NETO2 promotes progression of prostate cancer by inhibiting the cellular senescence pathway

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Abstract

Purpose: Prostate cancer (PCa) has become the most frequently occurring cancer among western men, due to the rapid progression of tumor, late events will inevitably occur, which leads to poor prognosis of patients. Therefore, it is of great significance to explore the molecular mechanism of tumor progression.

Methods: Herein, we conducted a transcriptomic analysis using public database to search for key genes that affect PCa progression. The correlation between gene expression and clinical pathological parameters and patient prognosis were examined, and it was verified in clinical samples. Next, we established cell lines by RNA interference, and studied its biological function by MTS, EdU, colony formation, wound-healing and transwell assay. The impact of gene on tumor growth was examined in nude mice xenograft model. Finally, high-throughput sequencing and signal pathway analysis were used to explore the molecular mechanism of gene action.

Results: By combining clinical samples with public databases, we found that NETO2 expression was markedly elevated in tumors especially in metastatic tumors compared with normal tissues, and the expression level of NETO2 was closely associated with the clinical pathological parameters and prognosis of the patients. Functionally, NETO2 drove PCa cell proliferation, migration and invasion in vitro, and promoted growth of PCa cells in vivo. Moreover, through transcriptome high-throughput sequencing and experimental verification, our data demonstrate that NETO2 inhibits PCa cellular senescence via modulating p53-p21 pathway.

Conclusions: Our results indicate that NETO2 plays an oncogenic role in PCa, which provides a new idea for clinical diagnosis and treatment of refractory PCa.

Introduction

According to the latest research report by the American Cancer Society, as the most common male malignancy, prostate cancer (PCa) was estimated about 268,490 new cases and over 34,500 deaths in the United States in 2022 (Siegel et al., 2022). Although the overall proportion of disease decreased at early stage due to interventions such as widespread prostate-specific antigen (PSA) testing and androgen deprivation therapy (ADT) (Wong et al., 2014), late events such as castration resistance and tumor metastasis will inevitably occur (Nevedomskaya et al., 2018;Smith et al., 2011), which lead to more than doubled the proportion of patients diagnosed with advanced disease in the last decade (Siegel et al., 2022). The quality of life and prognosis of patients with advanced cancer are very poor. In addition, up to 25% of patients undergoing surgical treatment experience biochemical recurrence (BCR), and these patients are at a higher risk of tumor metastasis and death (Brockman et al., 2015;Shao et al., 2020). In order to alleviate the enormous burden faced by families and society, it is of great significance to explore the molecular mechanism of early PCa progression.

One of the characteristics of malignant tumors is the high degree of heterogeneity, which is related to the changes of the expression and modification of specific genes by a variety of carcinogenic factors

(Roundtree et al., 2017). Neuropilin and tolloid like 2 (NETO2) gene is located on human chromosome 16, and encodes a transmembrane protein belonging to the CUB domain and LDLa-containing proteins subfamily (Stöhr et al., 2002). NETO2 could modulate the function of neuronal kainate receptors (KARs) as an auxiliary protein (Straub et al., 2011;Zhang et al., 2009a). In addition, it also able to interact with the K^+/Cl^- cotransporter by enhancing its recycling in hippocampal neurons (Pressey et al., 2017). Growing evidence has suggested that NETO2 can function as pro-cancer factors in many types of malignancies. In pancreatic cancer, osteosarcoma and nasopharyngeal carcinoma, NETO2 has been shown to play an important role in promoting tumor progression (Chen et al., 2022;Li et al., 2019;Wang et al., 2021). Furthermore, high levels of NETO2 were associated with clinicopathologic features and poor prognosis in many digestive system tumors (Hu et al., 2015;Liu et al., 2019;Villa et al., 2016;Xu et al., 2021). According to a recent study, NETO2 may be one of the key molecules which induce PCa (Sun et al., 2021). On this basis, we further explored the mechanism of its effect through the combination of clinical samples and experimental verification.

In the current study, first, public databases were used to search for key genes that affect tumor progression. We found that the expression level of NETO2 was closely associated with the clinical pathological parameters and prognosis of the patients, and this conclusion was further verified in our clinical samples. Next, the biological function of NETO2 affecting the malignant degree of PCa cells was studied in vivo and in vitro respectively, high-throughput sequencing and signal pathway analysis were used to explore the molecular mechanism of its action. In general, NETO2 is a tumor-promoting factor in PCa and may serve as a novel prognostic indicator as well as a potential therapeutic target for PCa.

Materials And Methods

Public database analysis

In the present study, the corresponding transcriptome data and clinical information of The Cancer Genome Atlas (TCGA) prostate adenocarcinoma (PRAD) were acquired from the UCSC Xena database platform (http://xena.ucsc.edu/). This dataset is comprised of 496 PCa samples (includes 488 primary localized PCa and 8 metastatic PCa) and 52 adjacent normal tissues (ANT). Subsequently, the expression profile of GSE21034 PCa mRNA sequencing dataset submitted by Taylor BS et al. was downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/), which contains 150 PCa samples (131 primary localized PCa and 19 metastatic PCa) and 29 normal tissues (Taylor et al., 2010). The “limma” R package was utilized to identify the differentially expressed genes (DEGs) in database (Ritchie et al., 2015). For TCGA dataset, DEGs were recruited with |log2FC| value > 1 and FDR < 0.05, but for GSE21034 dataset, DEGs were recruited with |log2FC| value > 0.59 and FDR < 0.05. A Venn diagram was constructed to identify the overlapping DEGs. The key genes in our subsequent studies have complete clinical data, including clinicopathologic parameters and follow-up information that match the gene expression. Because that patient mortality rate in the database is too low, we chose the BCR as the endpoint event of prognosis index.

Patients And Tissue Specimens

Total of 86 PCa patients' tissue specimen were collected at our institution from January 2009 to December 2019. All of these patients underwent surgical resection or biopsy, and none of them had received hormonal treatment, chemotherapy, or preoperative radiotherapy. Among them 50 patients had primarily localized disease and 36 cases had distal metastases. All enrolled patients underwent follow-up through telephone. This study was approved by the Ethics Committee of the First Affiliated Hospital for Guangzhou Medical University, and all patients have signed informed consents.

