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ORIGINAL RESEARCH

Neuropilin-2 Mediates Radioresistance in Nasopharyngeal Carcinoma via Wnt/β-Catenin Pathway

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Purpose: Neuropilin-2 (NRP2) is linked to poor prognosis in several malignant tumors. We elucidated the role of NRP2 in radiation resistance in nasopharyngeal carcinoma (NPC) and the underlying molecular pathways.

Material and Methods: CNE-2R cells of NPC were used for NRP2 knockdown. Stable NRP2 knockdown was achieved using three siRNAs targeting distinct regions of the NRP2 gene. The effect of NRP2 knockdown was confirmed through qPCR for mRNA and Western blot (WB) for protein levels. Assays for cell viability and colony formation were conducted to assess cellular responses to NRP2 knockdown and ionizing radiation. Bioinformatics analyses, including differential expression and pathway enrichment using GEO datasets and GSEA analysis, were performed to elucidate the molecular mechanisms underlying NRP2 function.

Results: After NRP2 knockdown in CNE-2R cells, NRP2 expression was significantly lower compared to non-knockdown cells by qPCR and Western blot analysis. NRP2 downregulation in CNE-2R cells led to decreased proliferation and clonal numbers post-radiation in comparison to the control group ($P < 0.001$). Analysis of the GEO database exhibited that NRP2 expression was notably elevated in nasopharyngeal carcinoma tissues vs normal tissues ($p = 0.012$). GSEA analysis showed a notable enhancement of the Wnt/β-catenin signaling pathway in NPC, with NES = 1.647, p adjust = 0.049, and FDR = 0.038. The WB analysis indicated that NRP2 knockdown notably reduced the level of WNT3a, Axin2, Cyclin D1, p-GSK3β, and β-catenin in the Wnt/β-catenin pathway in comparison to the negative control group ($p < 0.05$), with GSK3β expression remaining unchanged.

Conclusion: NRP2 is connected with radioresistance in NPC, potentially via Wnt/β-catenin pathway, and may be a potential therapeutic target.

Keywords: nasopharyngeal carcinoma, neuropilin-2, Wnt/β-catenin, radioresistance

Introduction

In southern China, nasopharyngeal carcinoma (NPC) occurs frequently, with approximately 30% of patients experiencing local recurrence and distant metastasis despite standardized treatment, largely due to radioresistance.[1,2] NPC radioresistance is due to the activation of signaling pathways like PI3K/Akt, NF-κB, and Wnt, alterations in oncogenes, hypoxia, cancer stem cell proliferation and resistance, and vasculogenic enhancement—all of which interact within the pathological ecosystem of NPC as an ecological and evolutionary unity.[3–5] Lymphatic metastasis is prevalent in NPC, with over 80% of individuals exhibiting regional lymph node involvement at the time of diagnosis, and approximately 50% presenting bilateral regional lymph node metastasis.[6] The degree of lymph node involvement significantly influences both treatment options and patient prognosis. Tumor invasiveness and metastasis are closely involved in the formation of tumor-associated vasculature and lymphatics, with vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) being pivotal in these processes.[7]

Neuropilin-2 (NRP2), a 130–140 kDa transmembrane glycoprotein, serves as an essential co-receptor for VEGFRs in tumor angiogenesis. Recent research highlights the crucial role of NRP2 in several cancers, where increased NRP2

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expression correlates with poor prognosis in prostate, thyroid, colorectal, breast, and bladder cancers, serving as an independent predictor of negative clinical outcomes.[8–11] NRP2 interacts with VEGF to enhance binding to VEGFRs, thereby promoting vascular formation. Additionally, NRP2 competes with class 3 semaphorins (SEMA3), which possess anti-angiogenic properties, facilitating angiogenesis.[12] VEGF overexpression in tumors, enabled by protease activation and basement membrane degradation, promotes endothelial cell proliferation and migration, resulting in new capillary formation that supports tumor growth and metastasis.[7] Clinical applications of recombinant human endostatin in lung and nasopharyngeal carcinoma have demonstrated efficacy in inhibiting VEGF/VEGFR-mediated angiogenesis, resulting in improved patient outcomes.[13] Experimental studies targeting NRP2 as a VEGFR co-receptor have also shown promising results, suggesting that inhibiting NRP2 to reduce tumor vasculature and enhance therapeutic efficacy is a viable strategy.[14] However, current research on the function of NRP2 in NPC remains limited. Given NRP2's dual role in vascular and lymphatic formation, targeting NRP2 presents significant potential as a novel therapeutic strategy for NPC.

