Long Non-coding RNA CRNDE Exacerbates NPC Advancement Mediated by the miR-545-5p/CCND2 Axis

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Abstract

Background: Previous studies indicated CRNDE to have a pivotal part within tumorigenesis. Notwithstanding, precise details on CRNDE activities within NPC are still uncertain. The investigation described in this article served to focus in greater depth on the mechanistics regarding CRNDE, together with all associated regulatory networks, on nasopharyngeal carcinoma (NPC) and its treatment possibilities.

Methods: Quantitative real-time polymerase chain reaction (RT-qPCR) analyzed CRNDE, miR-545-5p and CCND2 expression within NPCs and representative cell lineages. CCK-8 cell counting-, EdU-, wound-healing- / transwell-assays analyzed cellular proliferation, migrative, together with invasive properties. Apoptosis / cell cycle progression were scrutinized through flow cytometry. Dual-luciferase reporter assays validated CRNDE / miR-545-5p / CCND2 interplay. Proteomic expression of apoptosis-related protein, EMT-related protein and CCND2 protein were evaluated through Western blotting. In addition, Ki67 expression was evaluated through immunohistochemical staining. The effect of CRNDE in vivo was assessed by nude murine xenograft model studies.

Results: This study demonstrated up-regulated expression of CRNDE and CCND2 within NPC tissues /cell lines. Meanwhile, miR-545-5p was downregulated. CRNDE knockdown or miR-545-5p over-expression drastically reduced NPC proliferative, migrative and invasive properties, promoted apoptosis / altered cell cycle, and inhibited the expression of CCND2. However, miR-545-5p downregulation had opposing effects. All inhibiting functions generated by CRNDE downregulation upon NPC progression could be counterbalanced or synergistically exacerbated, depending on miR-545-5p downregulation or upregulation, respectively. Multiple-level investigations revealed CRNDE to serve as a sponge for miR-545-5p and can target CCND2 within NPCs.

Conclusions: CRNDE increases CCND2 expression by competitive binding with miR-545-5p, thus accelerating the development of NPC. This provides potential therapeutic targets and prognostic markers against NPC.

Background

Nasopharyngeal carcinoma (NPC) is an epithelial malignant tumor, commonly manifested within the pharyngeal recess[1]. Radio-and chemo-therapy form main therapies. Most patients having early symptoms are challenging to diagnose, and consequently, the disease develops rapidly. Due to the great differences in tumor biology among individual patients, the sensitivity of tumor cells to treatment is also different, poor patient prognoses prevail [2].Therefore, more detailed pathogenesis of NPC and advanced treatment methods must be unravelled.

Long non-coding RNAs (lncRNAs) are > 200 bp and lack an open reading frame that cannot encode proteins[3]. Abnormal expression and mutation of lncRNAs influences tumorigenesis and metastasis

within multiple tumor models, including colon,[4] prostate [5] and oral cancers[6]. Due to their vast spectrum of expression profiles and tissue-linked expression specificity, lncRNAs can be used as tumor markers and therapeutic targets[7]. Colorectal neoplasia differential expression (CRNDE) is a long noncoding RNA with high-sensitivity/-specificity within plasma and tumor tissues. CRNDE was found to be implicated within multiple tumor processes [8].Apart from colorectal cancer, CRNDE is also up-regulated in hepatic cancer[9],cervical cancer[10] and clear-cell-renal-cell carcinoma[11], promoting tumor expansion, invasiveness and metastases. However, the mechanism/s employed by CRNDE to influence NPC malignancy is lacking.

MicroRNAs (miRNAs) entail small non-coding RNAs (20-22 nucleotides long), that possess pivotal regulatory functions upon physiological / developmental processes within all cell-types [12].MiRNA dysregulation is linked to a vast spectrum of human conditions, such as tumors. Previous literature demonstrated miR-545-5p to be downregulated within colon adenocarcinoma and contributes to its proliferative, apoptotic, migrative and invasive properties [13]. LncRNAs have microRNA Responsible Elements (MRE),a sponge binding site for miRNAs, in order to regulate specific miRNA-orchestrated target transcript downregulation [14].Chen and colleagues highlighted lncRNA CRNDE to promote angiogenesis within hepatoblastoma through targeted activity on the miR-203 / VEGFA axis[15]. Zhu et al. confirmed that CRNDE promotes proliferative / angiogenesis properties by pancreatic cancer through regulation of miR-451a and CDKN2D[16]. Notwithstanding such evidence, the function/s of CRNDE and miR-545-5p within NPC are still uncertain and deserve further exploration.

Cyclin D2 (CCND2), has a periodicity in protein independence throughout the cell cycle. CCND2 is intimately associated with NPC tumorigenesis and progression[17]. It is reported that miR-150 can enhance apoptosis within NPC cells and thwart proliferative of NPC cells through CCND2 modulation[18]. This investigation focused on identifying CRNDE, miR-545-5p and CCND2 expression levels within NPC cells for establishing interplays. CRNDE influence upon NPC expansion and metastasis cells through miR-545-5p / CCND2 axis was also studied. The purpose of this study is to contribute novel options for NPC therapies.

Methods

1. Sample collection

Globally, 32 frozen NPC biopsies and healthy juxta-positioned tissues were collected from NPC patient cohorts that underwent surgical intervention at The First Affiliated Hospital of Bengbu Medical College. Patients were not treated prior to surgery. All procedures involving human samples in this study were conducted according to the Helsinki declaration and accepted by the Ethics Committee of The First Affiliated Hospital of Bengbu Medical College. Informed consent was collected from all individuals.

2. Cell culture and transfection

H.Sapiens NPC HNE-1, CNE-2Z, 5-8F and healthy nasopharyngeal epithelial cell line NP69 were from XiangYa School of Medicine of Central South University [Hunan,China]. All NPC cell lines were grown within RPMI-1640 medium [Gibco™, USA], augmented by 10 % fetal bovine serum [FBS, Gibco™, USA] and incubated at 37°C / 5% CO_2. NP69 cell cultures were grown within keratinocyte serum-free medium [Gibco™, Thermo Fisher Scientific, China]. Small interfering RNA against CRNDE (si-CRNDE) and relevant control (si-NC), miR-497-5p mimics and relevant controls (miR-NC), together with miR-497-5p inhibitor and relevant control (miR-inhibitor NC) were all procured from Sangon™ [Shanghai, China]. All transfections employed Lipofectamine 2000® [Thermo Scientific™, China].

3. Quantitative reverse-transcription polymerase chain reaction

Total RNA was collected from CNE-2Z / HNE-1 cells using TRIzol® [Ambion™, TX, USA] and reverse transcribed into cDNA through EasyScript® One-step gDNA Removal and cDNA Synthesis SuperMix kit [TransGen™, China]. Consequently, ABI StepOnePlus® [Bio-Rad™, USA] was utilized for 40 cycles of amplification at 95 °C for 30 s, denaturation at 95 °C for 5 s, and extension at 65 °C for 30 s. GAPDH and U6 served as normalization controls of mRNAs and miRNAs, respectively. The - 2ΔCT method was used to calculate relative expression for all investigated miRNAs / transcripts. Primer sequences for RT-qPCR were as follows:

CRNDE (Forward,5‘-GGAAAAATCAAAGTGCTCGAGTGG-3’;Reverse,5‘-TCTTCTGCGTGACAACTGAGGA-3’),

miR-545-5p (Forward,5‘-CGCGCGTCAGTAAATGTTTATT-3’;Reverse,5‘-AGTGCAGGGTCCGAGGTATT-3’),

CCND2 (Forward,5‘-TTTAAGTTTGCCATGTACCCAC-3’;Reverse,5‘-ACGTCTGTGTTGGTGATCTTAG-3’).