Cell Lines And Cell Transfection

The normal human prostate epithelial cell line RWPE-1 and human PCa cell lines LNCAP, PC-3 and DU145 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Human PCa cell line C4-2, 22RV1 and human embryonic kidney cell 293 T were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines had authenticated and were cultured as described previously (Zhao et al., 2021).

The timely and stable transfected cell lines were constructed to study the biological function of NETO2. We used the small interfering RNA (siRNA) targeting NETO2 (GenePharma, Shanghai, China) to silence endogenous NETO2 (named si-NETO2). Cells were transfected with Lipofectamine 3000 according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). In addition, to establish stable overexpression or knockdown of NETO2 (named NETO2 or shNETO2) in PCa cells, lentivirus (LANDM BIOTECH, Guangzhou, China) were synthesized and used to infect the cell. Stable cell lines were selected for 10 days with Puromycin (2μg/mL). The sequence information of siRNAs and shRNAs are listed in Supplementary Table S1. The transfection efficiency was identified by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting, and the most efficient sequence was used in the following experiments.

Rna Extraction And Qrt-pcr

Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, CA, USA). All-in-One First-Strand cDNA Synthesis kit (GeneCopoeia, Guangzhou, China) was used to reverse transcribe RNA into cDNA. PCR for quantification of gene expression was performed with SYBR green Premix Ex Taq II (Takara) on a CFX-96 system (Bio-Rad, Hercules, CA). The PCR thermal cycling conditions consisted of an initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 20 s, and a 30 s extension at 72°C. GAPDH was used as internal control. Relative expression was determined by $2^{-\triangle\triangle Ct}$ method. The sequence information of primers are listed in Supplementary Table S1.

Protein Extraction And Western Blotting

Total proteins were extracted from cells with RIPA lysis buffer (KeyGEN, KGP703) containing PMSF (1%), and its concentration was quantified using the BCA protein assay regents (#23225, Thermo Pierce, Rockford, IL, USA). The procedure of western blot was conducted as previously described (Zhao et al., 2021). Primary antibodies used in the study include: anti-NETO2, anti-GAPDH, anti-P53, anti-P21 and anti-P16 purchased from Abcam. Detection was achieved in Odyssey CLX Two-color infrared laser imaging system (LI-COR Biosciences, Nebraska, USA). Densitometric analysis of the bands was performed using ImageJ software.

Cell Proliferation Assays

The proliferation ability of PCa cells was measured by MTS assay, 5-ethynyl-2'-deoxyuridine (EdU) assay and colony formation assay, respectively. For MTS assay, the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA) was used according to the manufacturer's instructions. Briefly, transfected PCa cells were seeded into 96-well plates (5,000 cells per well in 200 μl of complete culture medium). Following culture for 24, 48, 72, 96, and 120 h, cells in each well were incubated with 10 μl of MTS reagent for 4 h, and the optical density (OD) value of each well was then measured at 490 nm. For EdU assay and colony formation assay, the process was performed as previously described (Liu et al., 2021).

Cell Migration And Invasion Assays

The migration and invasion ability of PCa cells were measured by wound-healing assays and transwell assay. The detailed procedure was conducted as described in our previous research (Liu et al., 2021;Zhao et al., 2021). It should be emphasized that, in the transwell assay, we simulated the invasion or migration of tumor cells by adding or not adding matrigel (BD Biosciences) in advance in the permeable support chamber (Corning Incorporated, Corning, NY, USA).

Animal Experiments

Thirty 4–5 weeks old male BALB/c nude mice were purchased from the Experimental Animal Center of Guangdong Province (Guangzhou, China). The animals were fed as described previously (Zhao et al., 2021). All procedures related to the experimental animals were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Guangzhou Medical University.

To evaluate the effects of NETO2 on tumor growth, mice were randomly divided into six groups (NC/NETO2/shNETO2 group, n = 5/group) and each mouse was subcutaneously injected with concentrated stable PCa cells 5×10^6 to establish xenograft tumors. The tumor sizes were monitored

weekly. After 4 weeks, the mice were sacrificed by cervical dislocation, and the tumors were dissected and weighed. The tissue was fixed and embedded in paraffin wax for histological examination and immunohistochemical (IHC) assay.

Histological And Immunohistochemical Assessment

To study tissue morphology and protein expression, histological and IHC analysis were performed in mice xenografts and clinical PCa samples. The procedure of H&E and IHC were carried out as described previously (Li et al., 2017). Primary antibodies used in IHC include anti-NETO2 (Abcam) and anti-KI67 (Servicebio). When the experiment complete, slices were scanned by PathScope digital scanner (Gene Tech, Shanghai, China), and the protein expression was quantified according to the protocol described previously (Li et al., 2017). For NETO2 IHC score, we comprehensively evaluated the protein staining intensity of each microscopic field and the proportion of positive staining cell, then the final IHC score is obtained by multiplying the results of the two indicators (Xiang et al., 2020). The IHC score ranged from 1 to 9, where < 6 was classified as the low-expression group, and ≥ 6 was classified as the high-expression group. The final score of all specimens were used for statistical analysis.

Gene Expression Profiling And Pathway Analyses

NETO2-overexpression (PC3-NETO2) and NETO2-knockdown (PC3-shNETO2) PC3 cells were used for gene expression profiling. RNA was isolated using RNeasy Mini kit (QIAGEN). Microarray processing was performed by SINOTECH GENOMICS (Shanghai, China) using GeneChip PrimeView Human Gene Expression Array (Affymetrix). Differentially expressed genes between stable transfected and control cells were calculated by “edgeR” package, and then selected by Q < 0.05 and absolute fold change (FC) value > 1.5. In order to analyze the biological functions involved in differential genes, Gene Ontology (GO, http://pgrc.ipk-gatersleben.de/misa/) enrichment analysis was performed. Then, the pathway enrichment analysis was performed by using KEGG (http://emboss.sourceforge.net/) database. The order of signaling pathways is based on the number of differential genes enriched in the pathway.