Our preliminary studies identified differential NRP2 expression between radioresistant CNE-2R and radiosensitive CNE-2 NPC cell lines, leading us to hypothesize that NRP2 contributes to NPC radioresistance.[15] This research seeks to reduce NRP2 expression in CNE-2R cells through gene knockdown methods and assess the subsequent impacts on cell viability and colony formation. Bioinformatics analysis will identify downstream pathways influenced by Neuropilin-2, while Western blotting will assess the expression of associated pathway proteins.

Materials and Methods

Cell Culture

As previously described, CNE-2R cells are a radioresistant cell line established from CNE-2 cells through repeated irradiation, whereas the parental CNE-2 cells were obtained from Fudan University Shanghai Cancer Center (Shanghai, China).[15] Cryopreserved CNE-2R cells were retrieved from liquid nitrogen storage, thawed, and subcultured. All cellular experiments utilized RPMI-1640 basal medium (Gibco) enriched with 10% FBS and 1% dual antibiotic solution, with incubation parameters strictly controlled at 37°C in 5% carbon dioxide environment with humidity regulation.

siRNA Transfection and Validation

siRNA transfection: CNE-2R cells were grown in 6-well plates until they reached 50–60% confluence. Three siRNAs targeting distinct sites on the NRP2 gene (NRP2 si-1: GUCUUGGUUUUAAUUAUUATT, NRP2 si-2: UCGGGAAAUACUCGUGAUATT, NRP2 si-3: CUCUGAAGAUUGCUCAAAAAA) were transfected following the manufacturer's instructions (Anernor Biotechnology Co. Ltd., Guangzhou, China). Negative control groups (NC) were transfected with empty vectors, while knockdown groups were named KD1-NPR2, KD2-NPR2, and KD3-NPR2. A FAM-labeled negative control was used to optimize and monitor transfection efficiency. Transfection was performed at a concentration of 50 nM, and efficiency was assessed 8 hours post-transfection.

qPCR validation: RNAiso Plus (Takara, Beijing, China) was utilized for the extraction of total RNA following the manufacturer's guidelines. RNA was precipitated with isopropanol, centrifuged, washed, air-dried, and dissolved in DEPC water. Spectrophotometric analysis was used to assess RNA concentration and purity. The PrimeScript™ RT reagent Kit (Takara, Beijing, China) was utilized for cDNA synthesis. Quantitative PCR was carried out using the SYBR Green method (YEASEN, Shanghai, China) on a ViiA7 Real-Time PCR System (ABI, USA). The thermocycling process started with an initial denaturation at 95°C for 5 minutes, subsequently, 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds were performed. The expression levels of genes were measured using the ΔΔCt method with GAPDH as the internal control.

Western blotting validation: Cells from the NC and KD1-NPR2 groups, in exponential growth, were washed 2–3 times with ice-cold PBS and left to lyse on ice for 30 minutes using a cell lysis buffer with PMSF. The Lysates underwent centrifugation at 14000 rpm for 10 minutes at 4°C to collect supernatants as total protein extracts, which were subsequently divided into portions and kept at −80°C. Proteins were combined with loading buffer, subjected to SDS-PAGE, and transferred onto PVDF membranes (Millipore, USA). The membrane was treated with 5% non-fat milk and incubated for 1 hour at normal room temperature with rabbit anti-NRP2 primary antibody (Thermo Fisher, 0.5 μg/mL)

and anti-GAPDH antibody (Abcam, 1:2500), then using HRP-conjugated secondary antibody goat anti-rabbit IgG (Abcam, ab6721, 1:10,000). The bands were visualized using chemiluminescence and quantified with ImageJ software.