4. EdU assay

Cells of each group of CNE-2Z and HNE-1 in logarithmic growth phase were seeded into 6-well plates (1.5×10^4 cells/well). The cells in each group were labeled with EdU detection solution and consequently fix-treated using 4 % paraformaldehyde at ambient temperature for 15 minutes. Following wash-steps using PBS, 200 µL / well click reaction solution was introduced, and plates placed into incubation for 30 minutes in darkness at ambient temperature. Following another PBS wash-step, 1 mL Hoechst 33342 was added to each well for DNA staining. Following a final PBS wash-step, wells were visualized by laser confocal microscopy.

5. CCK-8 assay

HNE-1 cells and CNE-2Z cells, with good growth status post-transfection, were inoculated in 96-well plates (5000 cells / well) .A 10 μL CCK-8 aliquot was introduced into all wells at 24-, 48-, 72- and 96-hours, respectively, and placed into incubation for 120 minutes. Absorbance (OD) values at 450 nm were measured for individual wells upon a microplate reader, and a proliferation curve was prepared.

6. Transwell assay

In the migration experiment, Materigel gel was not paved. In the invasion experiment, Materigel and Opti-MEM® I Reduced-Serum Medium were diluted at a ratio of 1 : 8, with 50 μL of each chamber spread at the base of the upper-chambers and incubated for 1h to render it semi-solidified. CNE-2Z and HNE-1 cells were inoculated into the upper-chambers within serum-free RPMI-1640 medium, and 600 μL RPMI-1640 medium carrying 10 % FBS was introduced into the lower wells. For the migration experiment, cells were grown for 24 hours. For the invasion experiment, cells were grown for 48 hours. The upper-chambers were consequently removed, fix-treated using 4 % paraformaldehyde for 15 minutes, dyed using crystal violet for 10 minutes, subjected to PBS wash-step, followed by careful removal of excess dye using a cotton swab, and finally observed / counted. The number of cells in five randomly chosen fields was calculated under the microscope, and all experiments were repeated three times.

7. Wound-healing assay

Cells were labeled at the bottom of the six-well plate pre-inoculation. 24 hours post-transfection, once cultures obtained 80 % confluence, cells were lined - perpendicular to the bottom - with a 100 μL sterile pipettor tip/gun. Linear changes at 0 h and 24 h were observed, and the cellular migrative ability was detected. The calculation used was:

Wound healing rate (%) = [(0-h scratch width – 24-h scratch width) / 0-h scratch width] × 100 %.

8. Flow cytometric analyses

In order to assess apoptosis, cultures were collected and resuspended into cell suspension, centrifuged, supernatant discarded, and pre-cooled at 4 °C with D-Hanks (pH=7.2~7.4) washing cell precipitation. Consequently, 1 × binding buffer was-step was performed on cultures cells to precipitate once, followed by centrifugation and cell collection. Consequently, 200 μL 1 × binding buffer was used for resuspending cell precipitation. A total of 2μL Annexin V-APC and PI staining were added, with cultures left at room temperature in darkness for 20–60 minutes. According to cell volume, 200-300μL of 1 × binding buffer were added, followed by on-line detection.

In order to detect cell cycle, samples were harvested / fixed overnight using 70 % ethanol, pre-cooled at 4 °C. Fixative was then removed and cells washed using PBS at 4 °C once. Cell staining solution was prepared by adding 0.5 mL propidium iodide staining solution to each cell sample, slowly and fully

resuspending cell precipitations, followed by 37°C in darkness for 30 min and temporary storage at 4°C or in an ice bath. Following staining, flow cytometry was used to complete the detection.

9. Dual-luciferase reporter assay

All 3 '-UTR CRNDE / CCND2 sequences were amplified using polymerase chain reaction (PCR) and ligated with GV272 vector to construct wild-type CRNDE reporter vector (CRNDE-wt) and mutant-type CRNDE (CRNDE-mut), wild-type CCND2 reporter vector (CCND2-wt) and mutant-type CCND2 (CCND2-mut) luciferase reporter plasmids, respectively. MiR-545-5p mimics together with corresponding negative control miR-NC were co-transfected with the two recombinant plasmids into CNE-2Z cells using lipofectamine® 3000 [Invitrogen™, USA]. Luciferase activities for individual study-groups were evaluated in order to analyze possible binding of CRNDE with miR-545-5p, and CCND2 with miR-545-5p.

10. Western blotting

Protein content from all tissue / cell line samples were extracted and quantified through BCA kit® [Beyotime™, China]. Following electrophoresis, samples were transferred onto PVDF membranes [Biosharp™, China], milk-blocked and consequently hybridized with a variety of primary antibodies [1:1,000 dilution, Proteintech Group, INC.™] and incubated overnight (4°C). This was followed by placing in incubation with in second antibodies [1:1,000 dilution, Proteintech Group, INC.™] at room temperature, membrane scanning and observation of band shifts.

11. Xenograft tumor Model

BALB /c nude murines (female) were segregated into two cohort-groups, sh-CRNDE NC (sh-NC) and sh-CRNDE. The tumor cells of each experimental group in the logarithmic growth phase were counted by blood cell counting plate, and finally suspended with required volume of D-Hanks or PBS. Following preparation of tumor cells (5.00 E +06 cells / murine),a disposable sterile syringe was utilized to aspirate absorb cells and consequently inject 200 μL into each nude murine. At 24-days following subcutaneous injection, each experimental animal was injected with 2% pentobarbital sodium (0.5mL) euthanasia, and cervical dislocation confirmed death. Selected tumor samples were fix-treated with 4% paraformaldehyde for immunohistochemical analyses. All other samples were kept at − 80 °C for Western blot assays.

12. Immunohistochemistry

The transplanted tumor was fixed with formalin for 48 hours, soaked in 4 % paraffin, sliced, dewaxed and hydrated. High pressure antigen repair consisted of 3 % hydrogen peroxide being introduced to tissue samples, followed by incubation at room temperature for 20 minutes. Following sample segment washing, drying, ki-67-treatment and 37 °C incubation for 60 minutes, a secondary antibody was added,

followed by incubation at 37 °C for 20 minutes. Following further wash/dry-steps, DAB coloring agent was added, with monitoring of color time under a microscope for any positive staining stop colorations. Once coloration step was stopped, samples were treated with hematoxylin staining for one minute, followed by 1 % hydrochloric acid treatment (a few seconds).Finally, samples were dehydrated, rendered transparent, and sealed for image capturing.

13. Statistical analysis

SPSS21.0® software was employed for analyzing experimental datasets, represented as mean ± standard deviation [SPSS, Inc™, USA]. One-/ two-way variance analyses were employed for comparative analyses. LSD-t test was employed for group comparisons. A p-value of <0.05 conferred significance.

Results

1. Downregulation of CRNDE inhibits NPC proliferative, migrative and invasive properties.

RT-qPCR was employed for gene expression analysis of CRNDE within 32 NPC and matching adjacent tissues. CRNDE expression in NPC tissues was found to be highly up-regulated in comparison to adjacent tissue levels ($p<0.01$) (Fig.1a). In comparison to healthy nasopharyngeal epithelial cells (NP69), up-regulation was observed for CRNDE in NPC cell lines CNE-2Z, HNE-1 ($P<0.01$) and 5-8F ($P<0.05$) (Fig.1b). Consequently, siRNAs (siRNA1,siRNA2 and siRNA3) were used for inhibiting CRNDE expression within CNE-2Z / HNE-1 cell lines. According to the best inhibitory effect, siRNA1 was selected for subsequent experiments ($P<0.01$) (Fig.1c).

CCK-8 assays indicated that CRNDE downregulation induced drastic regulatory influence on cellular viability in the NPC group, in comparison to the control group and NC ($P<0.01$)(Fig.1d). EdU assays revealed that CRNDE knockdown could significantly reduce the number of positive cells in CNE-2Z and HNE-1 cell lines ($P<0.01$)(Fig.1e). In addition, transwell / wound-healing assays demonstrated that CRNDE silencing in CNE-2Z and HNE-1 cell lines inhibited their migrative / and invasive properties ($P<0.01$)(Fig.2a,b).