β-galactosidase Staining

The cells were cultured in six-well plates until 90% confluence and then fixed and subjected to β-Galactosidase staining using a Senescence β-Galactosidase Staining Kit (Solarbio, China) following the manufacturer’s instructions. After staining for 12h, the cells were observed and recorded under microscope. The field of vision was randomly selected from three independent experiments.

Statistical Analyses

The SPSS V18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.0 software (San Diego, CA, USA) were used to perform statistical analyses. The association between NETO2 expression and PCa clinicopathologic characteristics was assessed by chi-squared test. Univariate and multivariate Cox regression models were constructed to estimate the hazard ratios (HRs) of independent factors affecting the overall survival in patients with PCa. Survival curves were plotted using Kaplan-Meier's method and compared between groups by the log-rank test. X-tile program was used to determine the cut-off values which optimized the significance of the split between Kaplan-Meier survival curves (Camp et al., 2004). For continuous variables, data presented as the means ± SD. Comparison between groups was carried out using the Student's t-test, paired t-test or one-way ANOVA. $P < 0.05$ was considered statistically significant.

Results

Identify and verify gene associated with PCa progression by bioinformatics analysis

By comparing the gene expression profiles of different patients in databases, we searched for genes associated with tumorigenesis or progression. In TCGA database, we identified 1003 genes with upregulated expression in tumors (Fig. 1A, Supplementary Table S2). In GSE21034 dataset, for further study the genes related to tumor progression, we subdivided the tumor patients to find key genes with progressively increasing expression in normal tissues, primary localized tumors and metastatic tumors (Fig. 1B, C, Supplementary Table S3). Combined with the co-upregulated DEGs selected from different databases, five genes that might play a key role in PCa progression were identified: NETO2, HOXC6, CACNA1D, SLC10A5 and ATP8A2 (Fig. 1D). Through reviewing relevant literature, we found that the mechanism of HOXC6, CACNA1D and ATP8A2 in promoting PCa has been widely reported (McCabe et al., 2008;O'Reilly et al., 2022;Paulo et al., 2012;Ramachandran et al., 2005;Zhou et al., 2019), while SLC10A5 has only been found to play a role in bile acid transport (Claro Da Silva et al., 2013). Although NETO2 has been proposed to be associated with the occurrence of prostate cancer (Sun et al., 2021), the specific mechanism of action remains unclear. Therefore, we chose NETO2 for verification in the follow-up study.

First, the NETO2 expression trends for adjacent normal tissues and PCa tissues (including primary localized PCa tissues and metastatic PCa tissues) were re-validated in TCGA (Fig. 1E), this is consistent with our previous conclusion in GSE21034 (Fig. 1J). In the paired samples of tumor tissue and paracancerous tissue, the expression level of NETO2 in tumor also had significant advantages (Fig. 1F). Next, we examined the correlation of NETO2 expression with clinicopathological characteristics in patients in TCGA and GSE21034. As shown in Fig. 1G, K, L, NETO2 expression level positively correlated with Gleason score and pathologic tumor stage in PCa patients. Finally, in order to test whether NETO2 can be used as a potential clinical prognostic indicator, we explored the association between NETO2 expression and patients' BCR and whether it has survival prognostic value. The results in GSE21034 dataset show that the expression of NETO2 and patients' BCR were obviously related, and high NETO2

expression associated with shorter BCR-free survival, which suggested that high expression of NETO2 predicted poor prognosis (Fig. 1M, N). A similar phenomenon was observed in TCGA (Fig. 1H, I).

NETO2 expression is correlated with PCa malignant degree and correlate with clinical outcomes of PCa patients

To further identify the clinical significance of NETO2 in PCa, immunohistochemical staining was performed on enrolled 86 clinical samples. By evaluating and counting the staining results, we obtained the respective NETO2 protein expression in patients with primary localized PCa and metastatic PCa (Fig. 1O). We then sought to explore the association between NETO2 expression and clinical characteristics. According to the result of IHC score, 86 patients were classified into low NETO2 expression group (n = 60) and high NETO2 expression group (n = 26). As listed in Table 1, NETO2 expression was significantly associated with Gleason score, pathologic tumor stage and metastasis status. We further explored whether NETO2 expression was associated with the prognosis of patients with PCa. Kaplan–Meier survival analysis revealed that patients with PCa with high NETO2 expression had shorter overall survival than those with low NETO2 expression (Fig. 1P). We also performed univariate and multivariate Cox regression analyses of prognostic indicators using collected clinical specimens (Table 2). Univariate analysis indicated that Gleason score ≥ 7 (HR = 1.693, p = 0.014), tumor metastasis (HR = 3.363, p = 0.007), surgical intervention (HR = 2.346, p = 0.046) and high NETO2 expression (HR = 2.709, p = 0.018) were independent risk factors for prognosis of patients with PCa. However, multivariate analysis suggested that only high NETO2 expression (HR = 2.517, p = 0.035) was the hazard factors predicting overall survival in patients with PCa.

Table 1
Correlation between NETO2 expression with clinicopathological characteristics in PCa.

Variables	n	NETO2		χ^2	P
		High	Low		
Age (year)				0.086	0.769
≤ 70	36	12	24		
> 70	50	14	36		
Gleason score					
< 7	25	4	21	6.287	**0.043**
= 7	22	5	17		
> 7	39	17	22		
Preoperative PSA level					
< 10	21	6	15	0.774	0.679
10–20	14	3	11		
> 20	51	17	34		
pT stage					
≤T2	52	10	42	7.548	**0.006**
>T2	34	16	18		
Metastasis					
M0	50	10	40	5.366	**0.021**
M1	36	16	20		
NETO2 expression level was determined by IHC score.					
pT, pathologic tumor stage.					