Cell Viability Assay

Cells from the NC, KD1-NRP2, and KD2-NRP2 groups were settled in 96-well plates at a concentration of 5000 cells for every well. The cells were left to incubate for a duration of 24 hours following exposure to X-ray doses of 0, 4, and 8 Gy. Cell viability was examined with CCK-8 solution (10 μg/mL; TransGen Biotech, China) in a 37°C, 5% CO_2 incubator for 60 minutes. The absorbance reading at 450 nm using a BioTek microplate reader (USA), and growth curves were generated by plotting OD values over time.

Colony Formation Assay

Cells from NC, KD1-NRP2, and KD2-NRP2 groups were settled at 500 cells for every single well in 6-well plates with 2 mL medium, evenly distributed by gentle rotation, and placed at 37°C with 5% CO_2 for 12 days. Cells forming visible colonies were fixed with 4% paraformaldehyde and stained with crystal violet, both from Beyotime, China, for 20 minutes and 10 minutes, respectively, at room temperature. Colonies were enumerated using a gridded transparent sheet for direct observation.

Bioinformatics Analysis

The NPC expression dataset GSE12452 was obtained and examined from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The gene expression differences were analyzed using the limma package in R (version 3.40.2), and NRP2 expression was further validated with the Wilcoxon rank-sum test. Functional pathways were identified using KEGG pathway enrichment analysis. The Gene Set Enrichment Analysis (GSEA) was conducted to discover enriched gene sets between NPC and normal tissues. The study analyzed the co-expression between NRP2 and essential genes in the signaling pathway of Wnt/β-catenin.

Western Blotting

Following the protocol in section 2.2.3, Western blotting was performed on NC, KD1-NRP2, and KD2-NRP2 groups. Primary antibodies included anti-Wnt3a (1:1000; Abcam, ab81614), anti-Axin2 (1 μg/mL; Abcam, ab32197), anti-Cyclin D1 (1:10,000–1:50000; Abcam, ab134175), anti-p-GSK3β (1:10,000–1:20000; Abcam, ab75814), anti-GSK3β (1:5000–1:10000; Abcam, ab32391), and anti-β-catenin (0.25 μg/mL; Abcam, ab16051).

Statistical Analysis

The statistical examination was accomplished using GraphPad Prism 9.0. One-way ANOVA was employed for continuous data. A p-value of 0.05 was adopted to evaluate the significance of results.

Results

Effective Knockdown of NRP2 in CNE-2R Cells

After siRNA transfection for 24 hours, qPCR and WB were used to evaluate NRP2 knockdown efficiency at the levels of mRNA and protein, respectively. Figure 1 illustrates that NRP2 mRNA expression significantly decreased in KD1-NRP2, KD2-NRP2 and KD3-NRP2 transfected groups in contrast to the negative control group ($p<0.001$, $p<0.001$, and $p<0.01$, respectively). In Figure 2, WB results showed a significant reduction in NRP2 protein expression in the KD1-NRP2 and KD2-NRP2 groups compared with the negative control group ($p<0.001$, and $p<0.001$, respectively). Therefore, siRNAs from the KD1-NRP2 and KD2-NRP2 groups were selected for further experiments.

NRP2 Knockdown Sensitizes CNE-2R Cells to Irradiation

CCK-8 assays were conducted to determine the impact of NRP2 knockdown on the survival rate of CNE-2R nasopharyngeal carcinoma cells post-irradiation. As shown in Figure 3A, 24 hours post-irradiation, survival rates in KD1-NRP2 and KD2-NRP2 groups at 0, 4, and 8 Gy were significantly reduced in comparison to the NC group ($P<0.001$). During

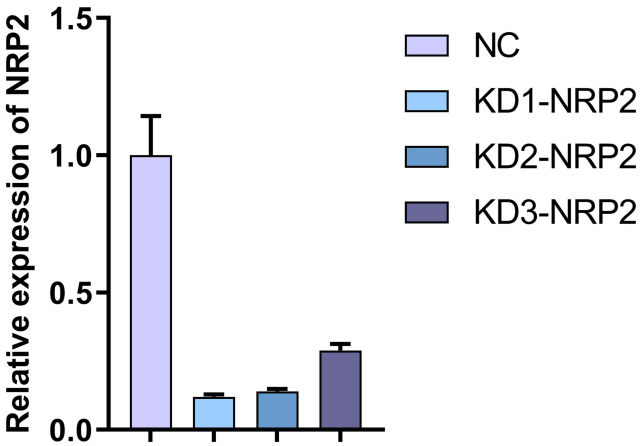

Figure 1 The NRP2 mRNA expression levels were analyzed in different groups using qPCR.
Abbreviations: NC, negative control group (empty vector group); KD1-NRP2/KD2-NRP2/KD3-NRP2, siRNA transfection groups.

