scientific reports

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NOX4 serves as a pan-cancer prognostic biomarker and therapeutic target in tumorigenesis

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NADPH oxidase 4 (NOX4) is a key regulator of intracellular reactive oxygen species (ROS) and plays a critical role in tumorigenesis and cancer progression. It contributes to cancer cell transformation, proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT). To elucidate the molecular mechanisms underlying NOX4-mediated tumorigenesis, we performed a comprehensive pan-cancer bioinformatics analysis, integrating data from The Cancer Genome Atlas (TCGA), and validated our findings with in vitro experiments. We systematically analyzed NOX4 expression patterns across various cancer types and explored the correlations between NOX4 expression and patient survival, immune infiltration, tumor mutational burden (TMB), and microsatellite instability (MSI). In vitro assays, including Wound healing, Transwell, and CCK-8 assays, were conducted to validate the biological functions of NOX4 in breast cancer cells. Pan-cancer analysis revealed that NOX4 is significantly upregulated in various cancers, including breast cancer. Elevated NOX4 expression is associated with poor patient prognosis, immune cell infiltration, TMB, and MSI. Functional experiments confirmed that downregulation of NOX4 can inhibit the proliferation and metastasis of breast cancer cells. Our pan-cancer analysis provides valuable insights into the role of NOX4 in tumorigenesis. These results highlight NOX4 as a promising biomarker for prognosis and a potential therapeutic target for anti-tumor treatments across multiple cancer types.

Keywords NADPH oxidase 4, Pan-cancer, Prognostic biomarkers

Cancer remains a major global health challenge, with approximately 20 million new cases and nearly 10 million cancer-related deaths reported worldwide in 2022[1]. A comprehensive understanding of cancer pathogenesis necessitates analyses that extend beyond individual tumor types to encompass diverse tumor types.

Reactive oxygen species (ROS) play a critical role in the initiation and progression of cancer. The NADPH oxidase (NOX) family, which catalyzes electron transfer at the cell membrane, is a key source of ROS generation[2]. The NOX family comprises six identified members: NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2[3]. NOX4, the most widely expressed member of the NOX family, plays a key role in the occurrence and development of diverse cancers[4]. It promotes tumor progression by influencing cancer cell transformation, proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT)[5,6]. NOX4 has been shown to be dysregulated in a variety of malignancies including Lung Cancer, Renal Cell Carcinoma (RCC), Colorectal Cancer (CRC), Gastric Cancer (GC), Pancreatic Cancer, Glioblastoma, and Ovarian Cancer, etc[7]. Despite extensive research on NOX4 in various cancer types, existing studies are generally focused on specific cancers and lack a comprehensive, pan-cancer analysis. Therefore, a more systematic investigation of NOX4 expression levels and their correlation with patient outcomes, including tumor immune infiltration, across different cancer types is needed. Such analyses will deepen our understanding of NOX4's role in tumor development and potentially uncover new therapeutic targets and prognostic biomarkers, leading to novel strategies for cancer treatment.

In this study, we performed a comprehensive pan-cancer analysis integrating multiple databases to systematically investigate NOX4 expression across various cancer types. We evaluated gene expression patterns, patient survival prognosis, tumor immune infiltration levels, and conducted gene enrichment analyses. The primary objective of this study is to elucidate the role of NOX4 in cancer pathogenesis and to explore its potential molecular mechanisms, which could provide valuable insights for cancer diagnosis and clinical prognosis.

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Results

NOX4 expression in pan-cancer

This study systematically analyzed the expression characteristics of NOX4 in pan-cancer based on the TCGA-GTEx database. The results revealed significant NOX4 expression differences in various tumor tissues. Specifically, NOX4 expression was significantly upregulated in multiple cancers, including GBM, GBMLGG, LGG, BRCA, LUAD, ESCA, STES, COAD, COADREAD, PRAD, STAD, HNSC, LUSC, LIHC, SKCM, BLCA, THCA, READ, OV, PAAD, TGCT, ALL, LAML, PCPG, ACC, and CHOL. In contrast, NOX4 expression was significantly downregulated in KIRP, KIPAN, KIRC, WT, UCS, and KICH compared to normal tissues. Additionally, no significant differences in NOX4 expression were observed in UCEC and CESC when compared to normal tissues (Fig. 1A). Further analysis based on the TCGA database revealed that NOX4 expression was significantly upregulated in multiple cancer types, including GBM, GBMLGG, LUAD, COAD, COADREAD, BRCA, ESCA, STES, STAD, PRAD, HNSC, LUSC, LIHC, THCA, READ, PCPG, BLCA, and CHOL. In contrast, NOX4 expression was significantly downregulated in CESC, KIRP, KIPAN, KIRC, and KICH. Additionally, no significant differences in NOX4 expression were observed in LGG, UCEC, and PAAD compared to normal tissues (Fig. 1B). The statistical analysis of NOX4 expression screened by the above two databases showed that NOX4 expression was consistent in 22 cancers (Fig. 1C).

The relationship between NOX4 and clinical parameters in tumor patients

This study thoroughly investigated the correlation between NOX4 and tumor stage and prognosis. Through the analysis of 21 types of malignant tumors (including BRCA, COAD, LUAD, LUSC, STAD, UVM, etc.), it was found that the expression level of NOX4 is significantly positively correlated with Tumor Stage, indicating that NOX4 expression increased with advancing tumor stage (Fig. 1D and Fig. S1). To evaluate the clinical prognostic value of NOX4, we employed the Kaplan-Meier method for survival analysis. As shown in Fig. 2A and C, among the 21 cancer types, high NOX4 expression in patients with BRCA, COAD, LUAD, LUSC, STAD, and UVM tumors is significantly associated with shorter overall survival (OS) (Fig. S2). Further analysis revealed that NOX4 expression levels are significantly correlated with disease-free survival (DFS) (Fig. 2B and D). Particularly noteworthy is that in specific cancer types such as BRCA, COAD, LUAD, LUSC, STAD, and UVM, patients with higher NOX4 expression exhibited poorer DFS. These findings suggest that NOX4 may serve as an adverse prognostic marker for various malignant tumors, providing a potential indicator for clinical prognosis evaluation.