Table 2
Univariate and multivariate Cox regression analysis for overall survival in patients with prostate cancer

Variables	Univariate			Multivariate		
	HR	95% CI	P Value	HR	95% CI	P Value
Age	1.308	0.563–3.039	0.533	-	-	-
Preoperative PSA level	1.267	0.738–2.173	0.390	-	-	-
Gleason score	1.693	1.114–2.571	**0.014**	1.290	0.784–2.122	0.316
pT stage	1.538	0.672–3.520	0.308	-	-	-
Metastasis status	3.363	1.390–8.134	**0.007**	2.325	0.842–6.420	0.104
Hormone therapy	0.306	0.072–1.308	0.110	-	-	-
Surgical intervention	2.346	1.014–5.428	**0.046**	2.071	0.881–4.868	0.095
NETO2 expression level	2.709	1.184–6.201	**0.018**	2.517	1.066–5.945	**0.035**

Age, between age ≤ 70 and age > 70; Preoperative PSA level, between < 10 and ≥ 10; Gleason score, among Gleason score < 7 and ≥ 7; pT stage, pathologic tumor stage between ≤ T2 and > T2. HR, hazard ratio; CI, confidence interval.

Neto2 Facilitates Pca Cells Proliferation, Migration And Invasion In Vitro

First, we detected the expression of NETO2 in PCa cell lines and prostate normal epithelial cell RWPE-1 through qRT-PCR and western blot, respectively (Fig. 2A, B). We found that its expression in tumor cells was significantly higher than that in normal cell, with the highest expression in PC-3 and DU145 and lower expression in C4-2. Next, in order to study the effect of NETO2 on the biological functions of PCa cells, we knocked down NETO2 expression in PC-3 and C4-2 respectively, and verified the knockdown efficiency at mRNA level and protein level (Fig. 2C, D).

MTS assay, EdU assay and colony formation assay were conducted to determine the function of NETO2 on proliferation capabilities. As shown in Fig. 2E-G, transfection of the NETO2-siRNA obviously suppressed PCa cell proliferation compared with the control cell. In addition, our clinicopathologic association findings revealed that higher levels of NETO2 were associated with tumor metastasis. To verify this phenomenon in vitro, the migration and invasion ability of PCa cells were measured by wound-healing assays and transwell assays. As a result, we found that the mobility of cells decreased with the down-regulation of NETO2 (Fig. 2H, I). All these results suggest that NETO2 acts as an oncogenic gene in PCa cells in vitro.

Neto2 Promotes Tumor Growth In Vivo

To further investigate the role of NETO2 in tumor progression, we observed the changes of tumor cells growth in vivo by establishing nude mice subcutaneous xenograft model. The efficiency of cells stable overexpression or knockdown of NETO2 was first demonstrated (Fig. 3A), consistent with the trend of accelerated or slower growth of subcutaneous tumors (Fig. 3B-D). H&E staining showed the histopathological features of the tumor tissues (Fig. 3E). The result of NETO2 immunohistochemistry confirmed the stability of gene expression in tumor cells in vivo environment (Fig. 3F). Furthermore, we further verified the change of tumor growth ability by cell proliferation marker KI67 immunostaining (Fig. 3G). Taken together, in vivo study also confirmed the correlation between NETO2 and tumor malignancy.

Mechanism Analysis Of Neto2 Promoting Prostate Cancer Progression

The clinical significance and biological function of NETO2 had been confirmed in previous studies, but the mechanism of its effect is still unclear. Therefore, we explored the molecular mechanism of NETO2 promoting tumor progression by transcriptome high-throughput sequencing of stable cell lines that interfere with NETO2 expression and combined with bioinformatics analysis. As seen in Fig. 4A, a total of 3629 DEGs (include 1651 upregulated DEGs and 1978 downregulated DEGs) were identified in NETO2 overexpresses PC-3 cell compared with the control cell, and there were 2619 genes (include 1340 upregulated DEGs and 1279 downregulated DEGs) expression changes when the NETO2 was knocked down. Thereafter, to further uncover the function and pathways of DEGs in cells interfering with NETO2, functional enrichment analysis, including GO and KEGG functional enrichment analysis, was conducted. In GO enrichment analysis, the top 30 significantly enriched terms of biological processes (BP), cell component (CC), and molecular function (MF) were shown in Fig. 4B. By comparing the two groups of terms, we found that the biological functions affected by the changed gene expression are mainly reflected in the DNA replication process. What is more, the results of the KEGG pathway analysis of DEGs showed that signaling pathways like metabolic pathways, PI3K-Akt signaling pathway, cellular senescence, MAPK signaling pathway, and so on were significantly enriched (Fig. 4C). Among them, the cellular senescence pathway was shown to have the smallest statistical Q value in common between the two groups (Supplementary Table S4).

Relationship Between Neto2 And Prostate Cancer Cellular Senescence

As KEGG pathway analysis suggested that NETO2 might play a role by regulating cellular senescence-related pathways. First, through β -galactosidase staining experiment, we observed that the number of senescent cells was significantly increased after the inhibition of NETO2 expression, and the proportion

of senescent cells was decreased after the gene was overexpressed (Fig. 4D). This suggested that the mechanism of NETO2 affecting the malignant degree of PCa cells might be related to cellular senescence. As cells mainly regulate the senescence process through the p53-p21 and p16-pRB pathways (Campisi and D'Adda Di Fagagna, 2007), we detected the classical molecules related to these pathways and found that p53 and p21 were negatively regulated by the changes in NETO2 expression, while p16 was no difference (Fig. 4E). Therefore, based on the above findings, we speculated that NETO2 affected the senescence process of PCa cells through p53-p21 pathway.

Discussion

Prostate cancer is biologically and clinically a heterogeneous disease whose risk varies with host and tumor characteristics. Although there are many effective interventions for early-stage tumor, the onset of PCa is generally insidious. Without routine screening, patients often have no special symptoms. In clinic, more than half of patients have distant metastasis when they visit a doctor, which means poor prognosis. Abnormal expression of genes in tumors often leads to changes in the biological functions of cancer cells, and then has a great impact on the occurrence and development of tumors (Baca et al., 2013;Berger et al., 2011). Therefore, finding effective tumor markers for diagnosis or treatment of diseases is of great significance.

