Targeting NOX4 Disrupts the Resistance of Papillary Thyroid Carcinoma to Chemotherapeutic Drugs and Lenvatinib

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Abstract

Background: Advanced differentiated thyroid cancer cells are subjected to extreme nutritional starvation which contributes to develop resistance to treatments; however, the underlying mechanism remains unclear.

Methods: We used 0.5% serum to mimic starvation during cell culture. A CCK8 assay, cell death Detection ELISAPLUS kit, PI staining were measured to determine cell viability, cell apoptosis and cell cycle respectively in BCPAP cells and TPC-1 cells expressing shRNA against NOX4. The cells were then treated with etoposide and doxorubicin, two chemotherapeutic drugs, as well as lenvatinib to determine the role of NOX4 in resistance. Lenvatinib-resistant BCPAP cells (LRBCs) were also established to confirm the role. Finally, GLX351322, a chemical inhibitor targeting NOX4, was used to inhibit NOX4-derived ROS and detect the the contribution of NOX4 to resistance in vitro and in vivo.

Results: NADPH oxidase 4 (NOX4) is highly expressed under serum starvation in BCPAP or TPC-1 cells. NOX4 knockdown impairs cell viability, increases cell apoptosis, extends G1 phase in cell cycle and modulates the level of energy-associated metabolites in starved cells. When these starved cells or Lenvatinib-resistant BCPAP cells (LRBCs) are treated with chemotherapeutic drugs or Lenvatinib, NOX4 knockdown inhibits cell viability and aggravates cell apoptosis depending on NOX4-derived ROS production. GLX351322, a NOX4-derived ROS inhibitor, has a significantly inhibitory effect on cell growth *in vitro* and the growth of BPCPA-derived even LRBCs-derived xenografts *in vivo*.

Conclusions: These findings highlight NOX4 and NOX4-derived ROS as a potential therapeutic target in resistance of PTC patients.

Introduction

Papillary thyroid carcinoma (PTC) is the most frequent histologic type of thyroid cancer, accounting for greater than 80% of cases [1]. The ten-year survival rate among patients with PTC that is refractory to radioiodine therapy is 10% from the time of detection of metastasis [2–4]. It has been reported that elevated levels of reactive oxygen species (ROS) as a risk factor for the development of PTC in patients with Hashimoto thyroiditis [5], and that increased aerobic glycolysis portends an unfavorable prognosis in follicular thyroid cancer [6]. Thus, it can be reflected that ROS level and glycolytic activity can be proposed as risk factors for the development of PTC.

Cells produce ROS through a variety of enzymatic systems, including NADPH oxidases, xanthine oxidases, NO synthase, cytochrome P450 reductase and the mitochondrial electron transport chain [7]. The involvement of NADPH oxidases (NOXs), which produce ROS as their primary and sole function, has become of particular interest in thyroid malignancy [8]. Among the NOXs, NOX4 was demonstrated to be upregulated in thyroid cancers such as PTC, so did its binding partner, p22phox [9, 10]. In a BRAF-mutant

PTC cell line, NOX4-dependent ROS generation was involved in $BRAF^{V600E}$-induced NIS (sodium/iodide symporter) repression via TGF-β/Smad3 signaling pathway [11]. In renal cancer, which more readily use glycolysis, NOX4 acts as a mitochondrial energetic sensor to modulate ATP levels in, providing hope for overcoming drug-resistance when combining with cytotoxic drugs [12]. Furthermore, we previously reported that NOX4 sustains cell growth in hypoxic PTC via ROS-HIF-glycolysis pathway [10]. Based on the effect of NOX4 on the pathway and drug-resistance, we wanted to address the role of NOX4 in resistance of PTC cells.

Lenvatinib has been approved by the US Food and Drug Administration (FDA) as a TKI for radioiodine-refractory differentiated thyroid cancer (RR-DTC) [13]. The approval was based on a study of 392 people with DTC that grew after they received radioactive iodine therapy. Patients in the study who received lenvatinib lived an average 18.3 months before their cancer started growing again compared to an average 3.6 months for patients who did not receive lenvatinib [14], demonstrating lenvatinib as a multitargeted tyrosine kinase inhibitor (TKI) improved median progression-free survival (PFS) in patients with thyroid carcinomas. However, lenvatinib has no significant impact on improving the overall survival rate for this disease so that almost 50% of patients with advance DTC treated with lenvatinib will develop significant disease progression after average of 18 months due to resistance mechanisms [15–17]. Importantly, lenvatinib could induce elevated ROS production [18] and resistance to lenvatinib leads to increased glycolysis [16]. Combining with the role of NOX4 in ROS and glycolysis in PTC [10], it still needs to be further investigated whether NOX4 is involved in the process of resistance to lenvatinib and how.

Methods

Cells and Cell Counting Kit-8

The human papillary thyroid carcinoma cell lines BCPAP was cultured in RPMI 1640 medium (Thermo fisher, 11875168) supplemented with 10% fetal bovine serum (FBS) (Thermo fisher,10437-028) and antibiotics in a 37°C incubator with 5% CO_2. For serum starvation, cells were cultured medium supplemented with 0.5% FBS.

Cell viability

Cell viability was determined by Cell Counting-Kit 8 assay (MCE), which was performed in 96-well plates and the absorbance was measured at 450 nm in a microplate reader (Thermo Varioskan Flash) after 2 hours of incubation. The assays were performed with N = 4 and repeated 3 times.

Cell apoptosis

Cell apoptosis was measured using the Cell Death Detection ELISAPLUS kit (MERCK, CAT #11774425001) following the manufacturers protocol.

Cell cycle

The cells (1×10^6) were fixed with 70% ethanol for 1 h at room temperature (25°C). The samples were then centrifuged at 1000g for 5 min. The 70% ethanol was removed, and the cells were then treated with 100 μL of RNase A (0.5 mg/ml) for 30 min at 37°C. Cell samples were then stained with 20 μg/ml of PI and analyzed with a BD FACSCalibur flow cytometer to obtain DNA content profiles. FlowJo 10.0 software was used for analysis of the cell cycle.

Glucose and Lactate quantification

Glucose and lactate in supernatants of LRBCs expressing shNOX4 or shCtrl under 10% or 0.5% FBS were measured turbidimetrically on a Cobas8000 (Roche). Briefly, cells were divided into 4 groups: shCtrl cultured in 10% FBS, shCtrl cultured in 0.5% FBS, shNOX4 cultured in 10% FBS and shNOX4 cultured in 0.5% FBS. Each group had 5 biological replicates. 500000 cells were seeded into each well of 6-well plates, and cultured in RPMI 1640 medium that contains 10 μM lenvatinib, 2000mg/L D-glucose, and none of lactate. After 48 hours, these supernatants were respectively collected and analyzed quantitatively by instrument.