EMT-linked proteins E-cadherin, N-cadherin and vimentin were identified via Western blotting. In comparison to the control group and NC, E-cadherin levels within the si-CRNDE group were elevated, with downregulated N-cadherin and vimentin levels ($P<0.01$)(Fig.2c).Such findings suggest CRNDE to exacerbate NPC progression.

2. Downregulation of CRNDE inhibits apoptosis of NPC cells and affects cell cycle.

CRNDE influences NPC pathology were studied by detecting apoptotic activities and cell cycle status of NPC. Flow cytometry demonstrated that apoptotic rates in the si-CRNDE group were elevated in comparison to control / NC groups, and the si-CRNDE groups of CNE-2Z / HNE-1 cell lines exhibited G0/G1 phase arrest (P<0.01)(Fig. 3a,b).

Apoptosis-related proteins were identified through Western blotting. Suppression of CRNDE promoted Bax / cleaved caspase 3 expression, and down-regulated Bcl-2 (P<0.01)(Fig. 3c). Such experimental results highlighted knocking down CRNDE to enhance NPC apoptotic activity via mitochondrial apoptosis-related pathway triggering.

3. CRNDE has a targeting relationship with miR-545-5p

Tiana Tool was used to predict any downstream regulatory correlation for miR-545-5p / CRNDE. The results highlighted that CRNDE possesses a binding site for miR-545-5p (Fig.4a). Firstly, it was confirmed by dual-luciferase reporter assay that over-expressing miR-545-5p blocked luciferase function within CRNDE-wt. This indicated that miR-545-5p binds onto CRNDE through sequence-specific manners (P<0.01)(Fig.4b). Furthermore, RT-qPCR was employed for analyzing relative expression levels of miR-545-5p within the control, si-NC, and si-CRNDE groups of CNE-2Z / HNE-1 cell lines. The assay outcomes demonstrated that miR-545-5p was upregulated within the si-CRNDE group in comparison to control / si-NC groups (P < 0.01) (Fig.4c). According to such outcomes, CRNDE can target and successfully downregulate miR-545-5p.

4. miR-545-5p regulates NPC proliferative, migrative and invasive properties

RT-qPCR was employed for analyzing miR-545-5p expression within NP69, CNE-2Z, HNE-1 and 5-8F. Consequently, miR-545-5p was downregulated within CNE-2Z, HNE-1 and 5-8F, in view of healthy nasopharyngeal NP69 cell line (P<0.01). Since miR-545-5p levels within CNE-2Z and HNE-1 lineages were relatively low, these two cell lines were selected for subsequent experiments.

CNE-2Z cultures were subjected to miR-545-5p mimics-transfection, while HNE-1 underwent miR-545-5p inhibitor transfection (P<0.01)(Fig.5b). CCK-8 assays demonstrated that, in comparison to the control group and NC, the cellular proliferative property was reduced following miR-545-5p upregulation, though increased following miR-545-5p downregulation (P<0.01)(Fig.6c).

EdU assays indicated that miR-545-5p upregulation significantly reduced cellular proliferative property, while miR-545-5p downregulation had opposing effects (P<0.01)(Fig.5e). In addition, transwell assays and wound-healing assays demonstrated that the migrative and invasive properties of CNE-2Z / HNE-1 cell lines were inhibited post-miR-545-5p upregulation, with the latter's downregulation having opposing effects (P < 0.01)(Fig.5d,6a).

E-cadherin, N-cadherin and vimentin were analyzed through Western blotting. E-cadherin level within the miR-545-5p mimic-group was higher in comparison to the control / NC groups, while N-cadherin / vimentin expression was reduced (P<0.01). Proteomic expression profile was reversed post-miR-545-5p (P<0.01)(Fig.6b). Overexpression of miR-545-5p, combined with CRNDE downregulation promotes si-CRNDE-mediated inhibition of cell migrative and invasive properties (P<0.01)(Fig.6c). Such outcomes suggest that miR-545-5p may affect the development of NPC.

5. miR-545-5p influences on apoptosis and cell cycle during NPC progression

Similar to CRNDE, miR-545-5p influences upon NPC pathology was studied by detecting the apoptotic activity and cell cycle status of NPC cells. Flow cytometry revealed that in comparison to the corresponding control / NC groups, the apoptotic rate of miR-545-5p-mimics-group in CNE-2Z was increased (P < 0.01), together with an increased degree of cells within G1 phase (P < 0.01). The apoptotic rate of miR-545-5p-inhibitor-group in HNE-1 cell lines was decreased (P < 0.01), with cellular degree in G1 phase significantly decreased as well (P < 0.01)(Fig.7a,b). Western blotting demonstrated that post-miR-545-5p upregulation of Bax and cleaved caspase 3 proteins were upregulated, with concomitant Bcl-2 downregulation. Conversely, miR-545-5p downregulation had opposing function (P<0.01)(Fig. 7c). Regarding CRNDE downregulation, miR-545-5p downregulation reduced inhibitory functions imposed by si-CRNDE on cellular proliferative property (P<0.01)(Fig.7d). Such outcomes indicated that miR-545-5p could affect NPC cellular cycle to promote apoptosis.

6. CCND2 is targeted by miR-545-5p and CRNDE acts as ceRNA regulating CCND2 expression by miR-545-5p.

Targetscan predicted that CCND2 can be a potential target of miR-545-5p. CRNDE and CCND2 have the same binding sequence as miR-545-5p, suggesting that CRNDE and CCND2 could compete to bind miR-545-5p (Fig.8a).The dual-luciferase reporter assay confirmed this, indicating miR-545-5p over-expression thwarted luciferase function within CCND2-wt (P<0.01) (Fig.8a). RT-qPCR demonstrated miR-545-5p upregulation within CNE-2Z, HNE-1 and 5-8F in comparison with NP69 (P<0.01)(Fig.8b). Furthermore, CCND2 downregulation was noted post-CRNDE downregulation or post-miR-545-5p upregulation. Such results gave opposing trends upon miR-545-5p downregulation (P<0.01) (Fig.8c).Western blot outcomes also demonstrated CCND2 downregulation post-CRNDE downregulation or miR-545-5p upregulation, with such influences being rescued post-miR-545-5p inhibitor treatment (P<0.01)(Fig.8d).

Furthermore, stemming from CRNDE downregulation, concomitant miR-545-5p downregulation weakens inhibitory influence by CRNDE downregulation upon CCND2 expression (P<0.01)(Fig.8e). In essence, CRNDE is a ceRNA whereby miR-545-5p regulates CCND2 expression.

7. Knockdown of CRNDE inhibits xenograft growth associated with miR-545-5p / CCND2 axis.

In order to study the effect of CRNDE knockdown in vivo, a murine xenograft model was developed via injection of sh-CRNDE or its related negative control (sh-NC)(Fig. 9a).In comparison to the sh-NC group, tumor volume / weight in sh-CRNDE group decreased (P<0.01)(Fig.9b). Immunohistochemistry demonstrated that the brown color of sh-CRNDE group was significantly reduced in comparison to NC group, post-ki67 staining and DAB coloring (Fig.9c).Western blotting demonstrated downregulation of CCND2 within sh-CRNDE group, together with upregulation of Bax / cleaved caspase 3; Bcl-2 was also downregulated. In addition, E-cadherin was upregulated while N-cadherin and vimentin were downregulated (P<0.01) (Fig.9d). It is further confirmed that knockdown of CRNDE inhibits the expression of CCND2 and inhibits the progression and metastasis of NPC in vivo.

Discussion

NPC (NPC) a highly prevalent head and neck malignant tumor with significant regionality[19]. The study of the pathogenesis and development mechanism of NPC can open a broader idea for the treatment of NPC. Following the above-described experiments, our investigation found that in NPC cells, miR-545-5p was downregulated, together with CRNDE and CCND2 upregulation. CRNDE can upregulate CCND2 through sponge binding of miR-545-5p, affecting NPC proliferation, migration, invasion and apoptotic activities.