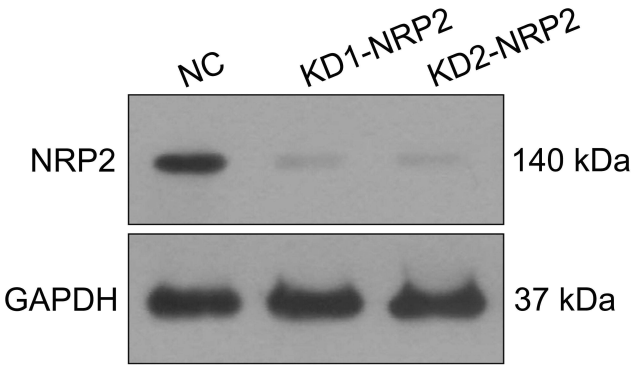

Figure 2 NRP2 protein expression was assessed across different groups by Western blot analysis.
Abbreviations: NC, negative control group (empty vector group); KD1-NRP2/KD2-NRP2, siRNA transfection groups.

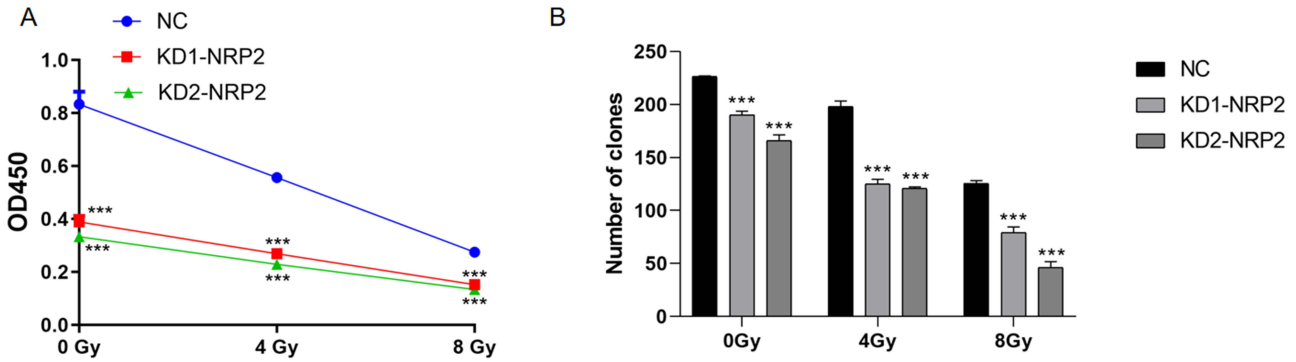

Figure 3 NRP2 knockdown increases radiosensitivity in CNE-2R cells. (**A**) CCK-8 assay results show reduced cell proliferation after NRP2 knockdown and irradiation, (**B**) Colony formation assay results demonstrate reduced colony numbers post-NRP2 knockdown and irradiation (***P < 0.001).
Abbreviations: NC, negative control group (empty vector group); KD1-NRP2/KD2-NRP2, siRNA transfection groups.

the colony formation test, as radiation dose increased (0 Gy, 4 Gy, 8 Gy), the number of colonies decreased significantly. The KD1-NRP2 and KD2-NRP2 groups also showed a notable reduction in colony numbers across radiation doses compared to the NC group (P<0.001) (Figure 3B). These findings imply that NRP2 knockdown elevates the radiosensitivity of NPC cells.