Plasmid and Lentivirus Production

Plasmids (pLKO.1) encoding for scrambled shRNA and human NOX4 shRNA were purchased from MISSION shRNA (Sigma-Aldrich). The shRNA targeting sequence was 5'- GCTGT ATATT GATGG TCCTT T-3'. Lentivirus was packaged in 293T cells using psPAX2 and VsvG, followed by viral transduction to BCPAP cells or TPC-1 cells with 5 μg/μl polybrene. Twenty-four hours post infection, single cells were selected and passed into 96-well plates in puromycin (1000 ng/ml) antibiotic for selection and maintained in puromycin (500 ng/ml).

Targeted metabolomics

BCPAP cells stably expressing shCtrl and shNOX4 in triplicate under 10%- or 0.5% serum condition for 48 hours were mixed with cold methanol/acetonitrile/H_2O (2:2:1, v/v/v) to remove the protein. Then all samples were sent to ShangHai Applied Protein Technology for further sample preparation and mass spectrometry analysis.

Xenografts

All animal experiments were approved by the Ethics Committee of the Third Hospital of Mianyang and the Ethics Committee for Animal Research BEIJING VIEWSOLID BIOTECH CO.LTD. Mouse xenografts models were established by subcutaneous injection into the flanks of NOD/SCID mice (6 to 9 weeks-old females, Charles River) with 5×10^6 BCPAP cells or LRBCs. When tumors grew to about 200~400 mm^3 after 10 days, the mice were randomly separated into four groups, one for water (untreated), one for GLX351322 (5mg/kg, Selleck, CAT #S0178), one for lenvatinib (30mg/kg, Selleck, CAT #S1164), and one for the combination of GLX351322 and Lenvatinib. All drugs were administered orally once per three days. There were at least 5 mice in each group. Tumor size was measured every 3 days, and the tumor

volume was calculated as Volume= Length x Width x Width x 0.52. To evaluate the survival rate, the number of mice in each group was increased up to no less than 15. Then these mice were monitored for 70 days.

Western blotting

The cells that stably expressing shNOX4 or shCtrl were harvested when the cell number was almost 1 million cells per well in 6-well plate by microscopical evaluation. Then the cells were lysed in RIPA buffer (MERCK, CAT #R0278) supplemented with the addition of protease and phosphatase inhibitors (Beyotime, CAT #P1051). After brief sonication, the mixture was pelleted at 12000g for 10 minutes at 4°C and the protein supernatant was transferred to another tube. Proteins from whole-cell lysates were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to PVDF membranes. Anti-NOX4 (Novus, CAT #NB110-58851) and anti-β-actin (Novus, CAT #NB600-501) antibodies were used at 1:1000 and secondary antibodies (Beyotime, CAT #A0208) conjugated with horseradish peroxidase (HRP) were used at 1:1000 dilutions in 5% non-fat dry milk. After the final washing, PVDF membranes were exposed for an enhanced chemiluminescence assay using the Gel imaging instrument (Shang Hai CLiNX, China).

ECAR

Extracellular acidification rate (ECAR) was measured using a Seahorse XF24 analyzer (Seahorse Bioscience). Briefly, ECAR was determined using the Glycolysis Stress kit (Seahorse Biosciences) according to the manufacturer's standard protocol. All assays were performed with N = 3 or more per condition and repeated 3 times. Injections were performed as follows: glucose (20 mM), oligomycin (4.5 μM), 2-DG (100 mM), GOD (100 μg/mL, MERCK, CAT #G7141), NAC (10mM, MERCK, CAT #A7250). Results were analyzed using Seahorse Wave vesion 2.4.

Database

Correlation between the transcriptive expression levels of LDHA, HIF1α, BCL2, Glut1, Ki67 and NOX4 was analyzed using data from TCGA by GEPIA (http://gepia.cancer-pku.cn/).

Statistical analysis

Student's t-test was used in all cellular experiments and the results from three independent experiments are presented as mean ± SD. All statistical analyses were performed using the GraphPad Prism software and means were compared by Welch's t test or unpaired t test. In order to evaluate whether the decrease in NOX4 show a higher effect under starvation conditions, we performed a two-way analysis of variance. For survival analyzes, log rank test was used for comparison. Asterisks denote statistical significance as follows: NS, no significance; $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

Results

NOX4 is required for cell survival of starved PTC cells.

Previously we reported that NOX4 as a sensitive protein plays an important role in hypoxic PTC cells [10]. Since serum starvation is also a common feature for solid tumors as similar as hypoxia [19], we then wonder whether NOX4 is responded to serum starvation. The results of immunoblot demonstrated that once the serum concentration in medium was decreased from 10–2.5% or 0.5%, NOX4 expressions in BCPAP cells (harboring BRAF mutant) and TPC-1 cells (harboring RET/PTC1 rearrangement) were significantly increased in a varying degree (Figure 1A), suggesting NOX4 is also sensitive to starvation in PTC cells.

Serum starvation is a widely used condition in cell biology to modulate cell viability and apoptosis [20, 21]. Thus, we investigated the role of NOX4 in cell viability and apoptosis under serum-starved condition. First, BCPAP and TPC-1 cells were infected with lentivirus expressing shRNA against NOX4 (shNOX4) or control shRNA (shCtrl) (Figure S1A). Then a CCK8 assay showed that NOX4 knockdown significantly repress cell viability in starved PTC cells (Figure 1B). Subsequently, cell death assays detected by Cell Death Detection ELISAPLUS kit showed significant increments of the apoptotic levels in NOX4-deficient PTC cells compared with the control cells under serum starvation (Figure 1C). In addition, cell cycle was examined by flow cytometry showing that NOX4 knockdown induced a significantly extended G1 phase in starved PTC cells (Figure 1D). Consistently, the increase of apoptotic sub G1 fraction confirmed our previous findings in cell apoptosis (Figure 1C). Altogether, these results underscore the importance of NOX4 in determining cell survival in starved PTC cells.

NOX4 deficiency modulates the levels of energy-associated metabolites of BCPAP cells.

To get metabolic insights in the role of NOX4 in starved PTC cells, the targeted metabolomics analyses involved in energy metabolism by mass spectrometry were performed upon serum-starvation or not in BCPAP cells. Principal component analysis (PCA) of targeted metabolomic analysis demonstrated a distinct separation between shCtrl and shNOX4 cells under 10% serum and 0.5% serum conditions (Figure S2A), which indicates the critical role of NOX4 in the levels of these metabolites. Under either 10%- or 0.5%- serum condition, the level of each metabolite was inconsistently altered by NOX4 deficiency (Figure S2B). In detail, the lactate level was reduced significantly in NOX4-dificient BCPAP cells under serum-starved condition (Figure S2C), which gives us a clue that NOX4 may involve in glycolysis of starved PTC cells. Furthermore, the ration of ADP/ATP and AMP/ATP, were significantly increased in NOX4-deficient cells under either 10%- or 0.5%- serum condition (Figure S2D), showing that NOX4 contributes to the energy demand of PTC cells. Besides, the ratio of NAD+/NADH was also upregulated by NOX4 deficiency under 0.5% serum condition as well (Figure S2E), reflecting a potentially relevant role of NOX4 in redox regulation. In all, these findings indicate a dispensable role of NOX4 in the maintenance of bioenergetic and redox metabolites in starved PTC cells.