Long-chain non-coding RNA (LncRNA) represents a non-protein-coding RNA molecular class which has pivotal parts within tumor development, treatment and prognosis. In recent years, CRNDE has been confirmed as upregulated within colon cancer and has promoted the development of other tumors. Notwithstanding, CRNDE roles within the process of NPC is rarely reported. This study demonstrated that CRNDE was upregulated within carcinoma tissues of NPC patients. In comparison to normal nasopharyngeal epithelial cells, CRNDE was upregulated within NPC lineages and promotes NPC proliferative, migrative and invasive properties, together with inhibiting apoptosis. This ability to promote cancer was suppressed when CRNDE was knocked down. In mitochondrial apoptosis-related pathways, anti-apoptotic gene Bcl-2 and pro-apoptotic Bax are two key influencing genomic factors[20].Caspases are essential components of the apoptotic mechanism, especially caspase-3[21]. Following knockdown of CRNDE, Bcl-2 was drastically downregulated, with concomitant upregulation of Bax and caspase-3. Epithelial-mesenchymal transition (EMT) describes the gradual event of epithelial cells obtaining mesenchymal profiles, with consequently serious contributions to cancer progression, metastasis and drug resistance[22][23].The reduction of E-cadherin, cytokeratin cytoskeleton replacement by vimentin together with mesenchymal cell morphological manifestations are all considered to be EMT characteristics. E-cadherin increase coupled with vimentin reductions can partially regulate NPC

metastasis and invasiveness.Therefore, we further studied the influence of CRNDE on EMT in NPC cell lines. This investigation demonstrated that post-CRNDE knockdown, E-cadherin was drastically downregulated, with concomitant upregulation of N-cadherin and Vimentin. In essence, such outcomes indicate that CRNDE accelerates NPC progression.

The competitive endogenous RNA (ceRNA) hypothesis of LncRNA shows that when miRNA response elements (MRE) bind to miRNA, miRNA-regulated genes can be silenced[24].Based on this, we launched a series of studies on CRNDE and ceRNA regulatory networks, revealing that miR-545-5p is intimately linked to tumorigenesis and development. In comparison to adjacent non-tumor tissues, miR-545-5p was significantly downregulated in colorectal cancer tissues[25].However, the roles of miR-545-5p over NPC development was rarely reported and required further exploration. Initially, miR-545-5p was validated as downregulated in NPC cells. When miR-545-5p was overexpressed, the proliferative, migrative and invasive properties of NPC were thwarted, and apoptotic activity was enhanced. When miR-545-5p was inhibited, the opposite result was obtained. Consequently, bioinformatics was used for predicting CRNDE and miR-545-5p downstream targeting interplays, and ultimately postulating that CRNDE can affect NPC development through miR-545-5p modulation – as confirmed by dual-luciferase reporter assay. In vitro and in vivo experiments validated CRNDE to target miR-545-5p.

CCND2 is a cyclin D family protein, and its kinase activity promotes tumorigenesis by enhancing signal transduction mediated by cyclin-dependent kinase (Cdk)[26]. Bioinformatics predicted CCND2 was a downstream target for miR-545-5p, and we further verified whether CRNDE regulated CCND2 as ceRNA. This study demonstrated miR-545-5p upregulation upon CRNDE knockdown. CRNDE downregulates CCND2,with this influence rescued by anti-miR-545-5p. Once miR-545-5p is overexpressed, it will form a synergistic effect with CRNDE knockdown. CRNDE can regulate tumorigenesis and development of NPC by serving as ceRNA of CCND2.

Conclusion

This study identified CRNDE as highly expressed within NPC biopsies and cellular lineages, acting as an oncogene for NPC by impeding miR-545-5p / CCND2 axis. In addition, CRNDE and CCND2 have binding sites with miR-545-5p. CRNDE knockdown can inhibit CCND2 within NPC, and this phenomenon is reversed when miR-545-5p is downregulated. Therefore, CRNDE acts as a ceRNA for sponging miR-545-5p, resulting in upregulation of CCND2 expression. In summary, CRNDE has potential application value in the treatment of NPC, which may provide new strategies for early diagnosis and targeted NPC therapy.

Abbreviations

LncRNAs: long non-coding RNAs; NPC: nasopharyngeal carcinoma; miRNAs: microRNAs; CCND2: Cyclin D2; EMT: epithelial-mesenchymal transition; RT-qPCR: quantitative real-time polymerase chain reaction; MRE: miRNA response elements; ceRNA: the competitive endogenous RNA.

Declarations

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Authors’ Contributions

GSC,JCY, LM, CZQ and FXJ performed the experiments, analyzed the data and wrote the paper. GSC and JCY designed the present study and provided experimental materials. All authors read and approved the fnal manuscript.

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Availability of data and materials

All the experimental procedures were approved and executed in accordance with the first affiliated hospital of Bengbu Medical College.

Consent for publication

All authors have consented to publication.

Competing interest

There are no conficts of interest to declare.

Ethics approval and consent to participate

All procedures in the current study involving human samples were performed in accordance with the Helsinki declaration and approved by the Ethic Committee of The First Affiliated Hospital of Bengbu Medical College. The informed consent has been obtained from each participant.

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Figures

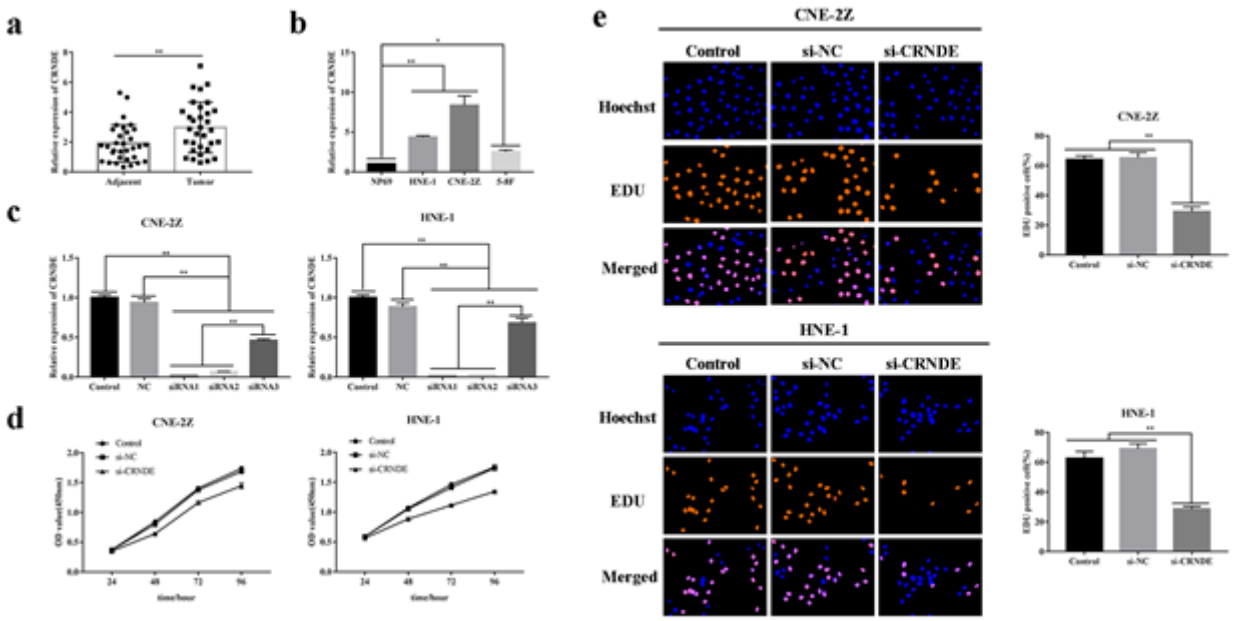

Figure 1

CRNDE knockdown blocks NPC proliferation. a.CRNDE was expressed in NPC adjacent tissue samples. b. CRNDE level within NP69, CNE-2Z,HNE-1, 5-8F cellular lineages. c. Relative expression of CRNDE in NPC cells (CNE-2Z,HNE-1) following transfection of siRNAs (siRNA1, siRNA2, siRNA3) and negative control (si-NC). d.CCK-8 assays analyzed NPC proliferative property (CNE-2Z, HNE-1). e. The EdU assays analyzed NPC proliferative property (CNE2Z, HNE-1) (×200)*p < 0.05; **p < 0.01.

