymal peculiarities. Generally, EMT has been associated with various tumor functions, including tumor cell migration, metastasis [18], and chemotherapy resistance [19]. Next, we investigated SNORD93's biological role in the metastasis and the expression of EMT-related genes. As shown in Fig. 4A&B, the migration capacity of CRC cells did not alter in response to SNORD93 pressure. Additionally, the expression of E-cadherin and β-catenin remained constant in contact with the control group (Fig. 4C). According to these results, the enforced expression of SNORD93 failed to influence the migration ability of CRC cells.

Discussion

Small nucleolar RNAs (snoRNAs), a subclass of non-coding RNAs, are typically distributed in the nucleolus and range in length from 60 to 300nt without protein-coding capacity [20]. Increasing evidence has revealed that the dysregulated snoRNA was tightly connected to the occurrence and progression of tumors, including CRC. By way of illustration, SNORD126 promoted the proliferation of CRC through increasing the expression of fibroblast growth factor receptor 2 (FGFR2) and activating the PI3K-Akt pathway [21]. Besides, combining with GAS5, the upregulated SNORD44 led to caspase-dependent apoptosis, which inhibited CRC cells from proliferating [22]. Moreover, SNORD1C expressed highly in CRC and enhanced the stem cell properties of CRC cells through activating the Wnt/β-catenin pathway [23]. Therefore, exploring the expression pattern of snoRNA in CRC and its regulatory role in the pathogenesis and progression of CRC may be beneficial in identifying new markers for the diagnosis, treatment, and prognosis of CRC.

Given the potential relevance of the dysregulated expression of snoRNAs in CRC, especially, SNORD93 exerts functions in multiple cancers. In this study, we investigated the expression pattern of SNORD93 and the possible molecular mechanisms underlying the CRC development. Indeed, we found that SNORD93 was obviously downregulated in CRC. Notably, CRC patients with lower expression of SNORD93 were related to poor prognosis. Adversely, Y. Zhao and colleagues found that SNORD93 was abundant in renal clear cell carcinoma tissues. Thus, SNORD93 might exhibit oppositely expression patterns in different cancers. Likewise, SNORD44 has been reported to be weakly expressed in prostate cancer while massively increasing in breast cancers [24][25].

Importantly, we explored the malignant features of SNORD93 in CRC cell lines. Initially, our findings suggested that SNORD93 blocked cell proliferation but failed to impact cell migration in CRC. Additional

evidence declared that exogenous SNORD93 did not influence the expression level of E-cadherin and β-catenin, which were respectively associated with EMT. These data led us to conclude that SNORD93 regulated cell proliferation rather than cell migration in CRC. Similarly, SNORD113-1 significantly suppressed cancer growth in hepatocellular carcinoma cells but neither affected their migration nor invasion [26]. While this outcome contradicts Patterson DG's discovery that MDA-MB-231 metastatic breast cancer cells exhibited elevated levels of SNORD93 expression, and the migration of MDA-MB-231 cells can be inhibited by using anti-sdRNA93 [14]. Overall, snoRNAs function as either oncogenes or tumor suppressor genes and regulate the biological behavior of malignancies in a tumor- and tissue-specific manner. As described, our recent results presented that SNORD93 functioned as a CRC suppressor, and on the other hand, SNORD93 activated the metastatic breast cancer cells.

All cancers depend on continuous cell division, and typically controlled cell cycle progression prevents the excessive proliferation of cancer cells [27]. According to the previous data, SNORD78 inhibited the proliferation of non-small cell lung cancer by inducing G0/G1 phase arrest, while SNORD76 delayed cell entry into the S phase in glioblastoma [28][29]. Meanwhile, SNORD52 enhanced the G2/M cell cycle transitions in hepatocellular carcinoma [30]. Consistently, our result presented that upregulated SNORD93 restrained CRC cell proliferation by arresting tumor cells into the G0/G1 phase. Expectedly, its upregulation was accompanied by increasing expression of the protein p21, blocking the G0/G1 phase, and a decreased expression of the proteins cyclinE2, cyclinD3, and CDK9, which were associated with driving G1 into the S phase. On the contrary, Shen L observed that SNORA24 enhanced the G1/S phase transition, contributing to colony formation, cell proliferation, and the growth of xenograft tumors in CRC [31].