The correlation of NOX4 expression and tumor immune microenvironment

The tumor immune microenvironment significantly influences tumor progression. To investigate the relationship between NOX4 and the pan-cancer immune microenvironment, we utilized the TIMER.2 database to analyze the correlation between NOX4 expression and immune cell infiltration. Heatmap analysis revealed that NOX4 expression levels were significantly positively correlated with macrophages, cancer-associated fibroblasts (CAFs), and endothelial cells. Taking the EPIC computational method for cancer-associated fibroblasts as an example, tumors that showed a significant positive correlation with NOX4 expression levels include BLCA (rho = 0.67, $p = 5.32E{-}50$), BRCA (rho = 0.78, $p = 1.28E{-}204$), CHOL (rho = 0.69, $p = 4.29E{-}06$), COAD (rho = 0.86, $p = 9.48E{-}82$), ESCA (rho = 0.85, $p = 2.26E{-}51$), and HNSC (rho = 0.86, $p = 2.34E{-}143$), among others (Fig. 3 and Supplementary file 1). Studies have shown that NOX4 is highly expressed in CAFs and drives CAF activation and pro-tumor functions through the production of ROS[8]. Studies have shown that the use of small-molecule NOX4/1 inhibitors (such as GKT137831) can effectively reverse the differentiation of CAFs, significantly promote the infiltration of $CD8^+$ T cells into tumors, and enhance the efficacy of immunotherapy[9]. These results suggest that NOX4 plays a significant role in the tumor immune microenvironment, with its

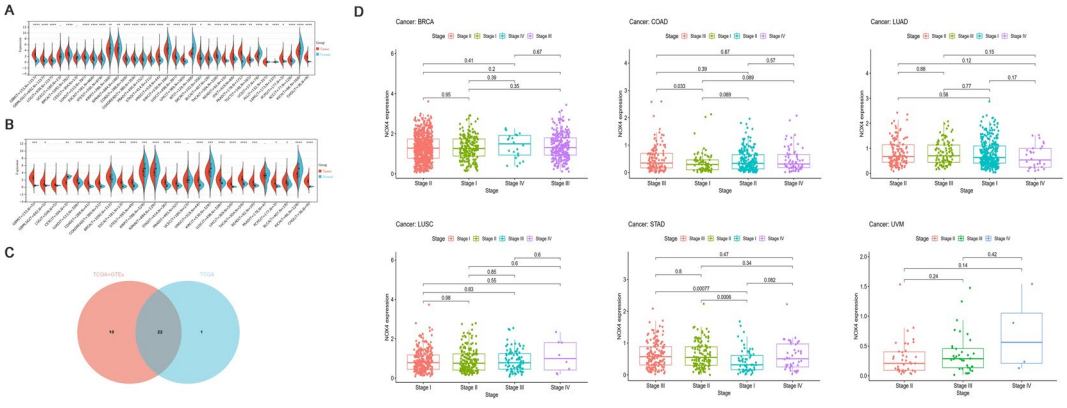

Fig. 1. NOX4 expression across different tumor types. (**A**) Expression levels of NOX4 in tumor and normal tissues across 34 cancer types using TCGA-GTEx combined data. (**B**) Expression levels from TCGA data alone. (**C**) Venn diagram showing overlap between the two datasets. (**D**) Association between NOX4 expression and tumor stage. Data are presented as mean ± SD. Statistical comparisons between tumor and normal tissues were performed using the Wilcoxon rank-sum test. $p < 0.05$ was considered statistically significant. Sample sizes for each group are detailed in Supplementary file 4.

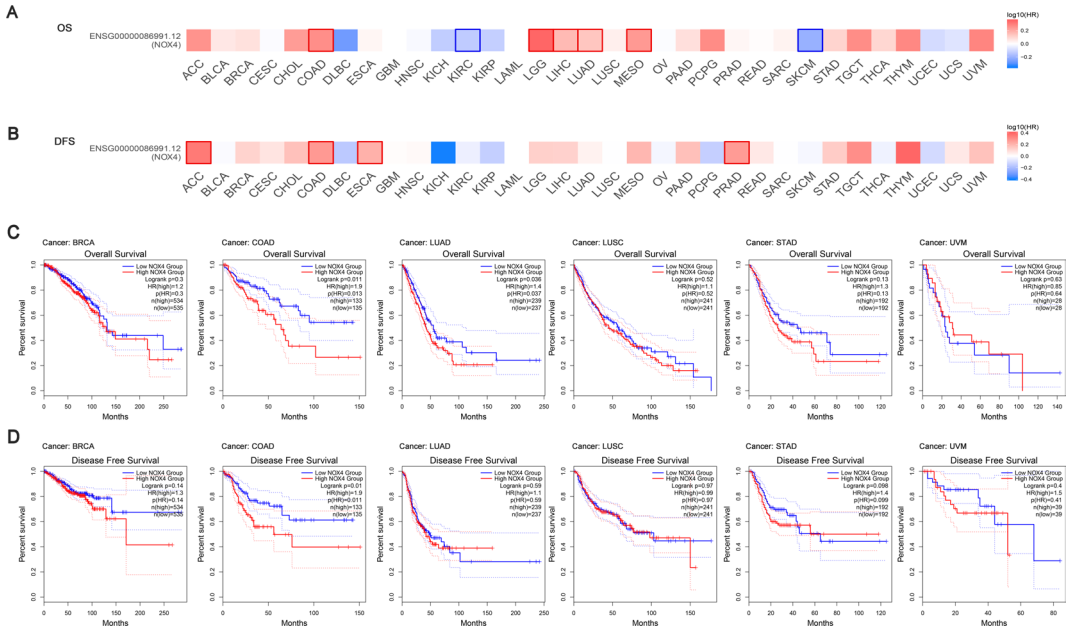

Fig. 2. NOX4 expression correlates with OS and DFS in pan-cancer survival analysis. (**A**) The overall survival map showing NOX4 expression in different cancers. (**B**) Disease-free survival map showing NOX4 expression in different cancer types. (**C**) Overall survival curve of NOX4 expression. (**D**) Disease free survival curve of NOX4 expression.