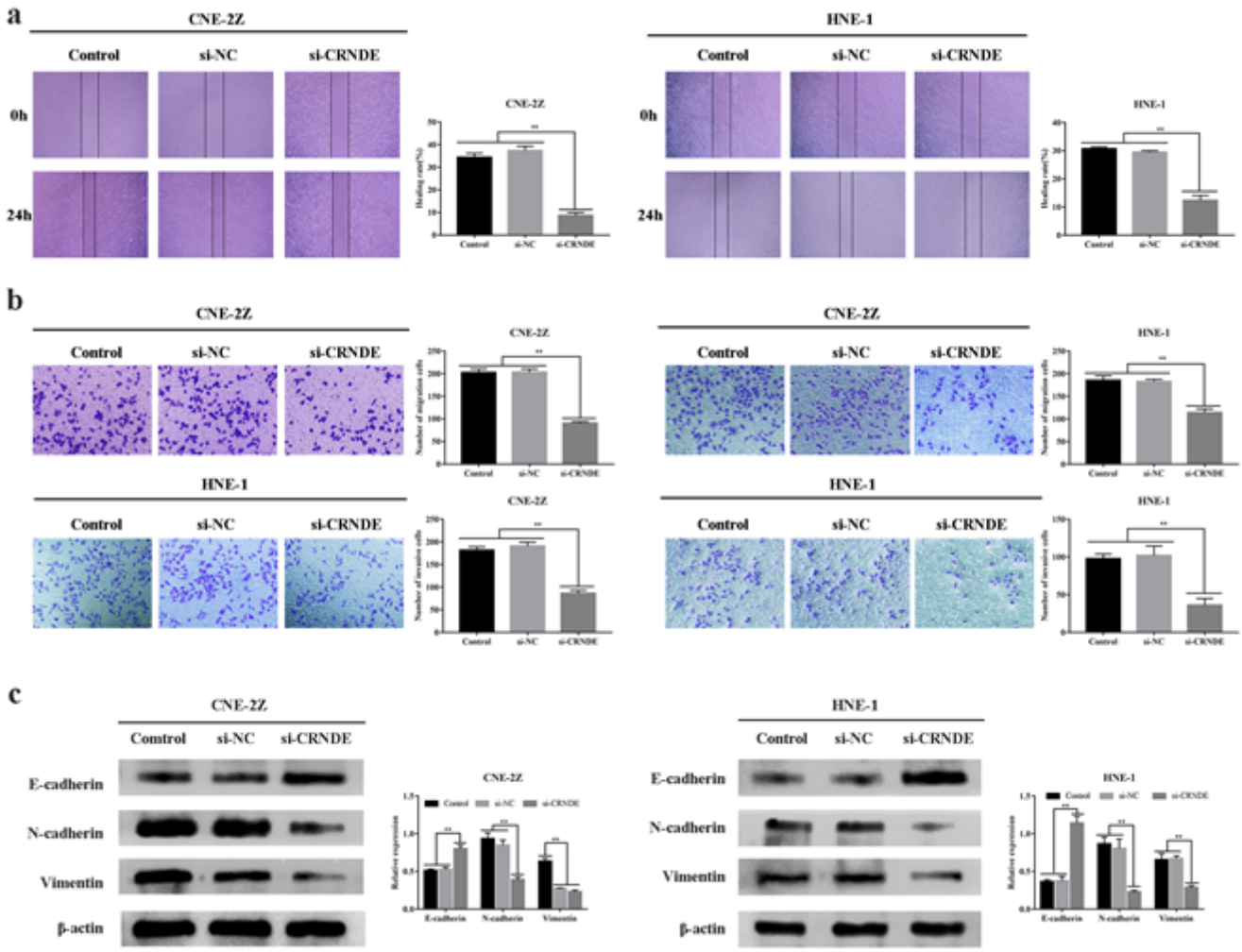

Figure 2

Downregulated CRNDE reduced NPC cell line metastases (CNE-2Z,HNE-1). a. Wound healing assays detected downregulated CRNDE inhibiting NPC migrative property (CNE-2Z, HNE-1).(×40). b. CRNDE knockdown reduced migration / invasion of NPC cells (CNE-2Z, HNE-1) as detected by Transwell assay (×200). c. Western blot analyzed proteomic content for EMT-related proteins in NPC (CNE2Z, HNE-1) when CRNDE was down-regulated. * p < 0.05; ** p < 0.01