Induction of cell apoptosis is inexorably linked with the development of cancer, and in particular, activation of caspases results in the programmed execution of cell apoptosis. To further understand the possible mechanism layed in SNORD93’s tumor suppressor function, we focused on caspase-dependent apoptosis. We elucidated that the upregulated SNORD93 effectively promoted cell apoptosis, together with the production of caspase8, Bim, caspase3, and caspase7 increased. Among them, caspase8 has been well-established in activating the death receptor signaling cascade [32]. Bim translocated and attached to Bcl-2 after receiving apoptosis signaling, triggering the release of cytochrome C and inducing mitochondrial apoptosis pathway, and they respectively activated the effector caspase3 and caspase7 to perform apoptosis. Likewise, the overexpression of SNORD44 with its host GAS5 stimulated cystatin-dependent apoptosis, which inhibited CRC cell proliferation [22].

Actually, caspase8 is irreplaceable in initiating not only exogenous apoptosis but also necroptosis [33]. Necroptosis is a novel identified form of programmed necrotic cell death. The ripoptosome is a protein complex whose contains FADD, RIPK1, caspase 8, and cFLIP-, and the formation of the ripoptosome is developed by RIPK1 recruiting and phosphorylating RIPK3 (pRIPK3), subsequently phosphorylates MLKL (pMLKL) to build the necrosome. Large pores generated by MLKL oligomers in phosphatidylinositol phosphate (PIP)-rich patches in the plasma membrane result in necroptotic cell death [34][35]. Notably, necroptosis was a double-edged sword with dual regulation of tumor occurrence and development [36]

[37]. For instance, Zeng found that necroptosis inhibited the CRC of proliferation, migration, and invasion [38]. However, Liu declared that necroptosis might participate in the growth of early CRC through enteritis mouse model experiments [39]. Surprisingly, our current data demonstrated that SNORD93 might prevent the proliferation of CRC cells by causing necrotizing apoptosis in cells.

Taking all these into account, our current work demonstrated the suppressor function of SNORD93 in CRC tumorigenesis, which might facilitate the development of snoRNA- directed-detection and treatment against cancers. Nevertheless, the mechanism underlying SNORRD93's oncogenic function is required for further investigation.

Conclusions

In summary, our findings demonstrated that inhibited SNORD93 was closely related to the poor outcome of CRC individuals. Notably, SNORD93 inhibited proliferation though delaying cells' entry into the S phase, inducing apoptosis as well as necroptosis in CRC. Collectively, our recent finding implied that SNORD93 might be a novel biomarker and potential therapeutic target of CRC.

Declarations

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Author Contributions

All authors contributed to the study conception and design. Conceptualization, Z.Z. and T.Z.; methodology, Y.X. and T.Z.; validation, Y.X., M.C.; investigation, J.L.; resources, M.C.; data curation, H.W.; writing—original draft preparation, M.C.; writing—review and editing, Y.X.; supervision, Z.Z. and T.Z; project administration, Z.Z. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was confirmed by the Ethical Committee of Quanzhou First Hospital and written informed consent was obtained from all patients.