NOX4 depends on ROS to oppose chemotherapeutic drugs-induced apoptosis.

Given that NOX4 functions as an energetic sensor coupling cancer metabolic reprogramming to drug resistance [12], and that serum starvation can render drug resistance of cancer cells [22], we then test the hypothesis that NOX4 contributes to the acquisition of drug resistance in serum-starved PTC cells. After BCPAP cells or TPC-1 cells stably expressing shRNA against NOX4 or control vector were treated with chemotherapeutic drugs (etoposide and doxorubicin (DOX)), we found that the cells exposed to etoposide or DOX reveal a normal enhancement of cell apoptosis compared to the control (buffer alone), whereas cells cultured with 0.5% serum showed a modest increase of the drugs-induced cell death (Figure 2A). Importantly, there was a significant increment of apoptosis in cells harboring NOX4 dificiency compared with previously drugs or serum-starvation-induced apoptosis (Figure 2A), which supports an inhibited role of NOX4 in the chemotherapeutic drugs-elicited cell death in either starved or normal PTC cells.

ROS generated by NOX4 was indispensable for metabolism and secretion of PTC cells [10, 23], we then asked whether the effect of NOX4 on drug-induced cell death is mediated by ROS. BCPAP and TPC-1 cells were treated with NAC (a ROS scavenger) and etoposide respectively or combinedly in completed medium. The results showed that NAC can not elicit additional apoptosis but does enhance etoposide-induced apoptosis (Figure 2B), indicating a protective role of ROS in response to etoposide. In starved PTC cells, the results showed that NAC combining with etoposide under serum-starved condition leads to more rates of apoptosis than etoposide-treated alone, almost equaling to that caused by NOX4 knockdown plus DMSO, whereas NOX4 knockdown plus NAC is disabled to aggravate etoposide-induced apoptosis compared with that caused by shCtrl plus NAC (Figure 2C). This indicates that NAC alone in the presence of etoposide increases apoptosis in starved cells, and that NOX4 knockdown does not change NAC effect. It can be further concluded that cell survival under starvation and etoposide treatment depends on ROS. Altogether these results suggest that the survival of PTC cells treated with etoposide depends on intracellular ROS specifically derived from NOX4.

Furthermore, these findings can be confirmed reversely by glucose oxidase (GOD) (a stable enzyme that oxidizes glucose into glucolactone converting oxygen into ROS) addition, showing a significantly inhibited apoptosis when comparing GOD with DMSO upon NOX4 knockdown (Figure 2C). Taken together, the data supports the critical roles of NOX4 and NOX4-derived ROS in the regulation of drug-induced apoptosis of starved PTC cells.

NOX4 is required for PTC cell survival in response to lenvatinib depending on NOX4-derived ROS

Next, we sought to use lenvatinib, a multi-targeted anticancer agent approved by The US Food and Drug Administration (FDA) for differentiated thyroid cancer that got worse even after they received radioactive iodine therapy [13], to investigate the influence of NOX4 in the process. A CCK8 assay demonstrated that lenvatinib does obviously decrease cell viability under both serum-full and serum-starved conditions, but NOX4 knockdown is capable of significantly reducing the cell viability caused by lenvatinib in starved PTC cells (Figure 3A). Correspondingly, in vitro studies on apoptosis showed that the lenvatinib-treated cells expressing shRNA against NOX4 in starved PTC cells has a higher apoptotic level than the cells expressing shCtrl (Figure 3B). Interestingly, it seems that the level of cell death induced by lenvatinib with

10% serum does not depend on NOX4 expression, different from the effects of etoposide and doxorubicin. This may be due to the fact that lenvatinib at 10% serum triggers apoptotic signal pathways different from chemotherapeutic drugs and independent of NOX4 expression.

Likewise, we used NAC and GOD to determine whether the role of NOX4 in Lenvatinib-induced cell death in starved PTC cells is mediated by NOX4-derived ROS. The results revealed that neither NAC nor GOD shows a significance between NOX4 knockdown and its control, whereas NOX4 knockdown did increase significant induction of apoptosis without NAC or GOD (DMSO) (Figure 3C). The data confirmed that NOX4 depends on ROS to regulate lenvatinib-induced apoptosis in starved PTC cells.

The survival of LRBCs requires NOX4 and NOX4-derived ROS.

We afterward characterized lenvatinib-resistant BCPAP cells (LRBCs) that had been exposed to doses of lenvatinib increased by 0.125 μM per week and until reaching a final concentration of 32 μM. IC50 of parent cells treated with lenvatinib in 72h was 2.91 μM, while those of LRBCs were 10.74 μM (Figure S3A), suggesting that LRBCs successfully acquired lenvatinib resistance. To determine the role of NOX4 in LRBCs, we still knocked down NOX4 expression by lentivirus-mediated shRNA. Immunoblots showed that the level of NOX4 protein is overexpressed in LRBCs compared with parent cells, but the level was dropped down when using shRNA agaist NOX4 (Figure S3B). The CCK8 and apoptosis assay together showed that nolenvatinb NOX4 knockdown significantly inhibits LRBCs survival under lenvatinib treatment independent of starvation in PTC cells (Figure 4A and 4B). Of note, starvation was helpful to break the resistance of LRBCs to lenvatinib, but NOX4 deficiency further aggravates the break of resistance.

Resistance to lenvatinib increases glycolysis [16], thus we want to assess the effect of NOX4 on glycolysis in LRBCs. The measurements of the extracellular acidification rate (ECAR) showed that LRBCs have higher glycolytic capacity than the parent cells, but the increment can be dropped close to the level of the parent cells by NOX4 knockdown (Figure 4C). Since the start and end port during glycolysis are represented by glucose uptake and lactate secretion, we investigated the influence of NOX4 on glucose uptake and lactate secretion in LRBCs. As expected, serum starvation decreased glucose levels and increased lactate levels in lenvatinib-treated LRBCs, whereas these differences were disappeared in NOX4-dificient LBRCs cells (Figure 4D), supporting an indispensable role of NOX4 in the start and end port of glycolysis under serum-starved condition in lenvatinib-resistant PTC cells.

Next, we performed modified assay to identify whether ROS mediates the role of NOX4 in the glycolysis of LRBCs by sequential injection with GOD and NAC post glucose. The results demonstrated that NOX4 knockdown leads to the downregulation of the basal glycolysis until the injection of GOD that rescues the effect of NOX4 deficiency, and both increments of ECAR were blocked by the addition of NAC (Figure 4E). Collectively, these findings suggest that NOX4 is required for the survival of lenvatinib-resistant PTC cells in which NOX4-derived ROS have a critical role in glycolytic activity.

GLX351322 leads to decreased cell survival of PTC cells and LRBCs.