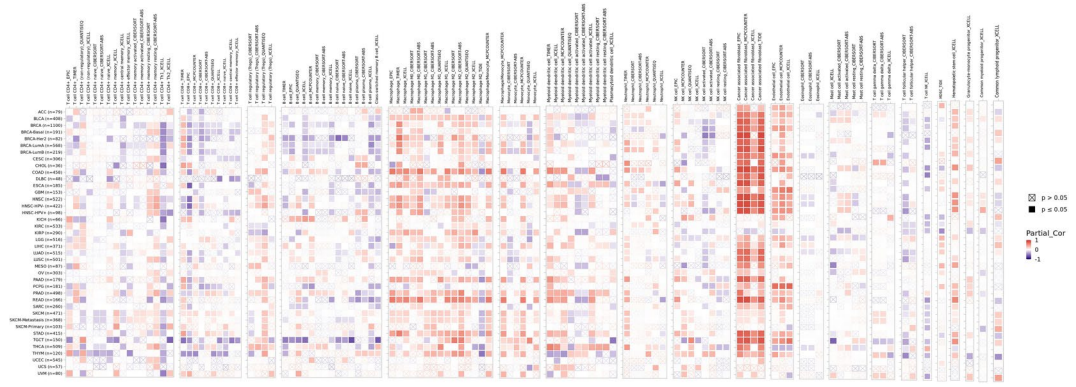

Fig. 3. Analysis of NOX4 expression and immune cell infiltration. The statistical analysis is detailed in Supplementary file 4.

regulatory mechanisms involving multiple aspects, including immune cell infiltration, expression of immune checkpoint molecules, and tumor metabolism, indicating that targeting NOX4 may provide a novel strategy to enhance immunotherapy efficacy.

Genomic heterogeneity and genomic expression

Studies have shown that genomic heterogeneity is a key factor in evaluating the potential effectiveness of tumor immunotherapy. Therefore, NOX4 was analyzed for its association with genomic heterogeneity indicators such as microsatellite instability (MSI), tumor mutation load (TMB), homologous recombination defect (HRD), mutant heterogeneity (MATH), tumor ploidy, loss of heterozygosity (LOH), and tumor purity (Fig. 4A–G). NOX4 expression was positively correlated with HRD in PRAD, ESCA, HNSC, COADREAD, COAD, OV, LIHC, and LGG. Further analysis reveals that NOX4 exhibits a positive correlation with the level of LOH in tumors including GBMLGG, PRAD, ESCA, LGG, HNSC, LAML, and TGCT. Concurrently, in tumor types such as THYM, LAML, TGCT, SARC, HNSC, and COAD, NOX4 expression is positively correlated with MATH. It is noteworthy that NOX4 expression shows a positive correlation with TMB in tumors such as ACC, KIRP, THYM, OV, LGG, COADREAD, and COAD. Moreover, in tumor types including DLBC, TGCT, and PRAD, NOX4 expression is positively correlated with MSI. In tumors such as PRAD, PCPG, and HNSC, NOX4 expression is positively correlated with tumor ploidy. Lastly, in tumor types including TGCT, LGG, and GBMLGG, NOX4