Consent for Publication

Not applicable

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Figures

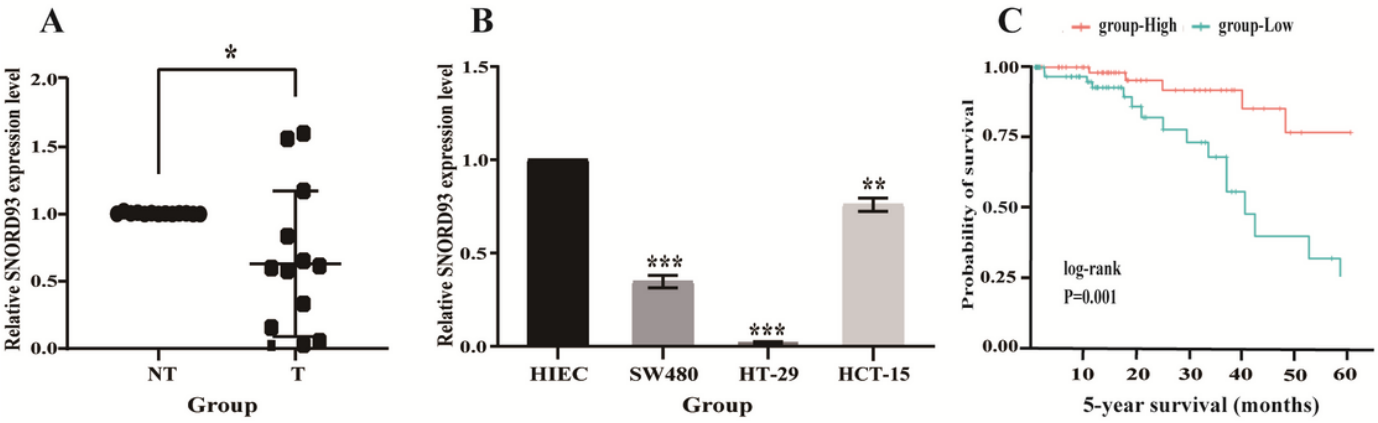

Figure 1

Low expression of SNORD93 in colorectal cancer. (A) Expression levels of the SNORD93 mRNA in 13 CRC tissues and adjacent tissues were determined by qPCR analysis, the expression levels of SNORD93 in each CRC tissue were normalized to 18S. * $p < 0.05$. (B) Expression levels of the SNORD93 mRNA normal cells (HIEC) and CRC cells (SW480, HT-29, and HCT-15), weredetermined by qPCR analysis. the expression levels of SNORD93 in each CRC cell were normalized to that in HIEC. ** $p < 0.01$; *** $p < 0.001$. (C) Correlation between the SNORD93 expression level and the 5-year survival rate of CRC patients. Green referred to rectal carcinoma patients with low SNORD93 expression, whilered referred to rectal carcinoma patients with high SNORD93 expression.