Since NOX4-derived ROS is vital for cell survival in starved PTC cells, we next used a chemical inhibitor targeting NOX4, GLX351322 [24], to inhibit NOX4-derived ROS. The results showed that GLX351322 significantly reduced cell viability in starved BCPAP and TPC-1 cells no matter lenvatinib was added or not (Figure 5A), which suggests that the mechanism of the inhibition of GLX351322 and lenvatinib to cell survival of starved PTC cells is not consistent, and the combination of both drugs is expected to have a superimposable antitumor effect in starved PTC tumors in vivo. This effect was also confirmed by the detection of apoptosis showing that GLX351322 enlarged lenvatinib-induced apoptosis in starved PTC cells (Figure 5B).

In LRBCs, GLX351322 significantly decreased the survival rates at a serum concentration of 10% in the presence of lenvatinib; GLX351322 could also significantly inhibit cell survival at a serum concentration of 0.5% independent of the presence of lenvatinib, but the combinatory usage of lenvatinib and GLX351322 is capable of maximizing the inhibitory effect (Figure 5C). A similar trend was confirmed in the detection of apoptosis in LRBCs as well (Figure 5D). These findings suggests that GLX351322 can effectively break the resistance of PTC cells to lenvatinib, and this effect is independent of serum concentration.

Combination of GLX351322 and lenvatinib completely suppresses PTC tumor growth even in LRBCs.

We further inoculated BCPAP cells or LRBCs into the dorsal tissue of NOD/SCID mice for 10 days. Thereafter, we used lenvatinib to target PTC tumors, and combined GLX351322 to break the resistance of PTC tumors to lenvatinib (Figure 6A). Lenvatinib or GLX351322 in these mice partially affected the progression of BCPAP-derived tumors, the combined usage of both drugs significantly reduced tumor volumes at each measurement time point from day 14, eventually eliminating the tumors *in vivo* (Figure 6B and 6C). Analogously, we obtained the same conclusion that the combination of the two drugs completely suppresses xenograft growth when using LRBCs-derived tumors as a model system,but the difference was that lenvatinib has no significantly inhibitory effect on LRBCs-derived tumors (Figure 6D and 6E). A Kaplan–Meier survival curve and log-rank test also showed the significantly better survival of the mice treated with the combined drug than that of single drug-treated mice (Figure 6F and 6G). Together, these findings signify the importance of NOX4 and the clinical significance of the combination against resistance to lenvatinib in PTC tumors. Of note, we also found that NOX4 expression positively correlated with resistant marker HIF1α, glycolytic markers Glut1 and LDHA, and proliferative marker Ki67, and negatively correlated with apoptotic marker BCL2 in tumor tissues in patients with PTC (Figure S4A). Taken together, these data demonstrate that GLX351322 exerts a suppressive effect on PTC tumor growth, and combination of GLX351322 and lenvatinib can completely suppress PTC tumor growth even in LRBCs.

Discussion

In this study, we have shown that NOX4 is highly expressed upon serum-starvation in PTC cells. NOX4 contributes to cell viability, cell apoptosis, cell cycle and energy-associated metabolites in starved PTC

cells. NOX4 is required for chemotherapeutic drugs-induced or lenvatinib-induced cell survival via NOX4-derived ROS. Either knockdown of NOX4 or usage of GLX351322 is capable of suppressing the progression of PTC tumors even of lenvatinib-resistant PTC tumors in vitro or in vivo.

TPC-1 and BCPAP cells are BRAF non-mutated and mutated cell lines respectively, but in the present study we did not evaluate the impact of BRAF mutation on NOX4 experimentally. Indeed, NOX4 expression levels are significantly increased in PTCs with a BRAF mutation in TCGA data [25]. Further study showed that NOX4 upregulation is controlled at the transcriptional level by the $BRAF^{V600E}$ oncogenic protein via the TGF-β/Smad3 signaling pathway dependent of MEK-ERK and that knockdown of NOX4 downregulates $BRAF^{V600E}$ -induced NIS repression [11]. A successful treatment for thyroid cancer is strictly correlated with an active Sodium Iodide Symporter (SLC5A5; NIS), which allows the retention of radioiodine in the tumor cells [26]. Based on these reports, it can be concluded that NOX4 expression is closely linked to BRAF mutant as shown exactly as $BRAF^{V600E}$-MEK-TGF-β/Smad3-NOX4-ROS-SLC5A5 (NIS) axis. Consequently, NOX4 is involved in the absence of radioiodine uptake by $BRAF^{V600E}$ PTC cells. While in our present study, the effect of NOX4 knockdown on cell viability, cell apoptosis, and cell cycle is likely to be independent of BRAF mutant, because all results of NOX4 knockdown in both cell lines harboring BRAF mutant or not presents similar tendency. In other words, BRAF mutant may not be involved the signaling of NOX4 in drug resistance of PTC cells.

Tumor cells including PTC cells face challenging physiological conditions, including hypoxia, low nutrient availability, and exposure to therapeutic drugs throughout their progression from tumor precursors to metastases [27]. They have to adapt to nutrient-deprived conditions for survival. Serum starvation resembles the growth factor deprivation characteristic of the poorly vascularized tumor microenvironment [28, 29]. This condition induces cell cycle synchronization arresting at the G1 phase [30] and increase of apoptosis [31]. Our results in this study are in line with these previous studies, further provide novel insights of the mechanisms underlying NOX4-driven adaptations to serum starvation. It has been also reported that serum starvation increases stemness of cancer cells [30], thus it can be concluded that NOX4 may involve multiple alterations for adaptation to serum starvation, not even limited to this stress. Further investigation would be required to expand our understanding of NOX4-responsive stresses among various tumor microenvironments and elucidate the detail mechanisms of adapting to these stresses.

Lenvatinib can suppress DTC albeit existence of adverse events [32], but tumor cells developed resistant mechanism involved alternative pathways for survival [33]. Metabolically, lenvatinib resistance leads to increased glycolysis [16], so overcoming this increase would be a potential strategy to solve the resistance. Previously we reported that NOX4 serves as a glycolytic regulator via ROS in hypoxia [10], the present study demonstrated not only the supportive effect of NOX4 on lenvatinib-induced glycolysis under serum-starved conditions via ROS in PTC cells *in vitro*, but also the surprising effect upon combinatory treatment of GLX351322 and lenvatinib *in vivo*, suggesting that PTC cells depend on NOX4 or NOX4-derived ROS to establish plasticity that is capable of responding to complex and changeable tumor microenvironments. So far, the understanding of NOX4 in tumor microenvironments remains

fragmentary, but it can be inferred that NOX4 is required for microenvironments-stressed PTC cells that needs ROS and glycolysis to serve as the major bioenergetic pathway. In future it is a challenge to develop new drugs associated with NOXs-derived ROS or drug combinations with less adverse effect and improve antitumor activity based on these findings.

Conclusions

In summary, these findings show that NADPH oxidase 4 (NOX4) is required for chemotherapeutic drugs- or lenvatinib-induced cell survival supporting a critical role of NOX4 and NOX4-derived ROS in the regulation of drug-induced apoptosis of papillary thyroid carcinoma (PTC) cells especially under serum-starved conditions. Moreover, we proposed a potential strategy to suppress the progression of PTC tumors including lenvatinib-resistant PTC tumors by knockdown of NOX4 or inhibition of NOX4 by GLX351322 chemical inhibitor.