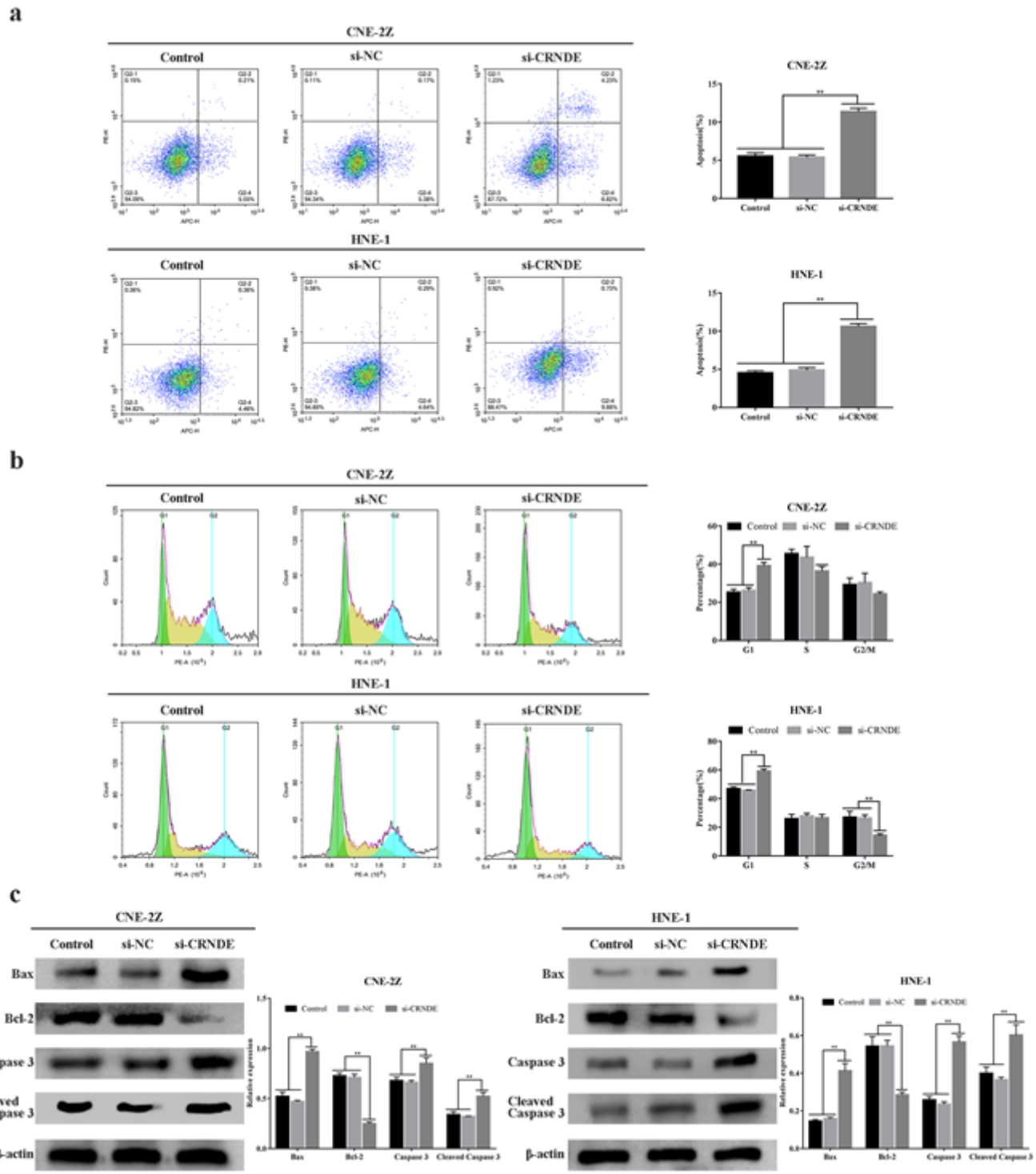

Figure 3

Reduced CRNDE level enhanced apoptotic rate and influenced NPC cellular cycle progression of (CNE2Z, HNE-1) a. Flow cytometry demonstrated that the apoptotic activity of NPC cell lines (CNE2Z,HNE-1) was enhanced following the down-regulation of CRNDE. b. The changes of cell cycle progression of NPC cells(CNE2Z,HNE-1) induced by down-regulation of CRNDE, as detected by flow cytometry. c. Western blot analyzed proteomic content for apoptosis-related proteins in NPC cellular lineages (CNE2Z, HNE-1) when CRNDE was down-regulated.*p < 0.05; **p < 0.01

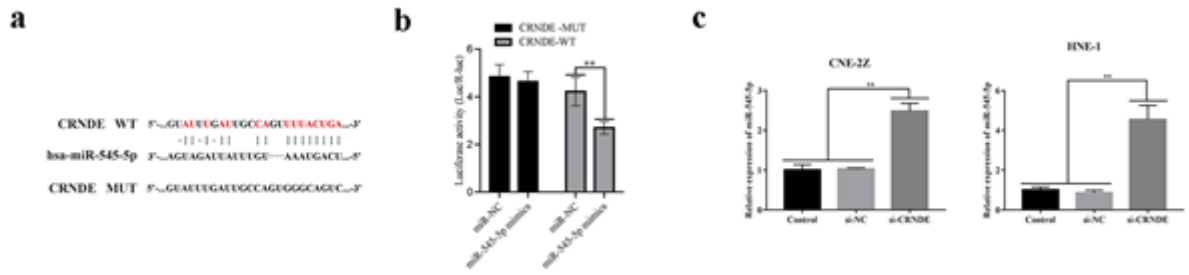

Figure 4

CRNDE sponges miR-545-5p within NPC. a. Prediction of targeted binding sites (CRNDE / miR-545-5p) by Tiana Tool. b. Following co-transfection of miR-545-5p or miR-NC, the luciferase function in CRNDE-WT / CRNDE-MUT in CNE-2Z were detected. c. The effect of si-CRNDE transfection over miR-545-5p expression in NPC (CNE2Z, HNE-1).*p < 0.05; **p < 0.01.

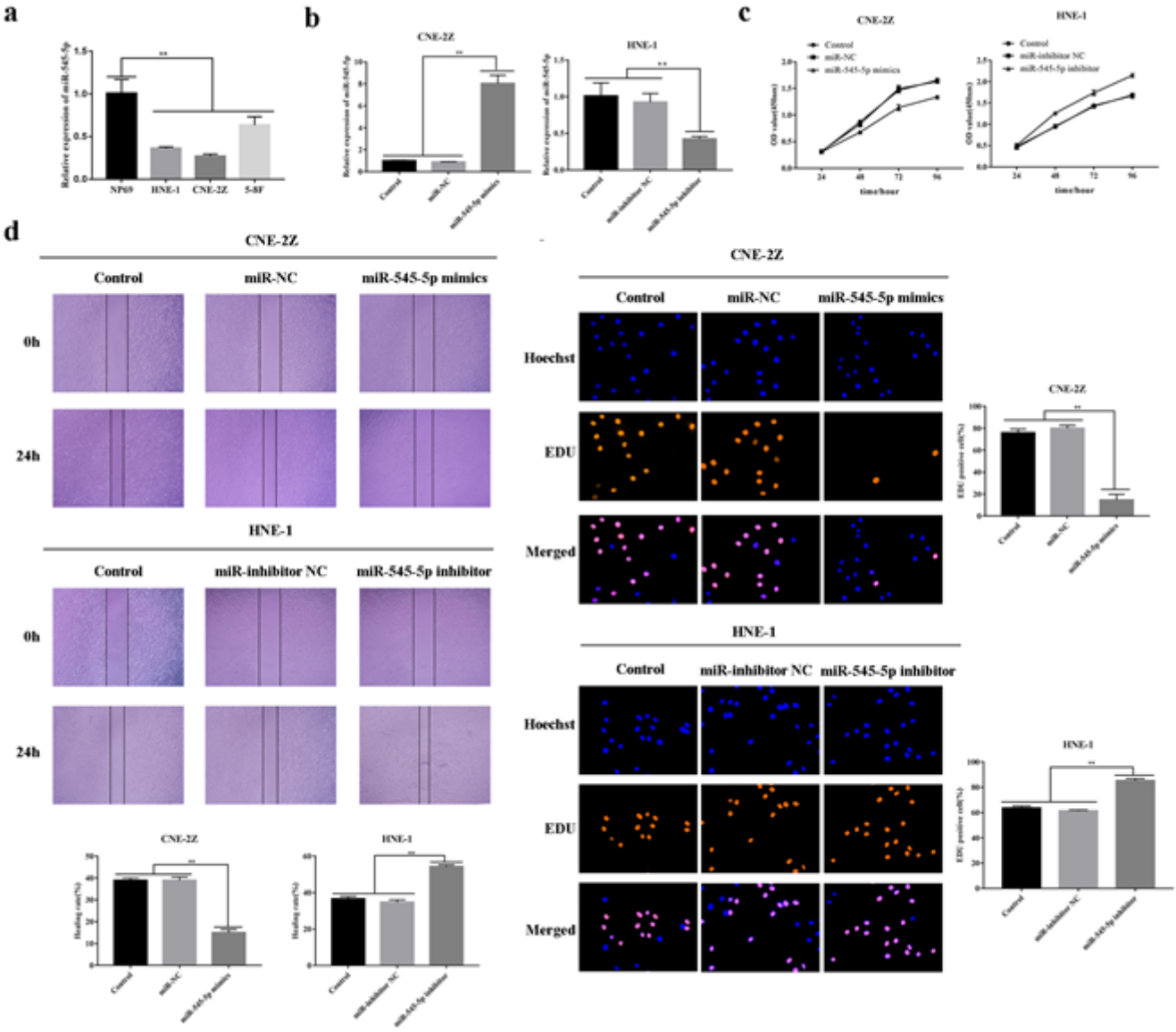

Figure 5

Up-regulation / down-regulation influences by miR-545-5p on NPC proliferative / migrative properties. a. miR-545-5p expression within multiple NPC cellular lineages (CNE-2Z, HNE-1, 5-8F). b. miR-545-5p expression within NPC (CNE-2Z, HNE-1) following transfection of miR-545-5p mimics / inhibitor. c. CCK-8 assays analyzed NPC proliferative property (CNE-2Z, HNE-1). d. The relationship between the expression of miR-545-5p and the migration of NPC cells (CNE-2Z, HNE-1) was studied by wound healing assay. (×40) e. The proliferation of NPC cells was analyzed by the EdU assay (CNE2Z, HNE-1).(×200)*p < 0.05; **p < 0.01