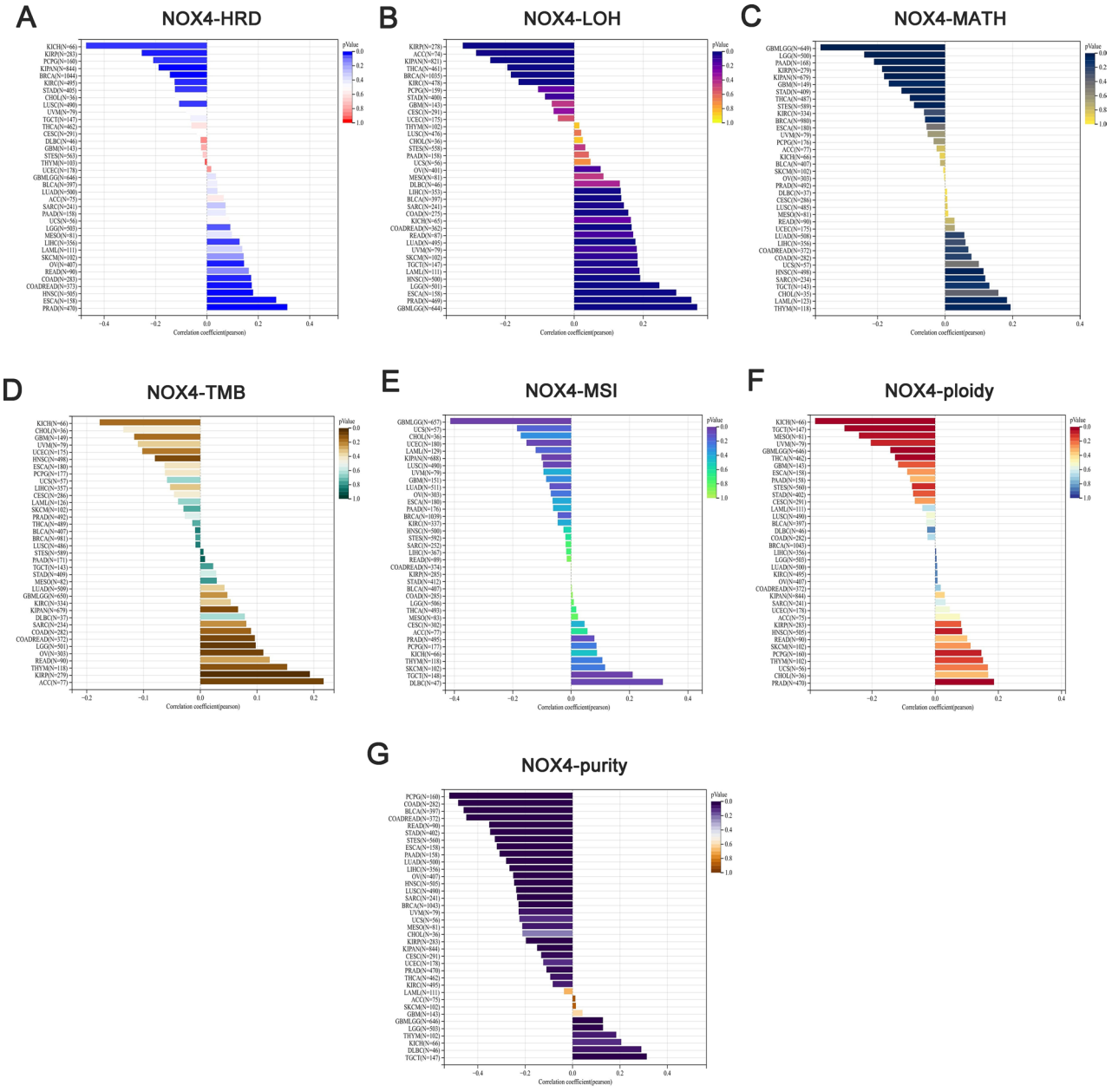

Fig. 4. Heterogeneity and NOX4. Pan-cancer correlation analysis of NOX4 with HRD, LOH, MATH, TMB, MSI, ploidy, purity.

expression is positively correlated with tumor purity. These findings suggest a potential role for NOX4 in regulating tumor genomic heterogeneity and provide a basis for future research into NOX4 as a biomarker for immunotherapy.

NOX4 co-expression patterns associated with specific biological processes

To further explore the potential roles of NOX4 in tumorigenesis, we analyzed its co-expression patterns with genes involved in specific biological processes. We observed that NOX4 expression was significantly positively correlated with genes related to EMT, m6A methylation, and immune checkpoints in pan-cancer tissues (Fig. 5A–F). For example, in COAD, NOX4 showed a significantly positive correlation with the expression of EMT-related proteins (FN1, ZEB2, ZEB1, TWIST1, SNAI2, VIM, and CDH2) and immune checkpoint-related proteins (PDCD1LG2 and HAVCR2).

Functional enrichment analysis of NOX4

To further explore the underlying biological processes of NOX4 in different cancer types, we first obtained 100 genes co-expressed with NOX4 (such as MIOX, AGXT2, GPX3, etc.) from the GEPIA database and mapped the protein-protein interaction (PPI) network using STRING (Fig. 6A and Supplementary file 2). Subsequently, we

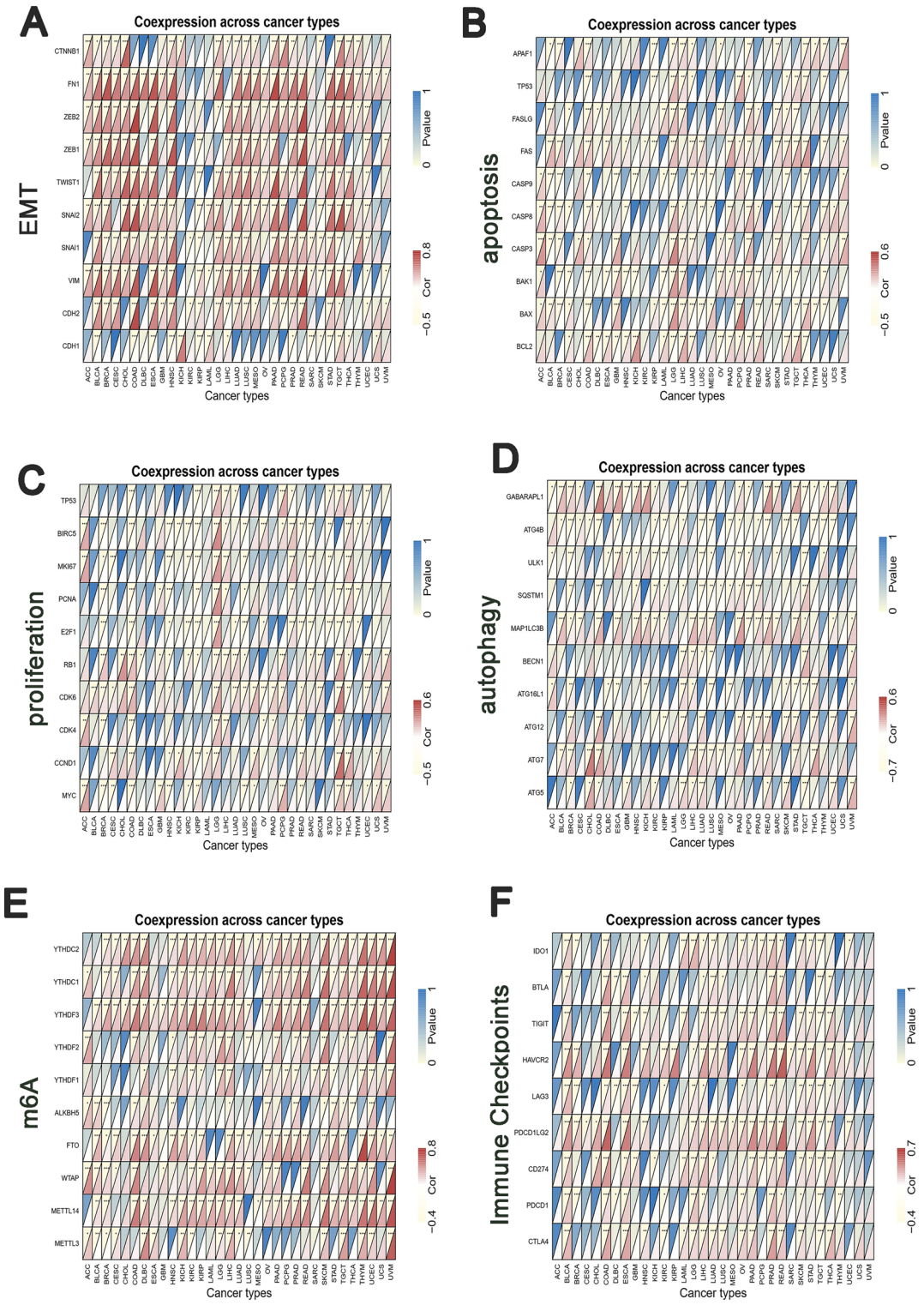

Fig. 5. NOX4 co-expression patterns associated with specific biological processes. Pan-cancer correlation analysis of NOX4 with EMT, apoptosis, proliferation, autophagy, m6A, Immune Checkpoints.

performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses to uncover the key roles of these co-expressed genes in tumorigenesis. Pathway analysis based on the KEGG database[10–12] demonstrated that the aforementioned genes were predominantly associated with metabolic pathways, with significant relevance to "Pentose and glucuronate interconversions", "Protein digestion and absorption", and "Parathyroid hormone synthesis, secretion, and action" (Fig. 6B). To further elucidate the molecular role of NOX4 in pan-cancer, we conducted GO enrichment analysis on these genes, categorizing

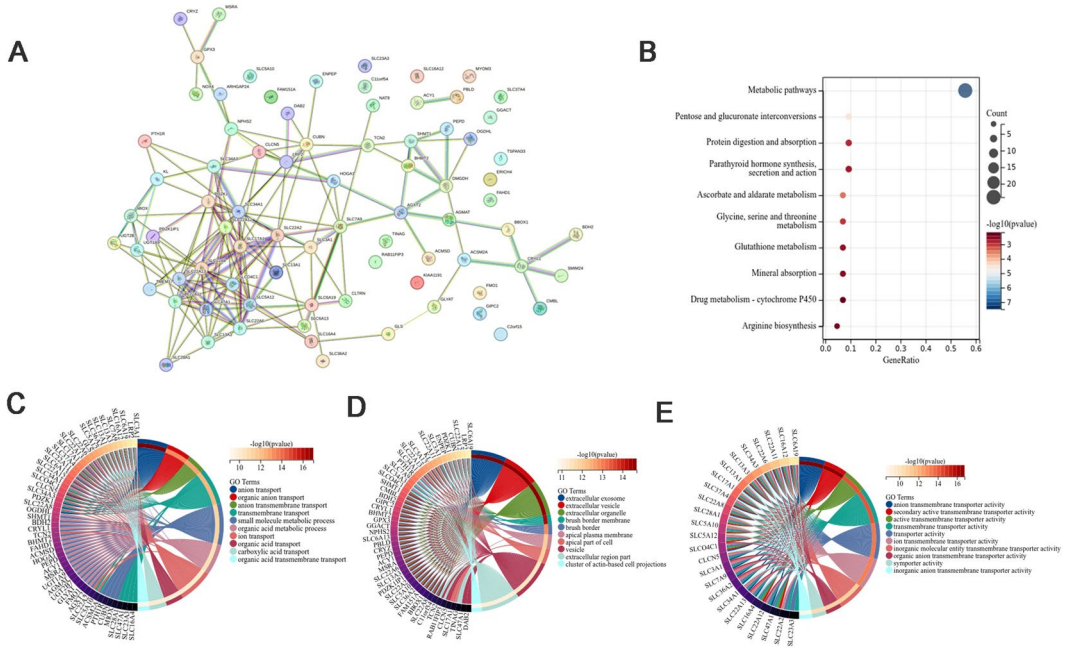

Fig. 6. NOX4-related genes functional enrichment analysis. (**A**) PPI networks of NOX4. (**B**) KEGG based on differential expression analysis. (**C**) BP based on differential expression analysis. (**D**) CC based on differential expression analysis. (**E**) MF based on differential expression analysis. Statistical analysis for panels **B**–**E** are detailed in Supplementary file 4.

them according to their functional annotations. The results were systematically classified into three categories: In the Biological Process (BP) category, these genes were primarily enriched in small molecule metabolic processes, transmembrane transport, ion transport, anion transport, and organic acid metabolic processes (Fig. 6C). Within the Cellular Component (CC) category, significant enrichment was observed in vesicles, extracellular region parts, extracellular exosomes, extracellular vesicles, and extracellular organelles (Fig. 6D). Regarding Molecular Function (MF), these genes were predominantly involved in transmembrane transporter activity, transporter activity, ion transmembrane transporter activity, inorganic molecular entity transmembrane transporter activity, and anion transmembrane transporter activity (Fig. 6E). See Supplementary file 5 for details. These findings suggest that NOX4 and its co-expressed genes may play a crucial role in cellular material transport, signal transduction, and material exchange within the tumor microenvironment. Specifically, extracellular exosomes are important mediators of intercellular communication, while transmembrane transporter proteins are responsible for substance transport across membranes. In summary, GO and KEGG enrichment analyses revealed that NOX4 and its co-expressed genes have important functions in metabolism and cellular material transport, providing valuable insights for further research into the biological roles of NOX4 in cancer.

NOX4 knockdown inhibits tumor cell migration and proliferation

To explore the biological functions of NOX4 in BRCA, we selected the MDA-MB-231 cell line as research models. First, we validated the efficiency of NOX4 siRNA interference using Western Blot and qRT-PCR experiments, and the results showed a significant downregulation of NOX4 expression levels (Fig. 7A,B). Subsequently, CCK-8 assays indicated that the proliferation capacity of MDA-MB-231 cells was significantly inhibited after NOX4 knockdown (Fig. 7C). Moreover, wound healing and Transwell migration assays further confirmed that the knockdown of NOX4 significantly reduced the migratory abilities of both cell lines (Fig. 7D–E). In summary, these experimental results suggest that NOX4 functions as an oncogene in BRCA by regulating tumor cell proliferation and migration, thereby contributing to tumor progression.