Declarations

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Author contributions

H.D. and J.S. conceived the initial concept and designed the study. P.T. and T.X. designed and performed the experiments and wrote the manuscript. P.T. generated and analyzed the bioinformatic data. X.P., R.Z., T.X. and J.H. assisted with experiments.

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Availability of data and materials

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Ethics approval and consent to participate

We confirm that all methods were carried out in accordance with relevant guidelines and regulations of the Municipal Committee for health and family planning of Mianyang, China. We confirm that all

experimental protocols in this study were approved by the Third Hospital of Mianyang (Sichuan mental health center), China.

Consent for publication

All authors consent for the publication of this study.

Competing interests

The authors declare that they have no competing fnancial interests.

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Figures

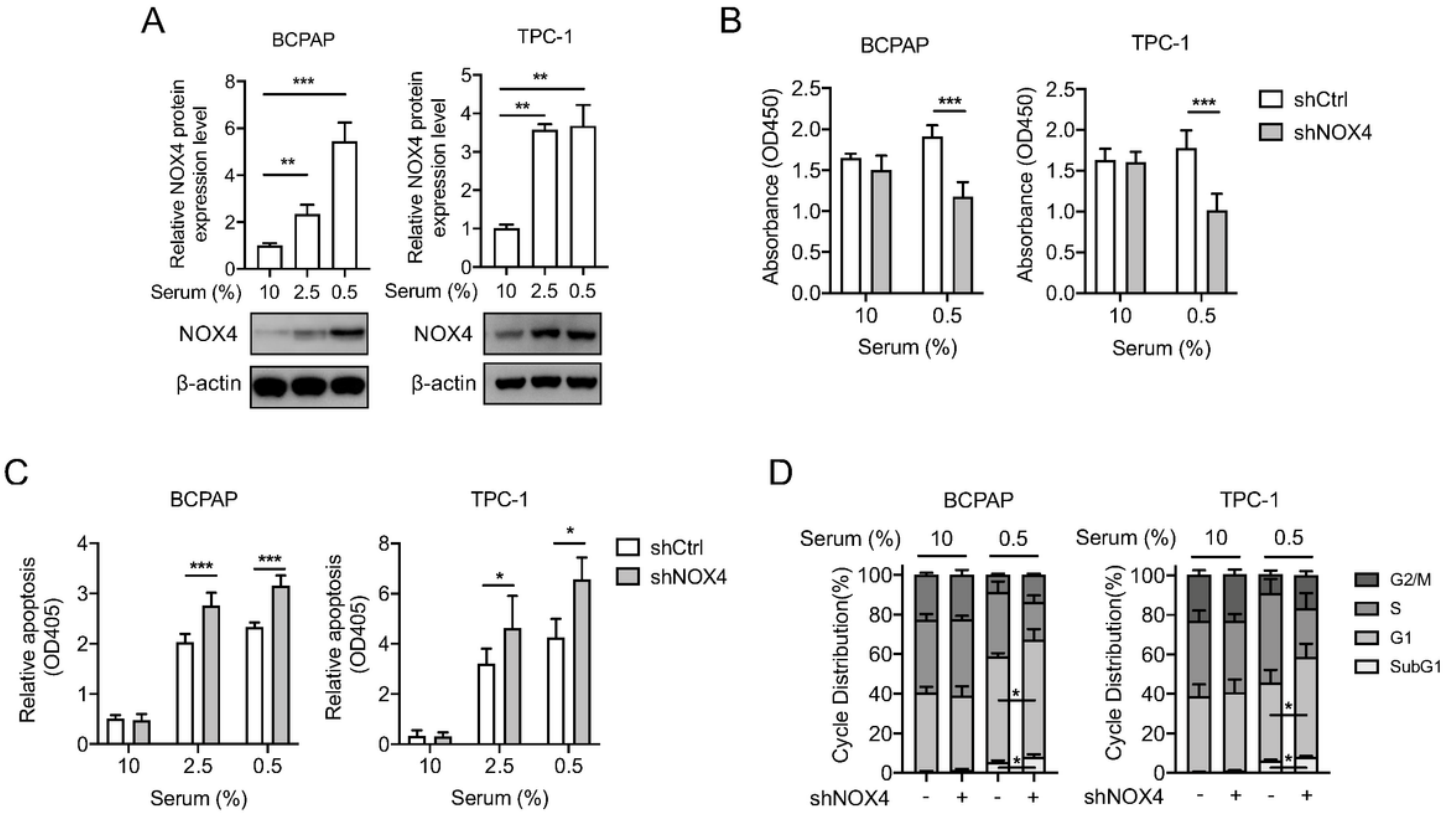

Figure 1

The role of NOX4 in cell biology in starved PTC cells. (A) Quantification of NOX4 protein by immunoblot in BCPAP and TPC-1 cells, which were cultured in 1640 and DMEM medium respectively supplemented with 10%, 2.5%, or 0.5% FBS for 48 h. n=3. (B) Cell viability was assessed in the indicated PTC cells expressing shRNA against NOX4 or not, which were cultured under 10% or 0.5% FBS for 48 h by CCK-8 assay. n=5. (C) Cell apoptosis was assessed in the indicated cells expressing shRNA against NOX4 or not, which were cultured under 10%, 2.5% or 0.5% FBS for 48 h by the Cell Death Detection ELISAPLUS kit. n=3. (D) Distribution of different phases during cell cycle in the indicated cells expressing shRNA against NOX4 or not under 10% FBS or 0.5% FBS condition for 48 hours (subG1, events with a <2N DNA content). n=3. Values are expressed as the means ± SD of biological replicates. *P<0.05, **P<0.01, ***P<0.001.