With the rapid development of genome-sequencing technology, an increasing number of promising biomarkers have been identified to have potential value in diagnosis or prognosis prediction. In the present study, by combining bioinformatics analysis and clinical research, our research reveal that NETO2 plays significant roles in PCa progression. Specifically, NETO2 expression was markedly elevated in tumors especially in metastatic tumors compared with normal tissues, and it was significantly associated with clinicopathological characteristics in patients with PCa. High levels of NETO2 were also a marker of poor survival. Additionally, our experiments observed that NETO2 could promote proliferation, migration or invasion of tumor cells in vitro or in vivo. Finally, through transcriptome high-throughput sequencing and experimental verification, our data demonstrate that NETO2 inhibits PCa cellular senescence via modulating p53-p21 pathway.

NETO2, also known as brain-specific transmembrane protein containing 2 CUB and 1 LDL-receptor Class A, previous studies of it have implicated this gene in neuron-specific processes (Zhang et al., 2009b). Recently studies have also linked NETO2 to an oncogene in various types of cancers (Chen et al., 2022;Hu et al., 2015;Villa et al., 2016). It should be emphasized that, according to the latest research, NETO2 could activate PI3K/AKT axis, thereby promoting osteosarcoma and esophageal squamous cell carcinoma cells epithelial-mesenchymal transition (EMT) (Wang et al., 2021;Xu et al., 2021). Similarly, a study of gastric cancer has demonstrated that NETO2 is an oncoprotein that activates the PI3K/Akt/NF-kB/Snail axis to contribute to tumor invasion and metastasis by inducing EMT (Liu et al., 2019). Interestingly, although the results of the KEGG pathway analysis showed that the PI3K-Akt signaling pathway ranked high in our research, the cellular senescence pathway was considered the most statistically significant. This suggested that cellular senescence might be specifically correlated with PCa progression.

Cellular senescence refers to the irreversible stagnation of cells at any period stimulated by various factors when they divide through the cell cycle. It is considered a major anticancer mechanism (Kumari and Jat, 2021). Common incentives such as telomere shortening, genomic damage, mitogens and proliferation-associated signals, epigenomic damage and activation of tumor suppressors have proved to induce cellular senescence (Campisi, 2013). Stimuli that induce senescence establish and maintain the growth arrest largely by engaging either or both of the p53/p21 and p16/pRB tumor suppressive pathways, which is recognized as a formidable barrier to malignant tumorigenesis (Adams, 2009;Campisi and D'Adda, 2007). In our experiment, we detected these key molecules and found that p53 and p21 were negatively regulated by the changes in NETO2 expression, while p16 was no difference. This indicated that NETO2 exerted its pro-cancer effect by inhibiting cellular senescence induced by the p53-p21 pathway, but it had no effect on the p16-pRB pathway. Similarly, Zemskova et al. found that P1M1 protein kinase could play an anti-cancer effect in PCa cells by inducing cellular senescence with p53, rather than through the p16-pRB pathway (Zemskova et al., 2010). Hu et al. pointed out that p21-dependent senescence induced by Riccardin D could be used as a new mechanism for inhibiting PCa (Hu et al., 2014). Besides, the common PTEN-deficiency in PCa restrains tumor development mainly by activating the cellular senescence process mediated by p53-p21 pathway (Parisotto et al., 2018).

Through the combination of bioinformatics analysis and clinical specimen verification, our work confirmed that NETO2 was closely related to the progression of PCa. Besides, based on the high-throughput sequencing, it has proved for the first time that NETO2 may exert biological function by inhibiting cellular senescence induced by p53-p21 pathway. However, there are still limitations in our current study that need to be taken into account. Although the connection between NETO2 and cellular senescence was found, it did not directly prove that NETO2 changed its malignant phenotype by affecting the senescence of PCa cells. In addition, the specific mechanism of NETO2 regulating p53-p21 pathway still needs further investigation.

In conclusion, the current study demonstrated that high levels of NETO2 expression in PCa tissues correlated with poor prognosis of the patients, and it might serve as a new prognostic indicator and a potential therapeutic target for PCa.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Yangzhou Liu: project development, data analysis and manuscript writing; Qian Xiang and Yuxiang Ma: perform experiment and data analysis; Zuomin Wang and Jinyou Pan: cell culture and data collection; Jiamin Wang and Mingda Zhou: histology examination and data collection; Jingwei Lin and Yingxin Cai: data analysis; Zhigang Zhao: project development and manuscript editing. All authors read and approved the final manuscript.

Data Availability

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

Ethics approval and consent to participate

This study involving human participants were approved by the Ethics Committee of the First Affiliated Hospital for Guangzhou Medical University, and all patients have signed informed consents. For animal experiments**,** all procedures related to the experimental animals were approved by the Animal Care and Use Committee of the First Affiliated Hospital of Guangzhou Medical University.

Consent to publish

Not applicable.