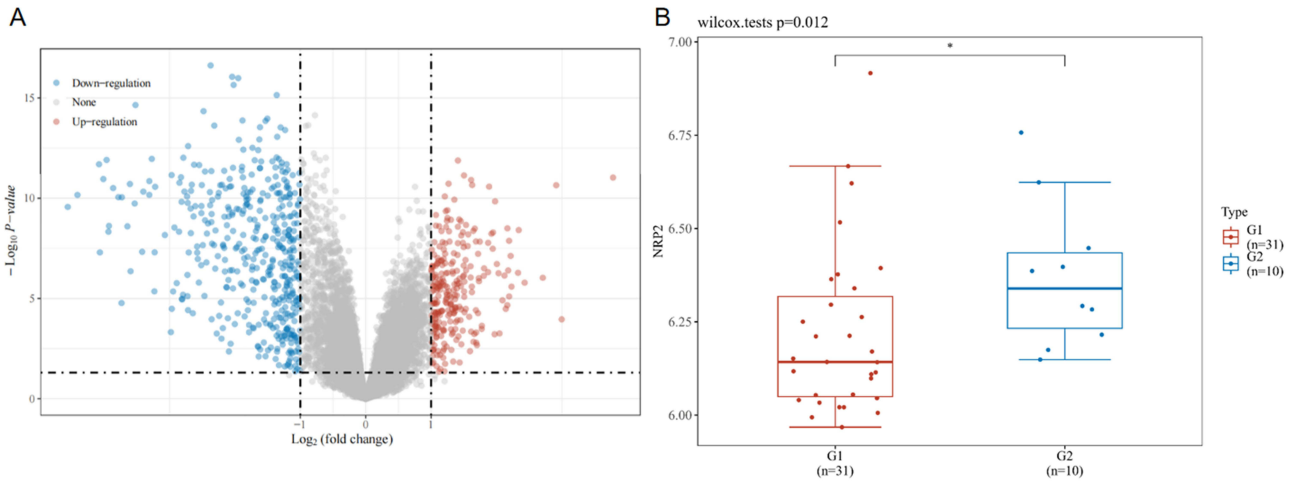

Figure 4 GEO Database Analysis Results. (**A**) Volcano plot illustrating differentially expressed genes in the GSE12452 dataset, filtered by adjusted p-value < 0.05 and absolute log2 fold change > 1. NRP2 was near significant: log2FC=−0.156, p=0.0558; (**B**) Wilcoxon rank-sum test demonstrates higher NRP2 expression in nasopharyngeal carcinoma (G1) vs normal tissues (G2; p=0.012, *p<0.05).

NRP2 Potentially Influences NPC Through the Wnt/β-Catenin Signaling Pathway

Differential gene expression in the GEO dataset GSE12452 was assessed with thresholds of an adjusted p-value < 0.05 and log2(fold change) > 1 or < −1. A volcano plot in Figure 4A shows 708 differentially expressed genes, with 263 upregulated and 445 downregulated. Although NRP2's log2FC was −0.156, it was close to significance with a p-value of 0.0558. The Wilcoxon rank-sum test revealed significantly elevated NRP2 expression in nasopharyngeal carcinoma tissues (G1) compared to normal tissues (G2) (p = 0.012, Figure 4B), indicating a potential biological role for NRP2 in this cancer.

KEGG pathway enrichment analysis revealed significant enrichment in eight pathways, such as amoebiasis, cell cycle, and cytokine-receptor interaction. Given the limited number of enriched pathways, GSEA was further conducted to explore other relevant pathways.

GSEA results showed significant enrichment of genes involved in REACTOME_CELL_CYCLE_CHECKPOINTS and REACTOME_DNA_REPLICATION pathways (Figure 5A and B). The Wnt/β-catenin signaling pathway showed significant enrichment in NPC, with a normalized enrichment score (NES) of 1.647, an adjusted p-value of 0.049, and a false discovery rate (FDR) of 0.038 (Figure 5C). Key genes such as FZD7, CTHRC1, KREMEN2, and WNT5A suggest that NRP2 influences NPC via the Wnt/β-catenin pathway. Although NRP2 is not directly engaged in Wnt pathway activation, it may play an indirect or synergistic role in this signaling pathway (Figure 5C).

Co-expression analysis was conducted on core genes identified in the GSEA analysis (FZD7, CTHRC1, KREMEN2, WNT5A, FZD6, LRP6, DKK1, FZD2, RYK, WNT2) alongside key Wnt/β-catenin pathway genes (CTNNB1, APC, AXIN1, GSK3B, TCF7L2, LEF1, WNT1, SFRP1, AXIN2, NKD1, MYC) with NRP2. The findings reveal a significant correlation between NRP2 and AXIN2 (R=0.497, p < 0.05) as well as DKK1 (R=−0.414, p < 0.05), suggesting that NRP2 may modulate the Wnt/β-catenin pathway by regulating these genes (Figure 5D).