Discussion

NOX4 expression and prognostic significance across diverse cancers

Cancer is a leading cause of death worldwide and remains a major research focus in the medical field. NOX4, an important member of the NADPH oxidase family, plays a key role in the development of a variety of tumors[3]. NOX4 catalyzes the electron transfer process to produce ROS, which influence the biological behaviors of tumor cells such as proliferation, migration, and invasion[13–15]. For example, in BRCA, NOX4 enhances the aggressiveness of tumor cells by promoting their EMT. In GC, NOX4 expression is significantly elevated in gastric cancer tissues compared to normal adjacent tissues and is associated with reduced overall survival and increased tumor aggressiveness[16–18]. NOX4 has been found to be an independent predictor of poor prognosis in GC[19]. NOX4 is also involved in regulating the metabolic reprogramming of tumor cells, providing the energy and material basis for their rapid growth[20]. In this study, we performed a pan-cancer analysis using bioinformatics

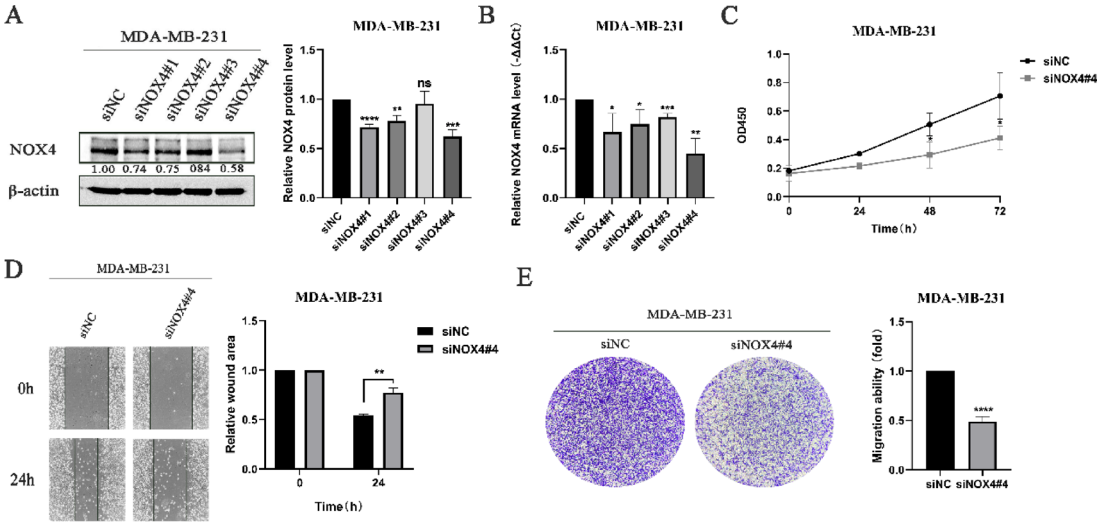

Fig. 7. Knockdown of NOX4 inhibits cell proliferation and migration in MDA-MB-231 cells. (**A**) Western blot analysis confirming NOX4 knockdown. (**B**) qRT-PCR validation of NOX4 mRNA suppression. (**C**) Cell proliferation assessed using the CCK-8 assay at 24, 48 and 72 h post-transfection. (**D**) Wound healing assay results at 0 and 24 h. (**E**) Transwell migration assay results after 48 h. Data are shown as mean ± SD from three independent experiments ($n = 3$ per group). Statistical significance was determined using unpaired Student's t-test. $p < 0.05$ was considered significant.

data, observing significant differences in NOX4 expression in tumor tissues and normal tissues based on TCGA GTEx, TCGA, and TCGA paired samples. We found that NOX4 is expressed higher in most tumor tissues than in paired normal tissues.

NOX4 has been shown to be a poor prognostic factor, but has lacked extensive analysis[21,22]. Based on the analysis of the TCGA database, we elucidated the multifaceted prognostic effects of NOX4 overexpression on overall tumor survival. By analyzing the relationship between NOX4 expression and clinical prognosis of tumor patients, this study found that high expression of NOX4 is associated with poor prognosis of patients with multiple tumors, including shortened OS and DFS. This suggests that NOX4 could be used as a potential marker to assess tumor patient prognosis. Clinically, detecting NOX4 expression levels in tumor tissue can help doctors make more accurate assessments of patient prognosis, informing the development of individualized treatment plans. For example, for patients with high NOX4 expression, more aggressive treatment measures, such as combination chemotherapy, radiotherapy, or targeted therapy, may be required to improve the efficacy of the treatment[7]. For patients with low NOX4 expression, a relatively conservative treatment strategy may be considered. Furthermore, NOX4's prognostic value can be used in combination with other clinical indicators to improve the accuracy and reliability of prognostic assessment.

NOX4's dual role in tumor immune escape and microenvironment regulation

Tumor immune escape is a key mechanisms of tumor progression, and NOX4 plays a complex role in this process[23]. Cancer-associated fibroblast-mediated immunotherapy resistance can be effectively overcome by NOX4 inhibition, improving outcomes in a variety of cancers[9]. In the tumor microenvironment, immune cells play an extremely important role as the soil of the tumor[24]. On the one hand, NOX4 promotes tumor immune escape by affecting immune cells in the tumor microenvironment[25]. In this study, the expression level of NOX4 was found to be correlated with the infiltration level of tumor immune cells, especially macrophages and CAFs. NOX4 may help tumor cells evade immune surveillance by regulating the function of these immune cells, such as inhibiting phagocytosis of macrophages or inducing the secretion of immunosuppressive factors by CAFs, thereby reducing the immune response in the tumor microenvironment. NOX4, on the other hand, may also promote an anti-tumor immune response in some cases. For example, NOX4 has been shown to induce tumor cells to express immunogenic molecules in certain tumor types, attracting immune cells into the tumor microenvironment and enhancing anti-tumor immune responses[20]. This bidirectional action suggests that immunotherapeutic strategies targeting NOX4 must carefully consider its specific mechanism of action in different tumor microenvironments to achieve precise treatment.