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Figures

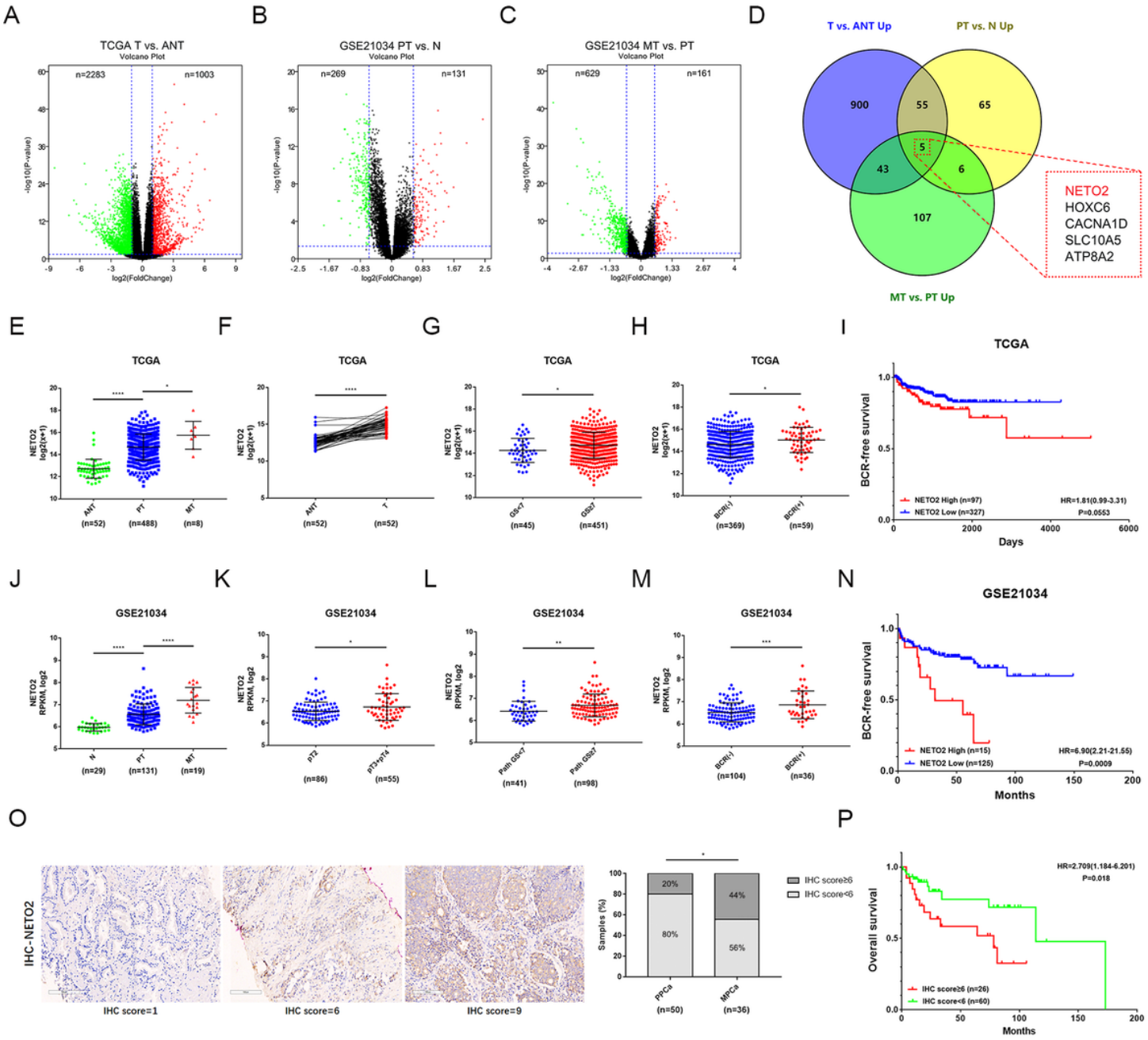

Figure 1

NETO2 expression is correlated with PCa malignant degree and correlate with clinical outcomes of PCa patients. (A) The differential expression gene of TCGA-PRAD database (1003 upregulated/2283 downregulated genes related to PCa formation, threshold set as |log2(Fold Change)| > 1, P < 0.05). (B, C) The differential expression gene of GSE21034 dataset (131 upregulated/269 downregulated genes related to PCa formation, 161 upregulated/629 downregulated genes related to PCa metastasis, threshold set as |log2(Fold Change)| > 0.59, P < 0.05). (D) Identify key genes related to PCa progression by combining TCGA database with GSE21034 dataset. (E) NETO2 expression increased in primary localized PCa tissues and metastatic PCa tissues compared with adjacent normal tissues in TCGA. (F) NETO2 expression level was upregulated in 52 paired PCa tissues compared with that in the matching adjacent

normal tissues in TCGA. (G, H) Correlations between NETO2 expression and patients' Gleason score and BCR status in TCGA. (I) The Kaplan-Meier survival analysis of BCR-free survival about NETO2 expression in TCGA. (J) NETO2 expression increased in primary localized PCa tissues and metastatic PCa tissues compared with normal tissues in GSE21034. (K-M) Correlations between NETO2 expression and patients' pathologic tumor stage, Gleason score and BCR status in GSE21034. (N) The Kaplan-Meier survival analysis of BCR-free survival about NETO2 expression in GSE21034. (O) NETO2 IHC staining was performed in clinical primary localized PCa tissues (n = 50) and metastatic PCa tissues (n = 36). Left: representative IHC score photographs of tumor tissue samples were shown. Right: the composition of NETO2 different expression in each group of tissue samples was counted. (P) The Kaplan-Meier survival analysis of overall survival of patients with different NETO2 expression levels. The data were presented as means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Student's t-test (E, G, H, J-M); paired t-test (F). T, tumor tissues; ANT, adjacent normal tissues; N, normal tissues; PT/PPCa, primary localized PCa tissues; MT/MPCa, metastatic PCa tissues; GS, Gleason score; BCR, biochemical recurrence; pT, pathologic tumor stage; HR, hazard ratio.