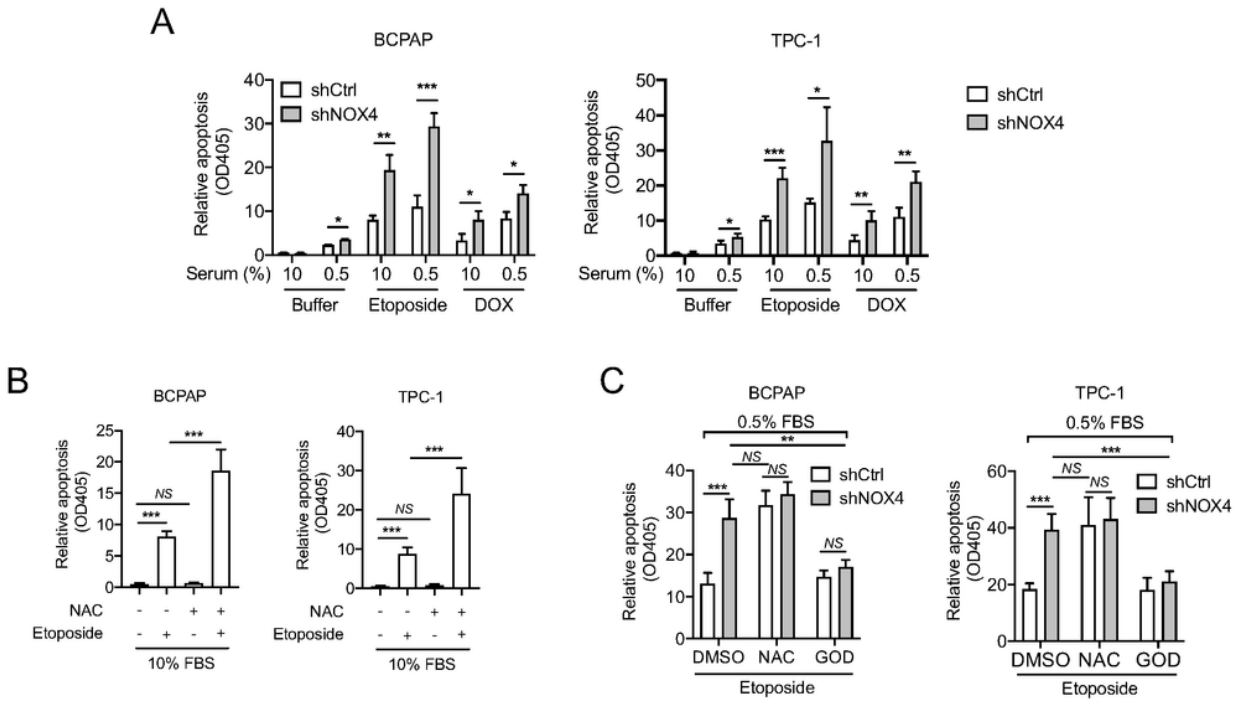

Figure 2

NOX4 attenuates apoptosis of starved PTC cells in response to chemotherapeutic drugs through NOX4-derived ROS. (A) Cell apoptosis was assessed by the Cell Death Detection ELISAPLUS kit. BCPAP or TPC-1 cells exposed to etoposide (20 μM) or doxorubicin (1 μg/ml) under 10% or 0.5% serum condition. n=3. (B) Cell apoptosis was assessed in the indicated PTC cells cultured under 10% FBS for 48 h and treated with the combination of NAC and Etoposide or each alone. n=3. (C) Cell apoptosis was assessed in the indicated PTC cells exposed to DMSO or NAC (10 mM) or GOD (100 μg/mL) at a serum concentration of 0.5%. n=3. Values are expressed as the means ± SD of biological replicates. *P<0.05, **P<0.01, ***P<0.001.

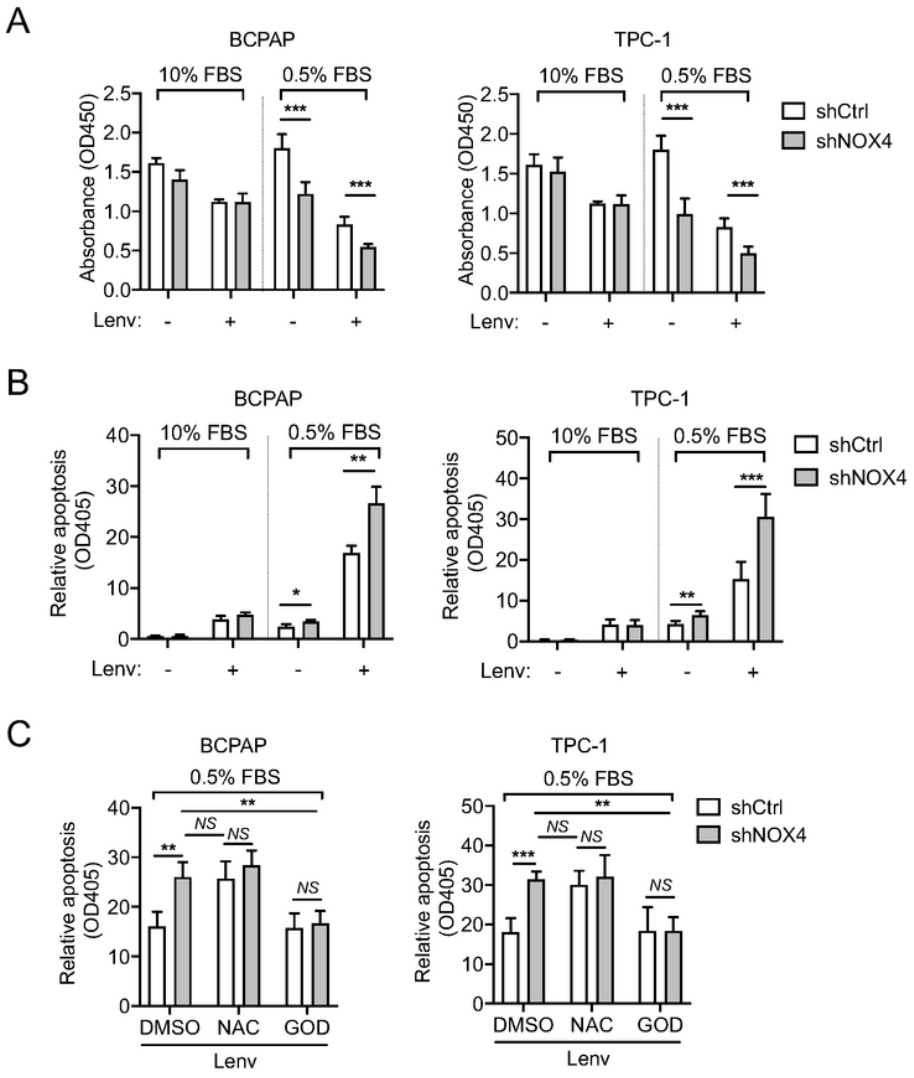

Figure 3

NOX4 and NOX4-derived ROS is required for survival of starved PTC cells treated with lenvatinib (A) Cell viability was assessed in the indicated PTC cells cultured under 10% or 0.5% FBS for 48 h and treated with DMSO or lenvatinib (3 μM) by CCK-8 assay. n=3. (B) Cell apoptosis was assessed in the indicated PTC cells exposed to DMSO or lenvatinib (3 μM) at a serum concentration of 10% or 0.5%. n=3. (C) Cell apoptosis was assessed in the indicated PTC cells exposed to DMSO or NAC (10 mM) or GOD (100 μg/mL) at a concentration of 3 μM Lenvatinib under 0.5% FBS condition. n=3. Values are expressed as the means ± SD of biological replicates. *P<0.05, **P<0.01, ***P<0.001. NS, no significance.