Western blot analysis demonstrated that NRP2 knockdown significantly reduced the levels of essential proteins in the Wnt/β-catenin signaling pathway, such as WNT3a, Axin2, Cyclin D1, p-GSK3β, and β-catenin, as opposed to the negative control (p<0.05). GSK3β exhibition, however, remained unchanged (Figure 5E).

Discussion

Despite evidence linking NRP2 to tumor development and progression, its specific role in nasopharyngeal carcinoma (NPC) is not well understood. Our study demonstrates that the downregulation of NRP2 in CNE-2R NPC cells, achieved via siRNA transfection, significantly reduces cell proliferation and colony formation following irradiation. Our findings suggest that NRP2 potentially promotes NPC progression through modulation of the Wnt/β-catenin signaling pathway.

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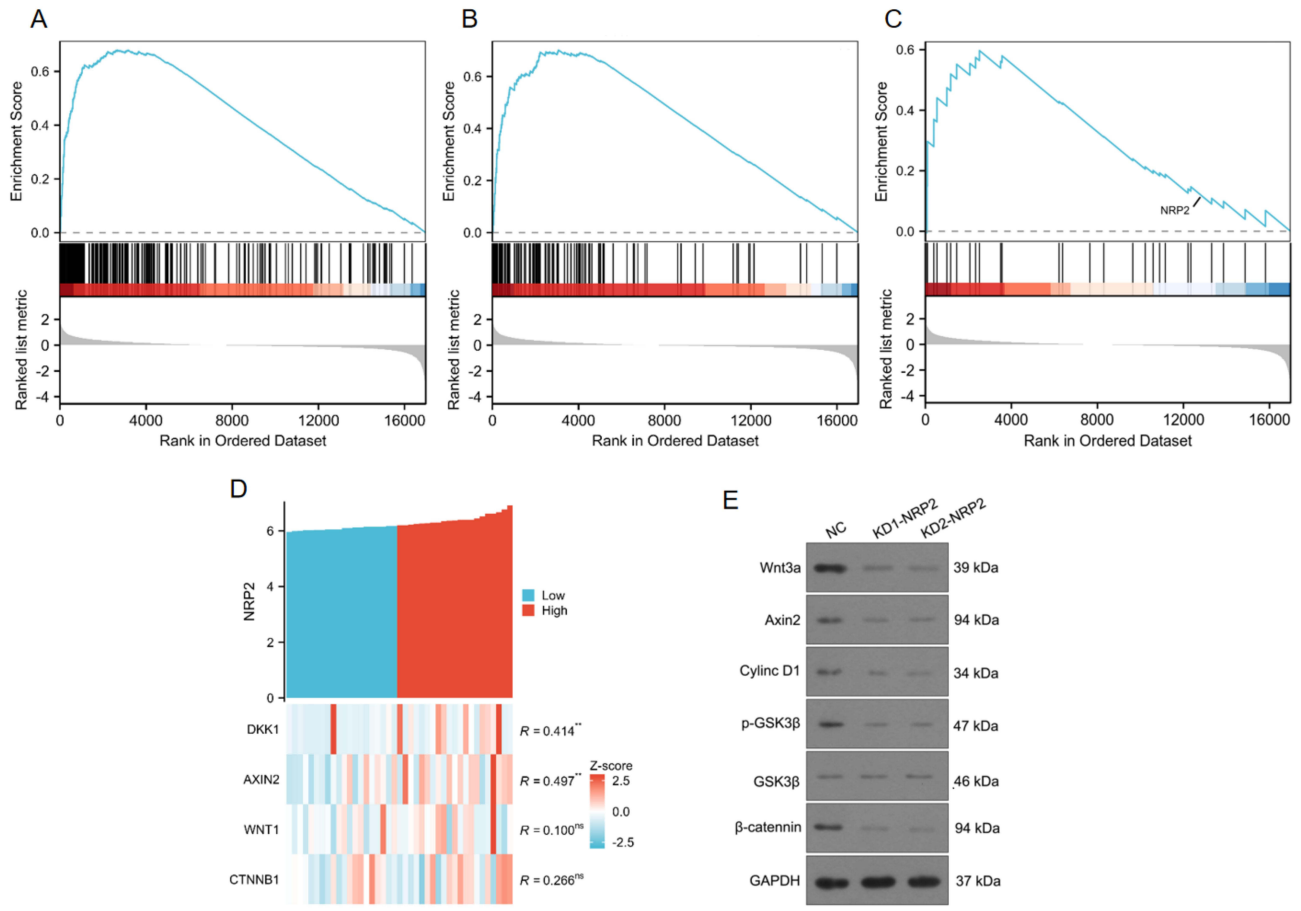