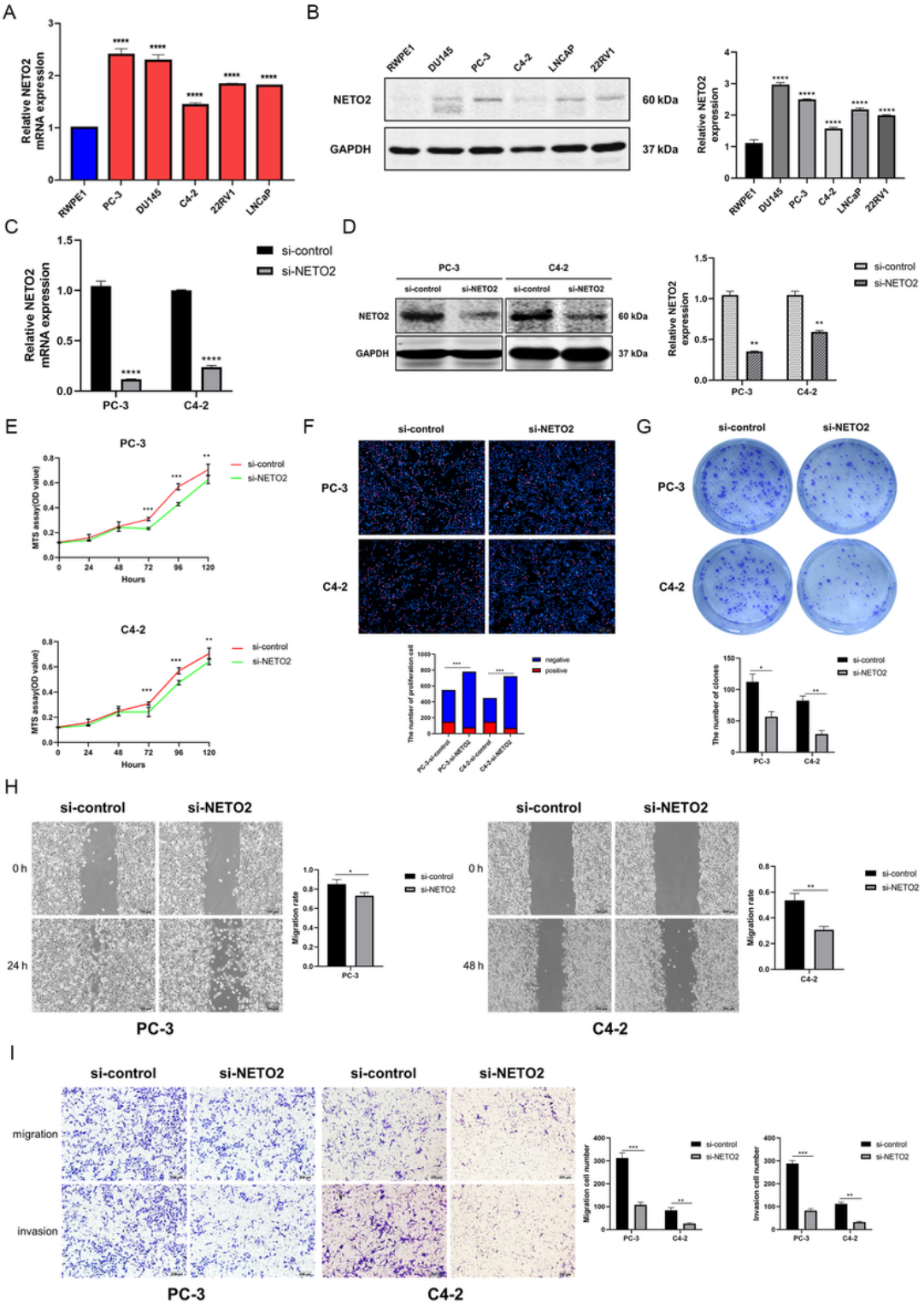

Figure 2

NETO2 facilitates PCa cells proliferation, migration and invasion in vitro. (A) Real-time PCR analysis of NETO2 mRNA expression in normal prostate epithelial cell (RWPE-1) and PCa cells. (B) Western blotting analysis of NETO2 protein expression in RWPE-1 and PCa cells. (C, D) NETO2 silenced PCa cell lines were established and confirmed by qRT-PCR and western blotting. (E-G) MTS assay, EdU assay and colony formation assay were performed to assess the proliferation ability changes in NETO2 silenced cell lines.

(H, I) Wound-healing assay and transwell assay were performed to assess the migration and invasion ability changes in NETO2 silenced cell lines. The data were presented as means ± SD from three biological replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Student's t-test.