NOX4 association with tumor heterogeneity and molecular signatures

Tumor heterogeneity plays an important role in tumor diagnosis, prognosis assessment and treatment decision. Recent studies have shown that homologous recombination deficiency (HRD) can serve as a predictive biomarker for the efficacy of immune neoadjuvant therapy in patients with non-small cell lung cancer (NSCLC)[26]. Studies have shown that HLA-I LOH may limit antigen presentation, thereby facilitating immune evasion, and is an important prognostic biomarker in triple-negative breast cancer (TNBC), associated with poorer recurrence-free survival[27]. TMB has also been shown to identify a subgroup of microsatellite stable (MSS) pancreatic cancers

with longer survival and a stronger anti-tumor immune response[28]. Our analysis results showed that NOX4 was associated with HRD, LOH, MATH, TMB, MSI, ploidy and purity. It is well known that EMT, apoptosis, cell proliferation, autophagy, m6A and immune checkpoint all play an important role in the occurrence and development of cancer[29–31]. Studies have shown that myricetin effectively inhibits NOX4 expression, thereby reducing the production of ROS, ultimately suppressing NF-κB activation and downstream Snail expression, thus blocking a key step in the EMT pathway[32]. Furthermore, other research has found that the demethylase FTO increases NOX4 expression by reducing NOX4 m6A methylation levels, leading to oxidative stress, inhibiting Wnt/β-catenin signaling, and ultimately impairing cardiomyocyte differentiation[33]. Additionally, studies indicate that knockdown of NOX4 and IL-8 can decrease the expression of programmed death-ligand 1 (PD-L1)[25]. Our experimental results also indicate that NOX4 can promote the proliferation and migration abilities of MDA-MB-231 cells. We analyzed the association of these phenotypically associated molecules with NOX4 in different cancers. In addition, NOX4-related genes were explored, and GO function and KEGG enrichment analysis were performed on these genes to provide a basis for subsequent mechanism studies.

Translational potential of NOX4 in immunotherapy

NOX4 holds significant promise in the field of tumor immunotherapy. Firstly, inhibiting NOX4 can enhance the effectiveness of tumor immunotherapy[9]. Given its role in promoting tumor immune escape, suppressing NOX4 activity can restore immune responses within the tumor microenvironment and enhance the ability of immune cells to target and destroy tumor cells. For instance, combining NOX4 inhibitors with immune checkpoint inhibitors could synergistically boost anti-tumor immune responses[8]. Secondly, the expression level of NOX4 can serve as a valuable biomarker for assessing the efficacy of immunotherapy[23,25]. Monitoring changes in NOX4 expression during immunotherapy can provide insights into the immune status of the tumor microenvironment, thereby aiding in the evaluation of treatment outcomes. Additionally, NOX4 represents a potential target for the development of novel immunotherapeutic drugs. By further elucidating the specific mechanisms through which NOX4 influences tumor immunity, researchers can design targeted therapies that directly intervene in tumor immune escape processes, ultimately improving the success rate of immunotherapy.

Study limitations and future research directions

While our pan-cancer analysis of NOX4 provides compelling evidence for its role as a prognostic biomarker and its association with immune infiltration, this study has certain limitations that warrant discussion. First, our bioinformatics analysis was primarily based on TCGA and GTEx databases, which, while comprehensive, may not fully capture the heterogeneity of real-world clinical populations. Future studies incorporating multicenter clinical cohorts and prospective patient samples would strengthen the translational relevance of these findings. Second, although we validated NOX4's functional impact in breast cancer cell lines, further investigation is needed across additional cancer types and in vivo models to elucidate its tissue-specific mechanisms. Importantly, the clinical utility of NOX4 as a biomarker will require rigorous validation using large-scale, real-world patient data with standardized assays. Despite these limitations, our integrated bioinformatics and experimental approach lays a solid foundation for future research into NOX4's therapeutic potential and its interplay with the tumor microenvironment.

Methods

Gene expression analysis

This study of the clinical data from The Cancer Genome Atlas (TCGA, https://www.portal.gdc.cancer.gov/) to obtain the relevant RNA-seq data, using the log2(x + 1) conversion method processing NOX4 FPKM expression values, so as to promote the follow-up analysis. The full names and appropriate acronyms of the tumors are listed below: GBM (Glioblastoma multiforme); GBMLGG (Glioblastoma multiforme and Lower grade glioma); LGG (Lower grade glioma); BRCA (Breast cancer); LUAD (Lung adenocarcinoma); ESCA (Esophageal carcinoma); STES (Stomach and Esophageal carcinoma); COAD (Colon adenocarcinoma); COADREAD (Colon adenocarcinoma and Rectum adenocarcinoma Esophageal carcinoma); PRAD (Prostate adenocarcinoma); STAD (Stomach adenocarcinoma); HNSC (Head and Neck squamous cell carcinoma); LUSC (Lung squamous cell carcinoma); LIHC (Liver hepatocellular carcinoma); SKCM (Skin cutaneous melanoma); BLCA (Bladder urothelial carcinoma); THCA (Thyroid carcinoma); READ (Rectum adenocarcinoma); OV (Ovarian serous cystadenocarcinoma); PAAD (Pancreatic adenocarcinoma); TGCT (Testicular germ cell tumors); ALL (Acute lymphoblastic leukemia); LAML (Acute myeloid leukemia); PCPG (Pheochromocytoma and Paraganglioma); ACC (Adrenocortical carcinoma); CHOL (Cholangiocarcinoma); KIRP (Kidney renal papillary cell carcinoma); KIPAN (Pan-kidney cohort); KIRC (Kidney renal clear cell carcinoma); WT (Wilms); UCS (Uterine carcinosarcoma); and KICH (Kidney chromophobe); UCEC (Uterine corpus endometrial carcinoma); CESC (Cervical squamous cell carcinoma and endocervical adenocarcinoma).