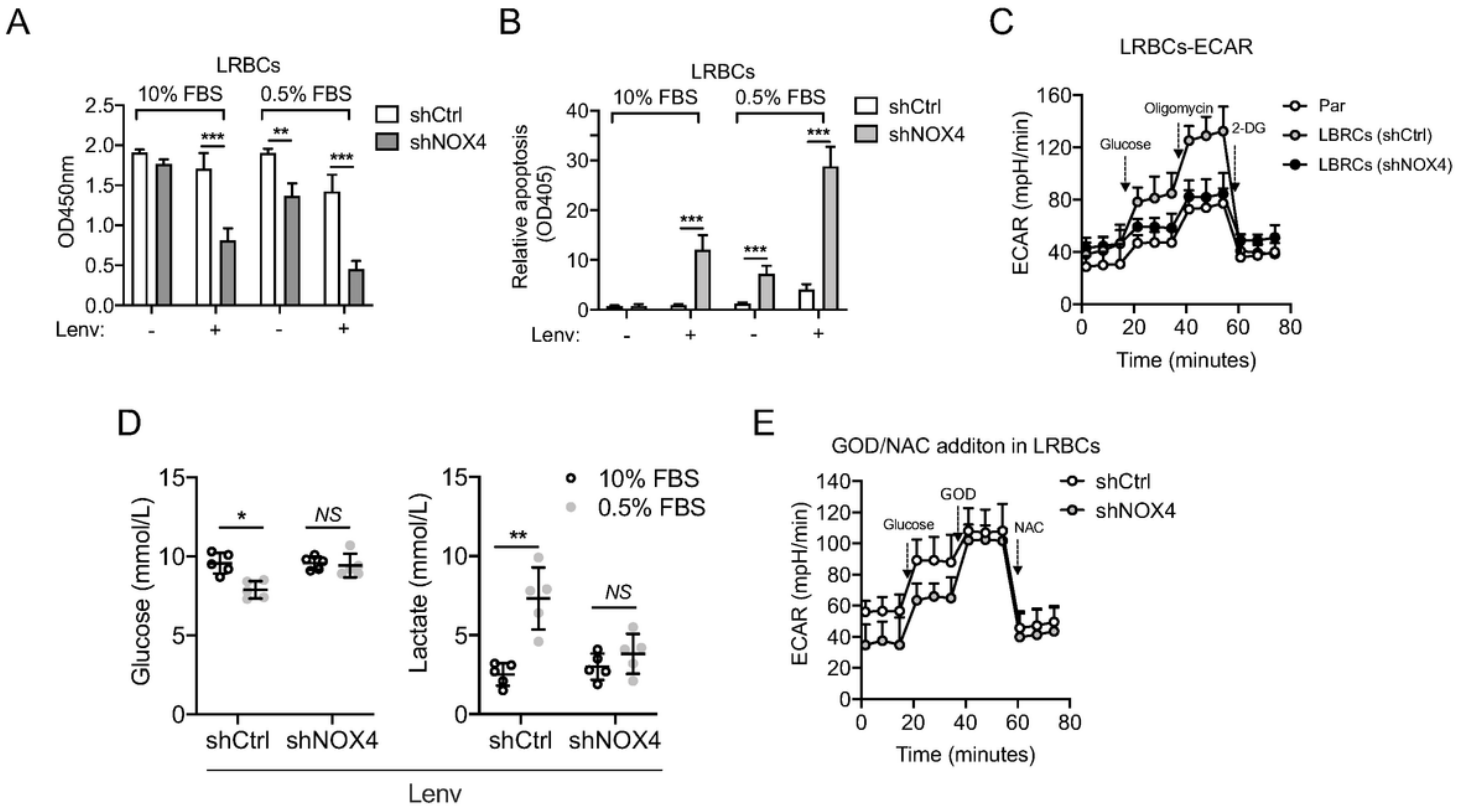

Figure 4

NOX4 deficiency weakens the resistance of LRBCs to Lenvatinib through NOX4-derived ROS. (A) Cell viability was assessed by CCK8 assay in LRBCs cultured under 10% or 0.5% FBS for 48 h and treated with DMSO or lenvatinib (10 μM). n=3. (B) Cell apoptosis was assessed in LRBCs cultured under 10% or 0.5% FBS for 48 h and treated with DMSO or lenvatinib (10 μM). n=3. (C) The ECAR analysis in the parental LRBCs (BCPAP cells) or LRBCs expressing shRNA against NOX4 or not. (n=3). (D) Supernatants of LRBCs expressing shRNA against NOX4 or not under 10% FBS or 0.5% FBS at a concentration of 10 μM Lenvatinib for 48 h were analyzed for glucose and lactate concentrations. (E) The modified ECAR analysis in LRBCs expressing shRNA against NOX4 or not. Values are expressed as the means ± SD of biological replicates. *P<0.05, **P<0.01, ***P<0.001. NS, no significance.