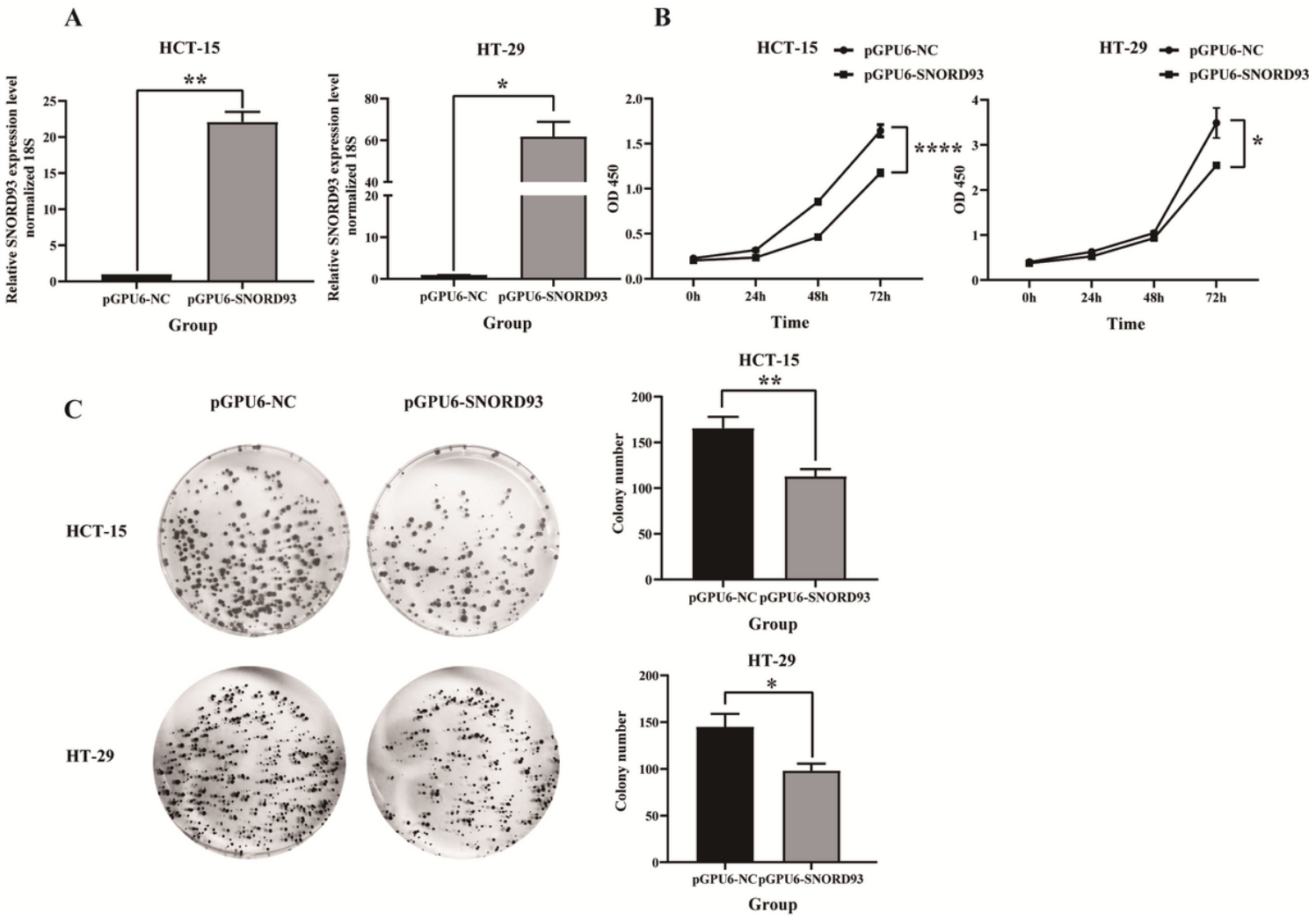

Figure 2

SNORD93 inhibited the proliferation of CRC cells. (A) The expression levels of SNORD93 in HCT-15 and HT-29 cells were upregulated after transfection with the SNORD93 overexpression vector compared with the pGPU6-NC group.* $p < 0.05$; ** $p < 0.01$. (B) The effects of SNORD93, with increased expression, on the proliferation of HCT-15 and HT-29 cell lines were detected by CCK8 compared with the pGPU6-NC group.* $p < 0.05$; **** $p < 0.0001$. (C) The effect of SNORD93, with increased expression, on the colony formation of HCT-15 and HT-29 lines was detected by clonal formation assay compared with the pGPU6-NC group.* $p < 0.05$; ** $p < 0.01$.