Survival prognosis analysis

Clinical information of NOX4 patients was obtained from the TCGA database. We performed prognostic analyses based on OS, disease-specific survival (DSS), and progression-free interval (PFI). We also through the single factor Cox regression analysis and Kaplan Meier survival analysis to evaluate the survival probability of different tumor patients.

Immune infiltration analysis

The relationship between NOX4 expression and immune infiltration was analyzed by The Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) online[34]. Immune cells such as T cells, macrophages and fibroblasts were selected as reference objects. Quantitative methods such as TIDE, XCELL and

EPIC were used to assess the extent of immune infiltration. P-value and correlation (cor) results were calculated using purity corrected Spearman test. The data obtained above is presented as a heat map.

Gene enrichment analysis

We used the Gene Expression Profiling Interactive Analysis 2 (GEPIA2, http://gepia2.cancer-pku.cn/) database to obtain the 100 genes most closely related to NOX4[35]. Functional enrichment analysis of Hyper-LGs was conducted using the DAVID database with Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses performed to identify significantly enriched terms (statistical significance threshold: $P < 0.05$)[10–12,36].

Cell lines, culture, and transfection

MDA-MB-231 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (ZETA LIFE, USA) and 1% penicillin-streptomycin, and maintained at 37 °C with 5% CO_2. NOX4 siRNA was custom-ordered from GenePharma (Shanghai, China). The siRNA sequences were as follows: GCUGAAGUAUCAAA CUAAUTT, AUUAGUUUGAUACUUCAGCTT; GCCCUUCAUUCAAUCUAGATT, UCUAGAUUGAAUGA AGGGCTT; GACCUGACUAUGUCAACAUTT, AUGUUGACAUAGUCAGGUCTT; GGGACAAGAUUUG AAUACATT, UGUAUUCAAAUCUUGUCCCTT. Negative control (NC): UUCUCCGAACGUGUCACGUT T, ACGUGACACGUUCGGAGAATT. The siRNA was transfected using Lipo3000, and the efficiency of NOX4 knockdown was detected by Western blotting.

Western blotting analysis

After digesting the cells with protein lysis buffer, total proteins were extracted from MDA-MB-231 cells. The BCA assay kit (Solarbio, PC0020) was used to measure the protein concentration. Proteins were separated using 10% SDS-PAGE, with 40 μg of protein loaded into each lane. The proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane and blocked with 5% milk. The primary rabbit antibodies used were anti-NOX4 (Cat#: DF6924; 1:1,000; Affinity) and anti-β-actin (Cat#: AC050; 1:1,000; Abclonal). A horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat#: AS014; 1:5,000; Abclonal) was used as the secondary antibody. An enhanced chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.) was employed for visualization, and the ChemiScope system was used to capture the images. We used β-actin as the loading control. The intensity of the protein bands was measured using ImageJ.

Wound-healing assay

MDA-MB-231 cells transfected with NOX4 siRNA were cultured in 6-well plates (37 °C, 5% CO_2 in an incubator). When the cells reached confluence, a linear wound was created by scraping the confluent cell layer using a 200 μL pipette tip. The cells were washed twice to remove detached cells and debris, and the medium was replaced with fresh medium containing 1% FBS. The wound size was observed and measured at 0 and 24 h.

Transwell assays

The migratory ability of the cells was measured using the Transwell assay kit (Cat. No. 3422, Corning, USA). MDA-MB-231 cells transfected with NOX4 siRNA were seeded at a density of 5×10^4 cells/well in serum-free medium, while the lower chamber was filled with medium supplemented with 20% fetal bovine serum (FBS). The cells transfected with siRNA were incubated for 48 h. Subsequently, the chambers were fixed with 4% paraformaldehyde for 30 min. The cells in the upper chamber were wiped off, and the cells attached to the bottom of the membrane were fixed and stained for observation under a microscope.

CCK-8 assays

MDA-MB-231 cells transfected with NOX4 siRNA were seeded into 96-well plates at a density of 5×10^3 cells per well and incubated overnight. Subsequently, the cells were cultured for 24, 48 and 72 h. Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Solarbio) at the specified time points. Ten microliters of CCK-8 reagent were added to each well, and after 2 h of incubation, the absorbance was measured at 450 nm using a microplate reader. A total of six replicates were set for each treatment group, and all experiments were repeated three times.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (BioFlux, Beijing), and cDNA was synthesized via reverse transcription, adhering to the manufacturer's protocol. Real-time PCR was performed using the SLAN-96P system with cDNA as the template. All reactions were performed in triplicate, and β-actin served as an internal control for normalization. The $2^{-\Delta\Delta Ct}$ method was used to assess the relative gene expression. The sequences of the specific forward and reverse primers were as follows: for NOX4, 5′-TGACGTTGCATGTTTCA GGAG-3′ and 5′-AGCTGGTTCGGTTAAGACTGAT-3′; for β-actin, 5′- CACCATTGGCAATGAGCGGTT-3′ and 5′- AGGTCTTTGCGGATGTCCACGT-3'.

Statistical analysis

Statistical analyses in this study were conducted using the above online database and ''R package (R studio version: 1.2.1335, R version: 3.6.3), as described above. GraphPad Prism 8.0 was used for the statistical analysis of experimental data. Differences were compared using a Student's *t*-test, and outcomes are shown as mean ± SD. Statistical significance was reported at $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

Data availability

The data used during the present study are available from the corresponding author upon reasonable request.

Received: 20 March 2025; Accepted: 30 June 2025
Published online: 09 July 2025

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Acknowledgements
This work was supported by Anhui Postdoctoral Scientific Research Program Foundation (No.2024B732); Key Projects of the Suzhou Municipal Health Commission (No.SZWJ2023a004); Suzhou Science and Technology Bureau (SZKJXM202320).

Author contributions
H.T. contributed to the conception. J.S. drafted the manuscript, and Z.G. provided revisions. J.S. and Z.G. conducted data investigation and analysis. L.Z., Z.D., L.W., Y.T., S.D. and Y.D. revised for important intellectual content. All authors have read and approved the final version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-09690-9.

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