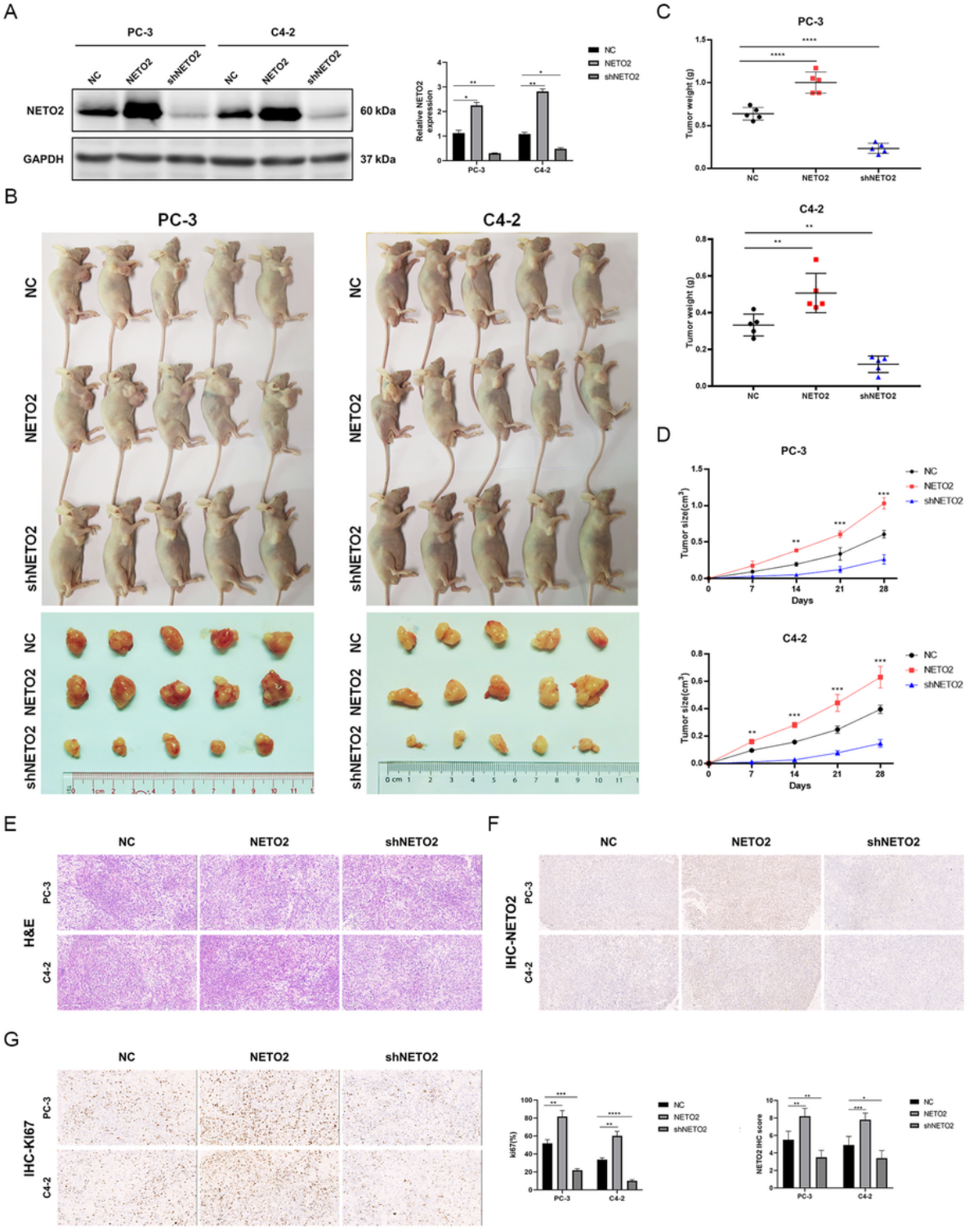

Figure 3

NETO2 promotes tumor growth in vivo. (A) Stable NETO2 overexpression or knockdown PCa cell lines were established and confirmed by western blotting. (B) The subcutaneous xenograft tumor model in nude mice was established, and the representative appearance of tumor mass resected from each group of mice. (C) Final tumor weights were measured at autopsy on day 28 after subcutaneous injection stable transfected PCa cells. (D) The tumor growth curves were measured with a calliper at the indicated days after cell injecting. (E) The xenograft tumor tissues were stained with H&E. (F) NETO2 IHC staining was performed in xenograft tumor tissues. (G) KI67 IHC staining was performed in xenograft tumor tissues to assess tumor proliferation. The data were presented as means ± SD from three biological replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; Student's t-test.

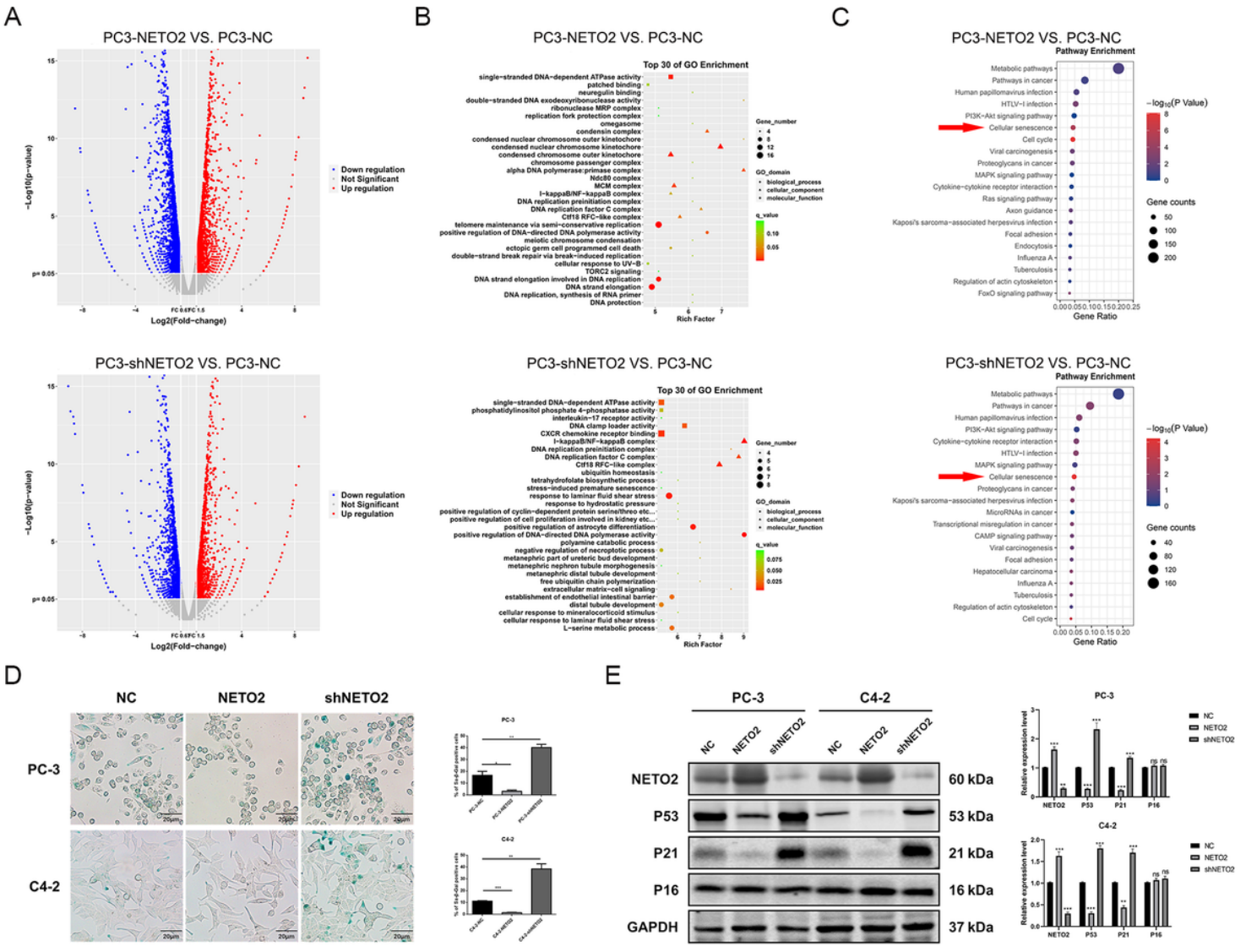

Figure 4

Relationship between NETO2 and PCa cellular senescence. (A) The differential expression gene of NETO2 overexpression or knockdown PC-3 cell lines (threshold set as |log2(Fold Change)| > 0.59, P < 0.05). (B) Top 30 GO enrichment analysis' terms of differential expression genes. (C) Top 20 KEGG pathway

analysis' terms of differential expression genes. (D) β -galactosidase staining experiment was performed to assess the cellular senescence of NETO2 stable interference PCa cell lines. (E) Markers of cellular senescence pathway were detected by western blotting. The data were presented as means ± SD from three biological replicates. $^{ns}P > 0.05$; *P < 0.05; **P < 0.01; ***P < 0.001; Student's t-test.

Supplementary Files

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