Figure 5 NRP2 influences NPC through the Wnt/β-catenin signaling pathway. (**A**) GSEA visualization for Cell Cycle Checkpoints (NES=2.736, P.adj=0.022, and FDR=0.017), (**B**) GSEA visualization for DNA Replication Process (NES=2.581, P.adj=0.021, and FDR=0.016), (**C**) GSEA visualization for Wnt Signaling Pathway (NES=1.647, P.adj=0.049, and FDR=0.038), (**D**) Heatmap illustrating the co-expression of NRP2 with key genes in the Wnt/β-catenin pathway (**p < 0.01, ns: p≥0.05), and (**E**) Western blot analysis indicates that NRP2 downregulation affects critical proteins (WNT3a, Axin2, Cyclin D1, p-GSK3β, and β-catenin) within the Wnt/β-catenin pathway in NPC cells.
Abbreviations: GSEA, gene set enrichment analysis; NES, normalized enrichment score; P.adj, adjusted p-value; FDR, false discovery rate; NC, negative control group (empty vector group); KD1-NRP2/KD2-NRP2, siRNA transfection groups.

NRP2 is a VEGF receptor involved in both developmental and tumor-associated angiogenesis.[16] NRP2 limits tumor lymphangiogenesis, thereby reducing the spread to sentinel lymph nodes and distant organs, without impacting normal lymphatic vessels.[17] VEGF, a highly specific growth factor for endothelial cells, facilitates endothelial cell proliferation, migration, and vessel formation. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF), all of which act as ligands to various growth factor receptors.[17] Previous studies indicate that VEGF receptors, including VEGFR1, VEGFR2, and VEGFR3, are present in both endothelial and tumor cells.[18] Researchers have also identified NRP2 as a growth factor receptor with dual roles in angiogenesis and lymphangiogenesis.[19] NRP2 is a transmembrane glycoprotein characterized by three extracellular subunits: two CUB subunits (a1a2), two Factor V/VIII homology subunits (b1b2), and an MAM subunit (c).[12] The a1a2 subunit binds SEMA3, which inhibits angiogenesis. NRP2 promotes angiogenesis by competitively binding VEGF ligands with SEMA3, thus counteracting SEMA3's negative regulation. The b1b2 domain binds VEGF, enhancing its affinity for VEGFR1, VEGFR2, and VEGFR3 and further promoting angiogenesis. The c domain facilitates homodimer or oligomer formation, essential for protein stability and receptor-ligand signaling.

Furthermore, NRP2 contributes to tumor cell proliferation through multiple signaling pathways. Initial research indicated that NRP2 facilitates esophageal cancer development and spread through the ERK-MAPK-ETV4-MMP-E-cadherin signaling pathway.[20] In prostate cancer, NRP2 enhances castration resistance via the androgen-receptor signaling pathway, where its depletion improves the efficacy of antiandrogen therapies.[21] Recent research shows that NRP2 enhances the development,