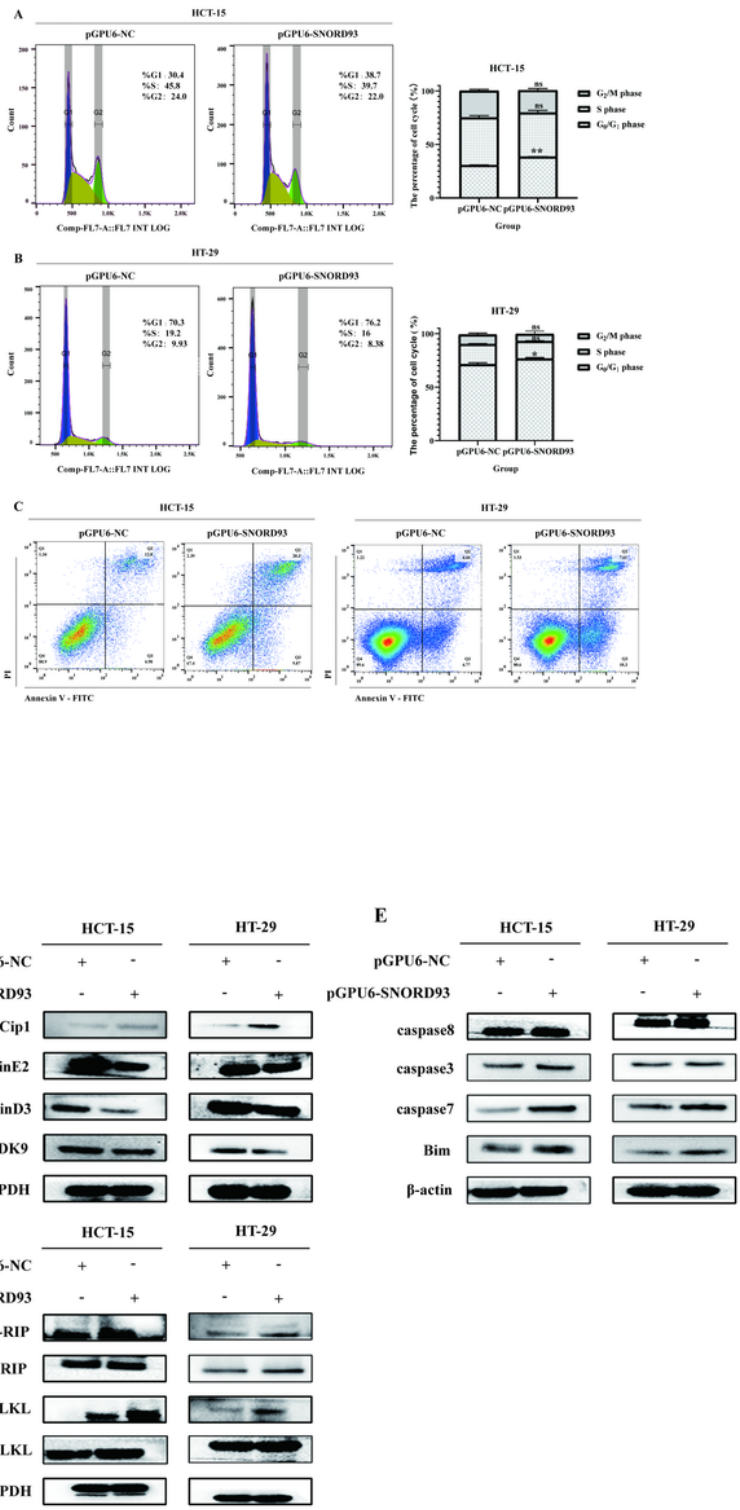

Figure 3

SNORD93 inhibited the proliferation of CR. (A, B). A flow cytometry analysis of the effect of cell cycle on CRC cell lines with increased expression of SNORD93 compared with the pGPU6-NC group. ns $P > 0.05$; ** $p < 0.01$. (C). A flow cytometry analysis of the effect of apoptosis on CRC cell lines with increased expression of SNORD93 compared with the pGPU6-NC group. ns $P > 0.05$; * $p < 0.05$. (D) Western blot analyses of the effects of overexpression of SNORD93 on the expression levels of p21, cyclinE2,

cyclinD3, and CDK9 in HCT-15 and HT-29 cell lines. (E) Western blot analyses of the effects of overexpression of SNORD93 on the expression levels of caspase8, caspase3, caspase7, and Bim in HCT-15 and HT-29 cells. (F) Western blot analyses of the effects of overexpression of SNORD93 on the expression levels of pRIP, RIP, pMLKL, and MLKL in HCT-15 and HT-29 cells.