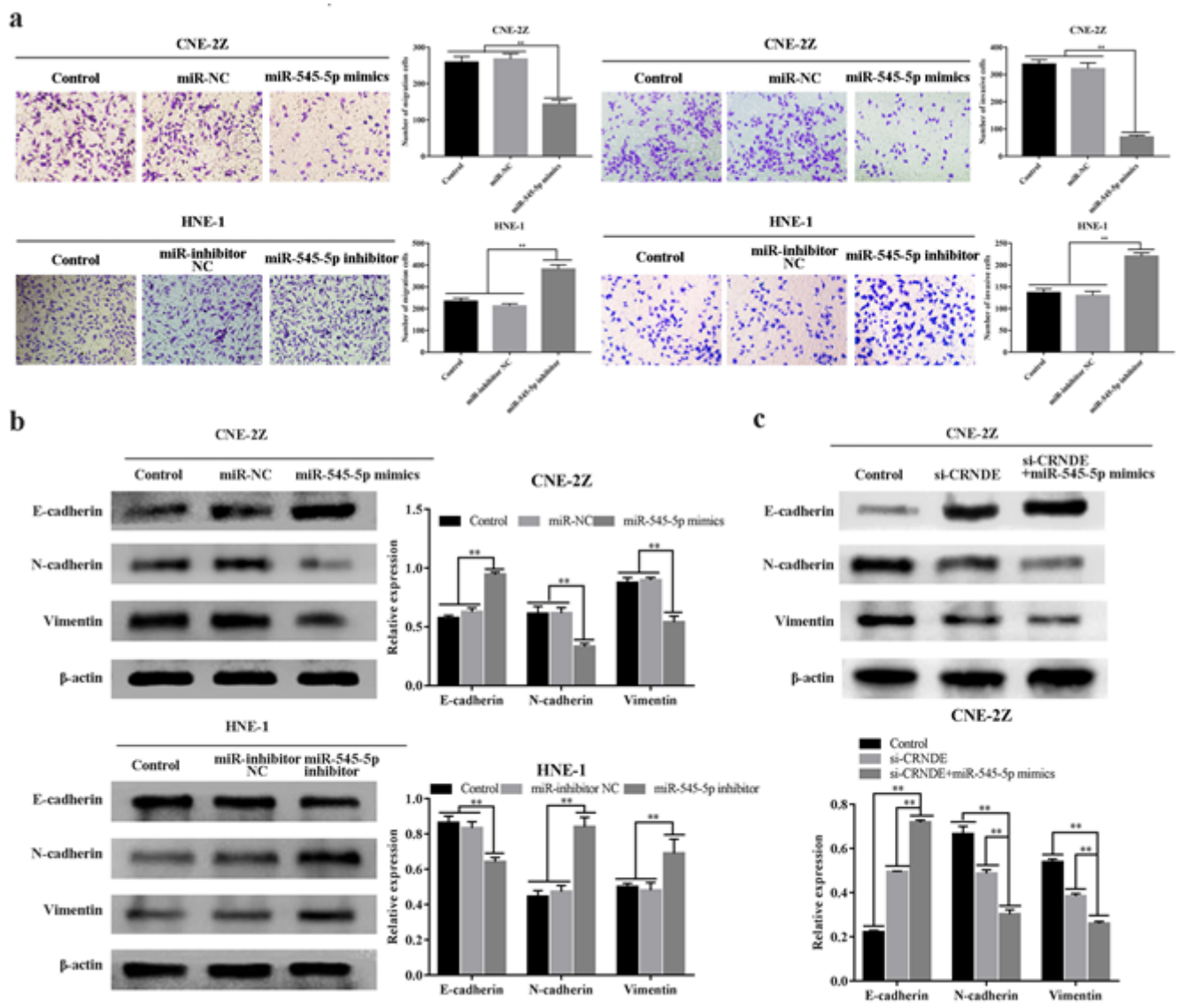

Figure 6

Effects of up-regulation / down-regulation of miR-545-5p upon invasion and migration of NPC cell lines a.The effect of miR-545-5p on migration and invasion of NPC cells(CNE-2Z, HNE-1) was detected by transwell assay. (× 200). b. Western blot was used to detect the effect of miR-545-5p mimics or miR-545-5p inhibitor transfection on the expression of EMT-related proteins in NPC cells (CNE-2Z, HNE-1). c. Western blot was used to detect the expression of EMT-related proteins when si-CRNDE was down-regulated and miR-545-5p mimics were co-transfected. *p < 0.05; **p < 0.01

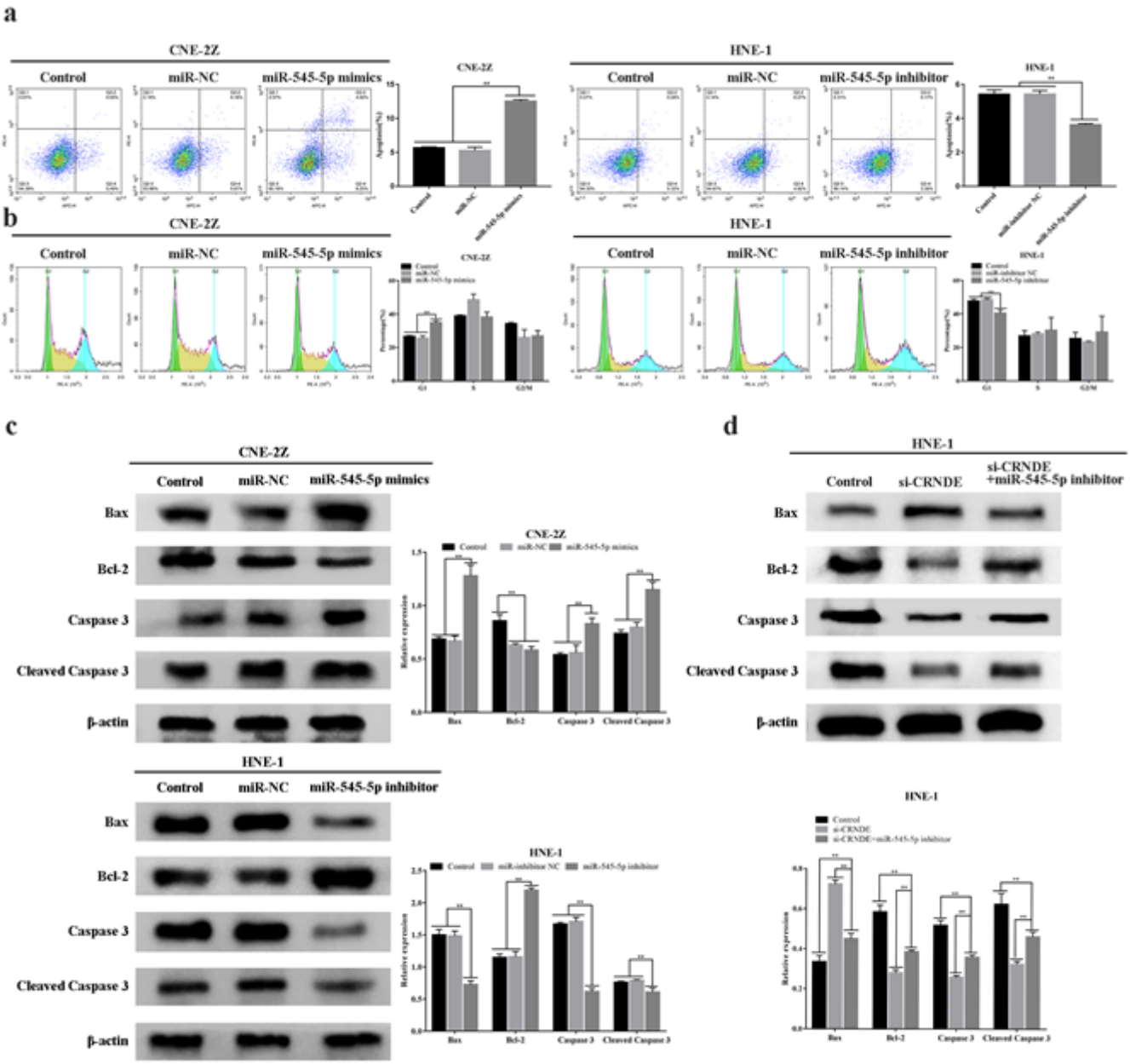

Figure 7

Effect of miR-545-5p on apoptosis and cell cycle progression of NPC cells.a.Flow cytometry was used to detect the effect of miR-545-5p on apoptosis of NPC cells(CNE-2Z, HNE-1).b.The effect of miR-545-5p expression on the cell cycle progression of NPC cells(CNE-2Z, HNE-1) was detected by flow cytometry.c.Western blot was used to detect the expression of apoptosis-related proteins when miR-545-5p was overexpressed or knocked down.d.The expression of apoptosis-related proteins was detected by Western blot following co-transfection of si-CRNDE and miR-545-5p inhibitor.*p < 0.05; **p < 0.01