movement, and invasion of pancreatic ductal carcinoma cells via the activation of the FAK/Erk/HIF-1a/VEGF signaling pathway.[22] NRP2 also mediates thyroid cancer growth via circRNAs and the miR-1287-5p/NRP2 pathway.[23] Additionally, NRP2 activation of the RSK1/Sox2/Zeb1 signaling pathway influences head and neck cancer cell proliferation and invasion.[24] Through differential gene expression, GSEA enrichment, and co-expression analyses, our findings indicate that NRP2 is markedly upregulated in NPC tissues, suggesting its pivotal role in NPC progression. NRP2 exhibited strong co-expression with AXIN2, a crucial element of the Wnt/β-catenin pathway, reinforcing the hypothesis that NRP2 facilitates tumor progression through this pathway. GSEA exhibited a marked enhancement of the Wnt/β-catenin pathway in NPC, indicating that NRP2 might indirectly or synergistically influence its regulation. To validate these findings, we conducted Western blotting analysis on pathway-related proteins. Results showed that in NRP2-expressing NPC cells, proteins such as WNT3a, Axin2, Cyclin D1, p-GSK3β, and β-catenin were significantly elevated, whereas NRP2 knockdown resulted in reduced expression of these proteins, with no observable change in GSK3β expression. These findings confirm NRP2's regulatory role on Wnt/β-catenin-related proteins, contributing to NPC progression. Our results align with the outcomes of Ji et al[19] and Kang et al.[25] Ji et al[19] demonstrated that the canonical Wnt signaling pathway regulates NRP2 expression in osteosarcoma, as also noted by Kang et al[25] showed that NRP2 functions as an oncogene in oral squamous cell carcinoma by triggering the Wnt/β-catenin pathway, which enhances cellular proliferation, migration, and invasion.

WNT ligands, such as WNT3a, activate the Wnt pathway by recruiting Dishevelled proteins. This disrupts the destruction complex, causing glycogen synthase kinase 3β (GSK3β) to dissociate or become inactive, allowing β-catenin to accumulate inside the cell and move to the nucleus. β-catenin interacts with transcription factors to activate Wnt target genes such as Cyclin D1 and Axin2. Dishevelled presence also prevents GSK3β from participating in β-catenin phosphorylation and degradation; thus, GSK3β itself undergoes phosphorylation, leading to an increase in p-GSK3β.[26] Our findings indicate that NRP2-expressing NPC cells exhibit increased levels of WNT3a, Axin2, Cyclin D1, p-GSK3β, and β-catenin, supporting the activation of the Wnt/β-catenin pathway.

A key function attributed to NRP2 is enhancing cancer cell survival.[27] Lee et al[9] showed that NRP2 knockdown via siRNA in thyroid cancer reduced cell proliferation, while Zhao et al[28] reported that miR-331-3p suppression of NRP2 decreased triple-negative breast cancer cell proliferation. Our CCK-8 and colony formation tests demonstrate that NRP2 knockdown markedly decreases NPC cell viability following radiation exposure. In 2015, research indicated that high NRP2 expression in bladder cancer correlated with advanced stages and poor prognosis, playing a role in therapy resistance, especially chemotherapy resistance. The VEGF-C/NRP2 axis reportedly induces autophagy, protecting cancer cells from chemotherapy-induced stress.[29] Research indicates that NRP1, a homolog of NRP2, contributes to radiotherapy resistance in medulloblastoma, non-small cell lung cancer, and esophageal squamous cell carcinoma by influencing stem cell populations, immune responses, epithelial-mesenchymal transition (EMT), and the PI3K/Akt signaling pathway.[30,31] Notably, these processes—including Wnt/β-catenin pathway activation, stemness-related traits, and EMT—are embedded within the tumor ecosystem, where multi-level interactions between cancer cells and their microenvironment drive adaptive evolution, with therapeutic resistance arising as an ecological survival strategy under selective pressure.[32] However, literature on NRP2's involvement in radiotherapy resistance is limited, necessitating further research to clarify its role and potential mechanisms. This study has certain limitations. Due to funding and experimental design constraints, in vitro migration, invasion, and in vivo experiments were not conducted. Future studies are necessary to confirm NRP2's role in NPC cell invasion and migration, as well as to conduct in vivo experiments to elucidate potential mechanisms underlying radiotherapy resistance.

Conclusion

NRP2 knockdown significantly inhibits proliferation and enhances radiosensitivity of CNE-2R cells. Mechanistically, NRP2 may activate Wnt/β-catenin signaling, and its suppression may attenuate this pathway. These findings support NRP2 as a promising therapeutic target in NPC.

Ethical Approval

This study was approved by Institutional Review Board of the Hainan General Hospital (Approval No.: Med-Eth-Re [2024] 754).

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Disclosure

The authors declare no relevant financial or non-financial interests in this work.

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