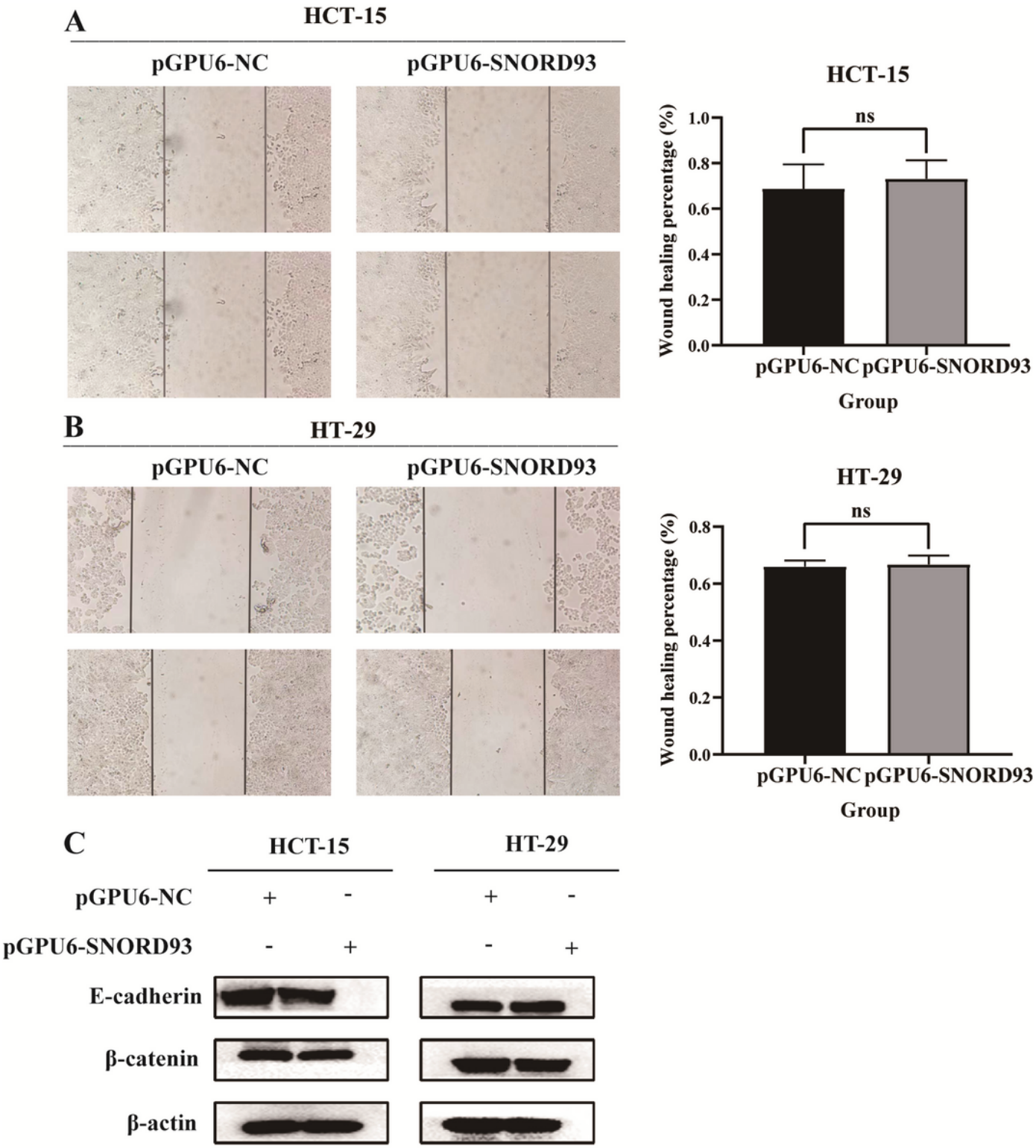

Figure 4

SNORD93 had no significant effect on the migration of colorectal cancer cells. (A, B) The effects of increased expression of SNORD93 on the apoptosis of HCT-15 and HT-29 cell lines were detected by scratch test compared with the pGPU6-NC group. Ns meant $P > 0.05$ (C) Western blot analyses of the effects of overexpression of SNORD93 on the expression levels of E-cadherin and β-catenin in HCT-15 and HT-29 cells.