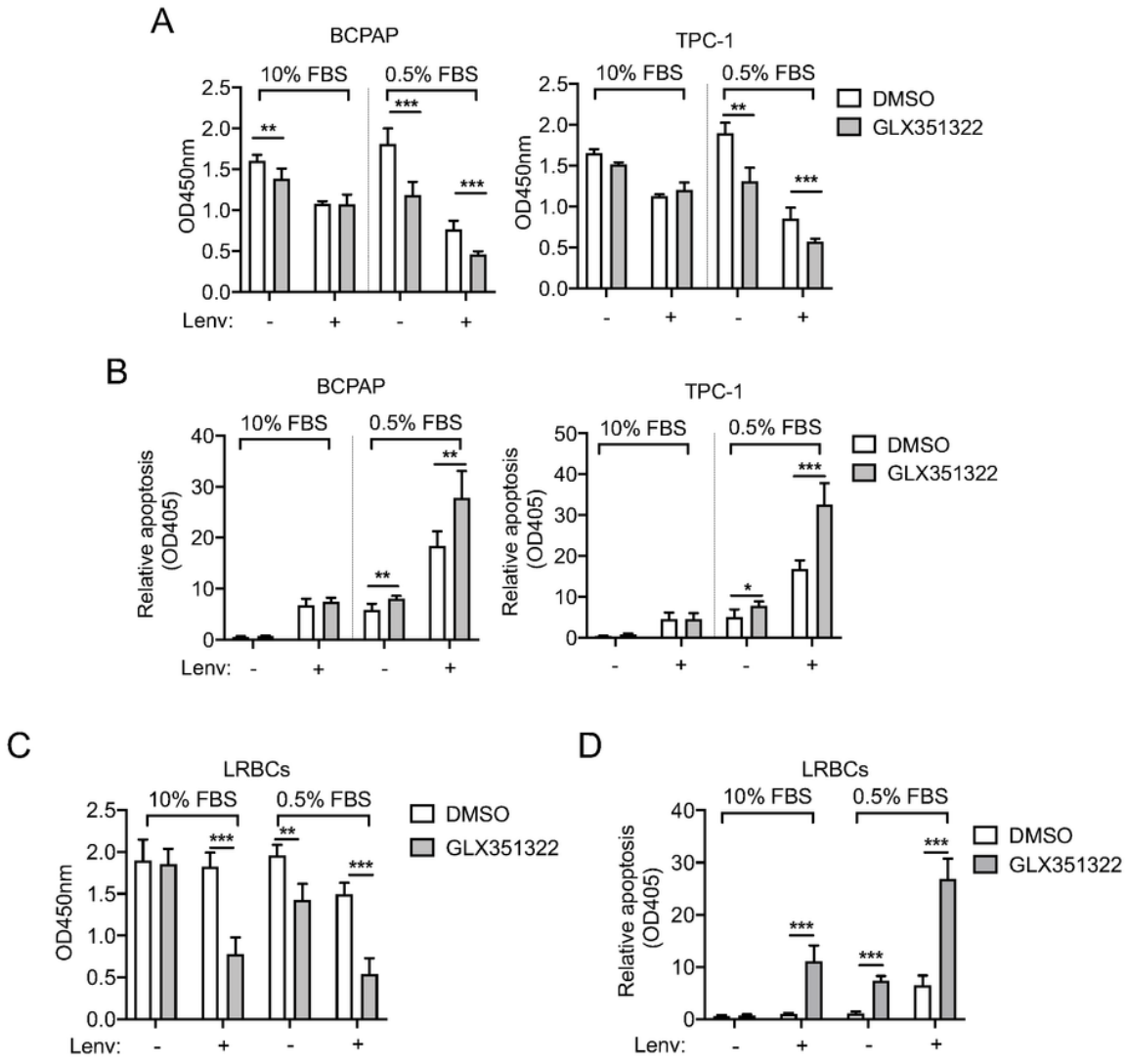

Figure 5

GLX351322 enhance cell death of PTC cells and LRBCs treated with lenvatinib. (A) Cell viability was assessed in the indicated PTC cells cultured with medium containing 10% or 0.5% FBS, and DMSO or GLX351322 (5 μM) for 48 h by CCK-8 assay. n=3. (B) Cell apoptosis was assessed by the Cell Death Detection ELISAPLUS kit using the indicated PTC cells cultured in medium containing 10% or 0.5% FBS, and DMSO or GLX351322 (5 μM) for 48 h. n=3. (C) Cell viability was assessed in LRBCs cultured with medium containing 10% or 0.5% FBS, and DMSO or GLX351322 (5 μM) for 48 h by CCK-8 assay. n=3. (D) Cell apoptosis was assessed using LRBCs cultured in medium containing 10% or 0.5% FBS, and DMSO or GLX351322 (5 μM) for 48 h. n=3. Values are expressed as the means ± SD of biological replicates. *P<0.05, **P<0.01, ***P<0.001. NS, no significance.

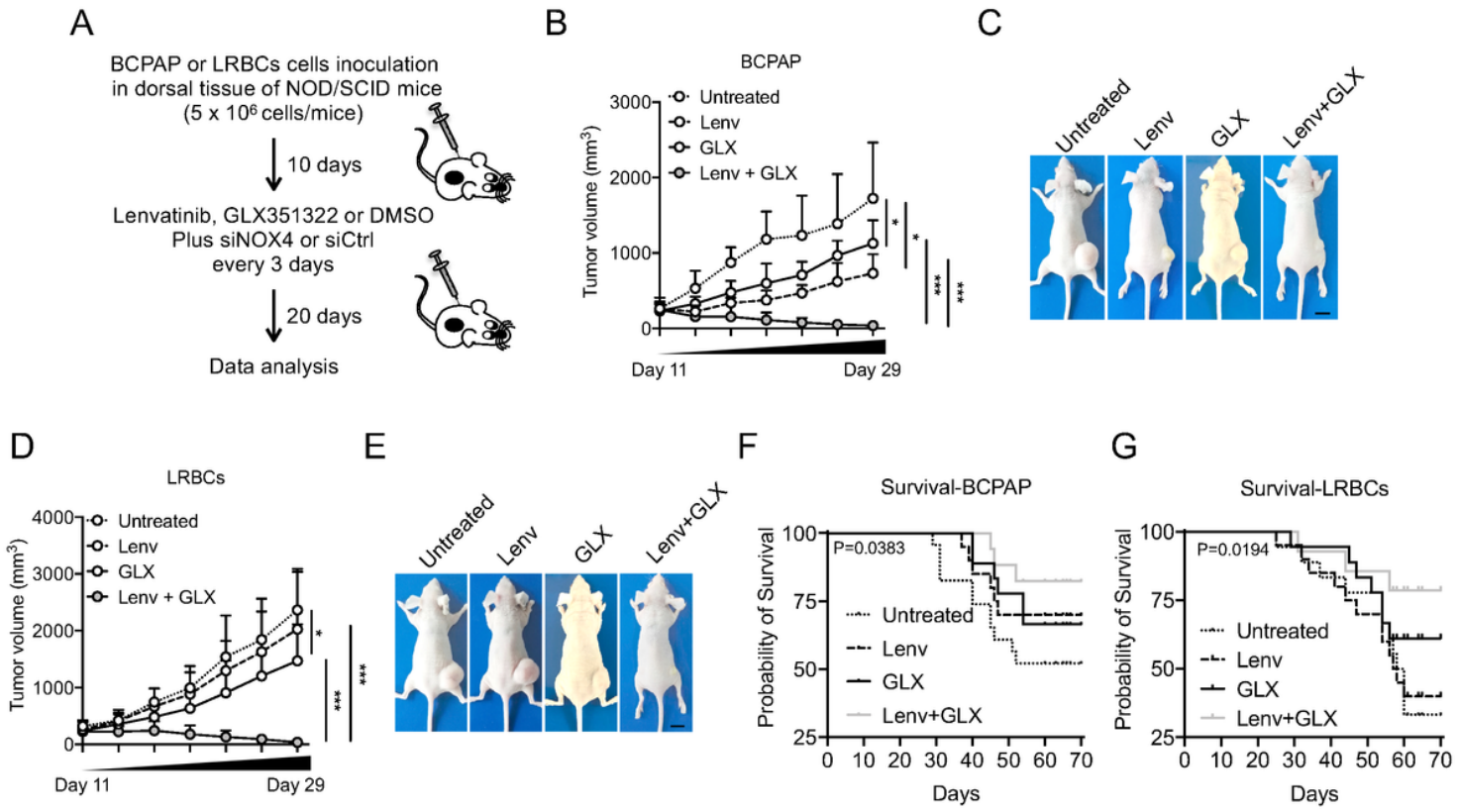

Figure 6

Combining GLX351322 with Lenvatinib completely suppresses PTC tumor growth. (A) Schematic diagram of inoculation and therapy in a mouse xenograft model. (B-E) Growth curve or representative gross morphology of xenografts on mice bearing BCPAP cells (B and C) or LRBCs (D and E) in dorsal tissues for 10 days were left untreated or were treated with lenvatinib (30mg/kg), GLX351322 (5mg/kg), or lenvatinib plus GLX352322 as described. n=5 to 7. (F-G) Kaplan-Meier survival curve showing significant improvement in survival of the mice treated with the combined drug as compared with single drug-treated mice for 70 days after inoculation. Values are expressed as the means ± SD of biological replicates. *P<0.05, **P<0.01, ***P<0.001. NS, no significance.

Supplementary Files

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- SuppFigsNOX420210915.pdf