

Nasopharyngeal carcinoma with non-squamous phenotype may be a variant of nasopharyngeal squamous cell carcinoma after inhibition of EGFR/PI3K/AKT/mTOR pathway

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Summary. Nasopharyngeal carcinoma (NPC) is a cancerous tumor that develops in the nasopharynx epithelium and typically has squamous differentiation. The squamous phenotype is evident in immunohistochemistry, with diffuse nuclear positivity for p63 and p40. Nonetheless, a few NPCs have been identified by clinicopathological diagnosis that do not exhibit the squamous phenotype; these NPCs are currently referred to as non-squamous immuno-phenotype nasopharyngeal carcinomas (NSNPCs). In a previous work, we have revealed similarities between the histological appearance, etiology, and gene alterations of NSNPC and conventional NPC. According to ultrastructural findings, NSNPC still falls under the category of non-keratinized squamous cell carcinoma that is undifferentiated. NSNPC has an excellent prognosis and a low level of malignancy, according to a retrospective investigation. Based on prior research, we investigated the molecular mechanism of NSNPC not expressing the squamous phenotype and its biological behavior. IHC was used to determine the expression of EGFR, PI3K, AKT, p-AKT, mTOR, p-mTOR, Notch, STAT3 and p-STAT3 in a total of 20 NSNPC tissue samples and 20 classic NPC tissue samples. We obtained human NPC cell lines (CNE-2,5-8F) and used EGFR overexpression plasmid and shRNAs to transfect them. To find out whether mRNA and proteins were expressed in the cells, we used Western blotting and qRT-PCR. Cell biological behavior was discovered using the CCK-8 assay, cell migration assay, and cell invasion assay. EGFR, PI3K, p-AKT and p-mTOR proteins were lowly expressed in NSNPC tissues by immunohistochemistry, compared with classical NPC. In the classical NPC cell lines CNE-

2 and 5-8F, overexpression EGFR can up-regulate the expression of p63 through the PI3K/AKT/mTOR pathway, and promote the proliferation, migration, and invasion of nasopharyngeal carcinoma cells. At the same time, knockout of EGFR can down-regulate p63 expression through the PI3K/AKT/mTOR pathway, and inhibit the proliferation, migration, and invasion of nasopharyngeal carcinoma cells. The lack of p63 expression in NSNPC was linked with the inhibition of the EGFR/PI3K/AKT/mTOR pathway, and NSNPC may be a variant of classical NPC.

Key words: Biological function, Epidermal growth factor receptor, Nasopharyngeal carcinoma, Non-squamous immunophenotype nasopharyngeal carcinoma, P63

Introduction

Nasopharyngeal carcinoma (NPC) is a cancerous tumor that develops in the nasopharynx mucosal epithelium (Chen et al., 2019). It is one of China's most common malignant tumors, and the incidence rate is the first in head and neck malignant tumors (Chen et al., 2016). The Agency Organization for Research on Cancer's GLOBOCAN 2020 estimates of global cancer incidence and mortality show that there were 80008 deaths and 133,354 new confirmed cases of nasopharyngeal tumors (Sung et al., 2021). According to current theories, Epstein-Barr virus infection, genetics, and environmental variables have a major role in the development of nasopharyngeal carcinoma (Tsao et al., 2014; Liu et al., 2017). Radiotherapy, chemotherapy, and targeted therapy are currently the most common forms of treatment (Lee et al., 2019).

The p63 gene, which belongs to the p53 transcription factor family, is crucial for the growth of the squamous epithelium (Soares and Zhou, 2018). P63

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contains two protein subclasses with T/A or amino-terminal Δ /N domains and a variety of isomers at the same time, wherein Δ Np63 α (p40) is the main p63 isomer in multilayer squamous epithelium, located in basal cells, and is highly expressed in multiorgan squamous carcinoma of the skin, head and neck, and lung (Ghioni et al., 2002). Different p63 subtypes have been shown to affect different cells, tissues and diseases, simultaneously acting along multiple pathways to achieve these functions (Kouwenhoven et al., 2015; Yoh and Prywes, 2015). Epidermal growth factor receptor, one of four members of the ErbB tyrosine kinase receptor family, plays a critical role in cell proliferation and differentiation, especially in epithelial cells (Sibilia et al., 2007; Kovacs et al., 2015). EGFR is abnormally activated through a variety of mechanisms and has been implicated in the development of a variety of human cancers. EGFR is overexpressed in more than 90% of head and neck malignancies and is associated with poor prognosis (Gomez et al., 2020). EGFR activation leads to autophosphorylation of receptor tyrosine kinases, which initiates a series of downstream signaling pathways. EGFR functions through a variety of signal transduction pathways, the most important of which are the PI3K/AKT/mTOR pathway and Ras/Raf/MEK/ERK-MAPK pathway (Sibilia et al., 2007). These widely studied signal cascades affect gene expression, cell proliferation, apoptosis, metastasis, adhesion, and angiogenesis (Sabbah et al., 2020).