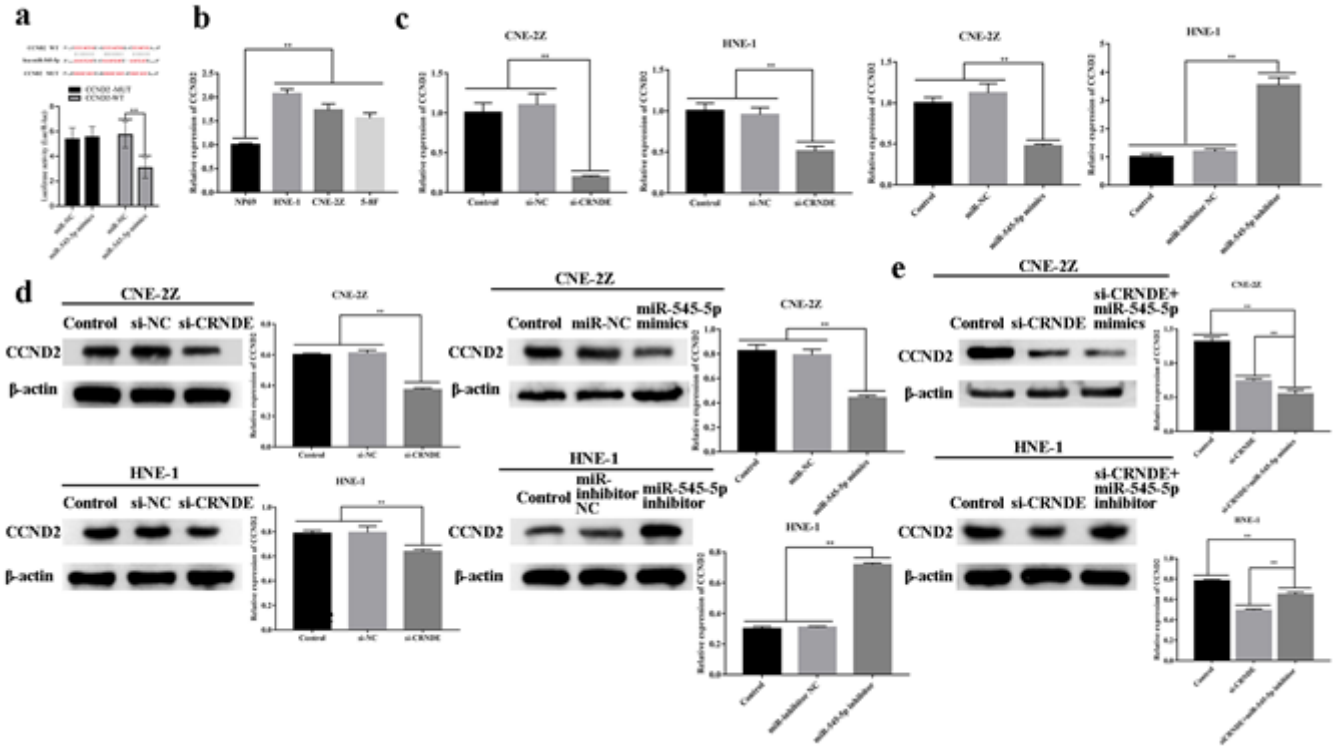

Figure 8

CCND2 expression was modulated by the CRNDE/miR-545-5p axis. a. The prediction of TargetScan and the detection of dual luciferase reporter assays demonstrated that CCND2 was a target of miR-545-5p. b. CCND2 was expressed in a human normal nasopharyngeal epithelial cell line (NP69) and NPC cell lines (CNE-2Z, HNE-1, 5-8F). c. Downregulating CRNDE or upregulating miR-545-5p can affect expression of CCND2 in NPC cells (CNE-2Z, HNE-1). d. Western blot was used to detect the changes of CCND2 expression in NPC cells (CNE-2Z, HNE-1) when miR-545-5p was upregulated or downregulated. e. Western blot was used to detect the expression of CCND2 when CRNDE was downregulated and miR-545-5p was upregulated or downregulated.*$p < 0.05$; **$p < 0.01$

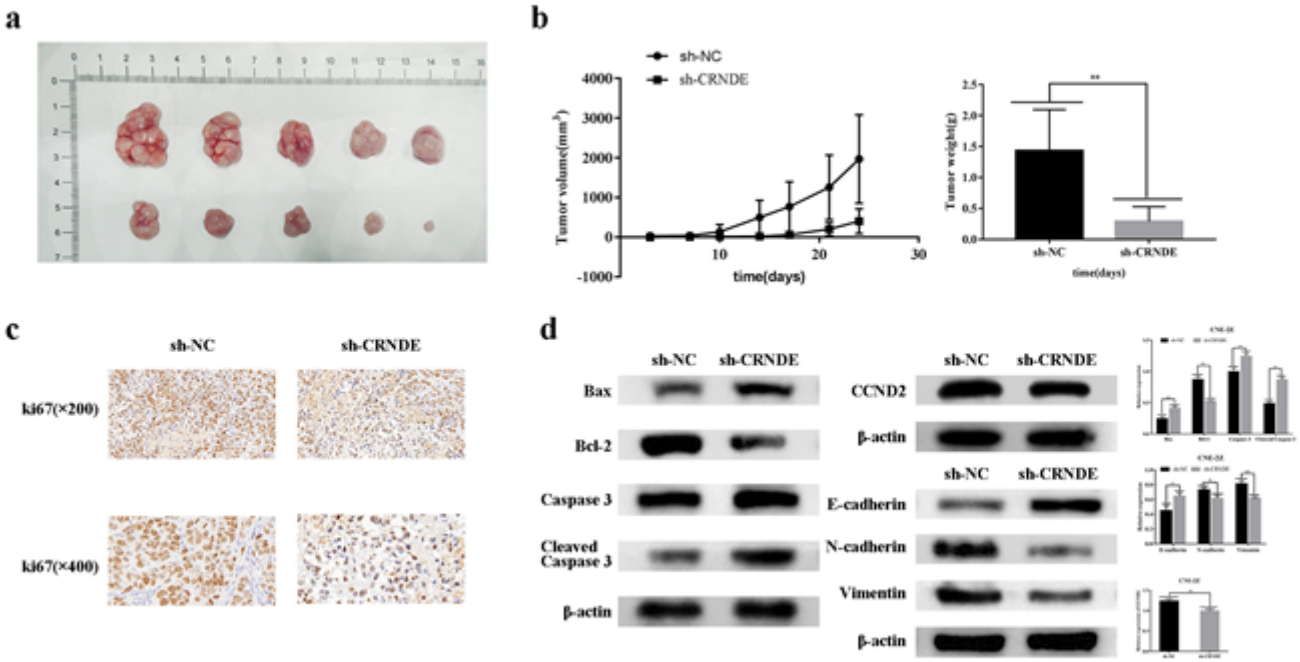

Figure 9

CRNDE silencing hinders tumour growth in vivo. a. Xenograft tumours were removed from sh-CRNDE or sh-NC groups. b. Tumor volume and weight in the sh-CRNDE or sh-NC groups were measured and calculated. c. Immunohistochemical staining for Ki-67 in xenografts .d.Western blotting was used to detect the expression of EMT-related proteins, apoptosis-related proteins and CCND2 protein in each group.*p < 0.05; **p < 0.01