Nasopharyngeal carcinoma expresses squamous phenotype in immunohistochemistry, that is, p63 and p40 show diffuse nuclear positive. However, in clinicopathological diagnosis, a small number of nasopharyngeal carcinoma did not express squamous phenotype, thus causing difficulties in the classification and typing of this type of nasopharyngeal carcinoma. This type of nasopharyngeal carcinoma is called non-squamous immunophenotypic nasopharyngeal carcinoma. Previous studies have found that the histological morphology, etiology, and gene changes of NSNPC are similar to those of classical nasopharyngeal carcinoma. Ultrastructural results showed that NSNPC still belongs to the undifferentiated type of non-keratinized squamous cell carcinoma. NSNPC has a low degree of malignancy and a good prognosis, but the reason why it does not express p63 and p40 remains unclear. Based on previous research results, this study further explored the molecular mechanism and biological behavior of NSNPC, providing a theoretical basis for related research.

Materials and methods

Tissue samples

Tissue samples diagnosed with NSNPC from 2011 to 2021 in the Pathology Department of the First Affiliated Hospital of Zhengzhou University were

collected, and the tissue samples were selected as poorly differentiated or undifferentiated carcinomas with microscopic morphology, and the co-negative expression of p63 and p40 was the diagnostic criteria for NSNPC, included after re-examination by a professional group of diagnosing physicians. At the same time, 20 patients with classic nasopharyngeal carcinoma (both p63 and p40 positive expression) were collected as the control group.

Cell culture Human NPC cell lines (CNE-2 and 5-8F) were purchased from Shanghai QuiCell Bioscience Co., Ltd. (Shanghai, China) and Roswell Park Memorial Institute-1640 (RPMI-1640, Gibco) medium containing 10% fetal bovine serum (Biological Industries, Israel) and 1% penicillin-streptomycin mixture (Solarbio Science&Technology Co., Ltd., Beijing, China) was used to cultivate the cell lines. Every cell line was grown in a 37°C, 5% CO₂ environment.

Cell transfection

The EGFR overexpression plasmid and shRNAs were designed and synthesized by GenePharma Biotechnology Co., Ltd. (Shanghai, China). The plasmid or shRNA was transfected into cells using the jetPRIME transfection reagent. For cell transfection, dilute 2 μ g of plasmid with 200 μ l jetPRIME buffer, add another 4 μ l jetPRIME reagent, mix thoroughly, let stand at room temperature for 10 minutes, and then add it to the six-well plate. An inverted fluorescent microscope (BX5; Olympus) was utilized to monitor the transfection effectiveness after 6 hours. The effect of gene silencing was discovered at the mRNA and protein levels after 48 hours.

Immunohistochemistry (IHC)

After the sections were deparaffinized, antigen retrieval was performed by high-pressure thermal repair, and incubated with immunohistochemical hypersensitivity kits and primary antibodies. Between each incubation, sections were washed 3 times with phosphate-buffered saline (PBS) for 3 min each. Sections were incubated with 3,3-diaminobenzidine (DAB). The antigen was discovered using the following antibodies: EGFR monoclonal antibody (Immunoway, Plano, TX, USA), PI3K monoclonal antibody (Immunoway, Plano, TX, USA), AKT monoclonal antibody (Immunoway, Plano, TX, USA), anti-phospho-AKT (p-AKT) monoclonal antibody (Abcam, Cambridge, UK), mTOR monoclonal antibody (Abcam, Cambridge, UK), anti-phospho-mTOR (p-mTOR) monoclonal antibody (Cell Signaling Technology, Boston, MA, USA), Notch monoclonal antibody (Abcam, Cambridge, UK), STAT3 monoclonal antibody (Abcam, Cambridge, UK), anti-phospho-STAT3 (p-STAT3) monoclonal antibody (Cell Signaling Technology, Boston, MA, USA). The level of staining and its intensity were used to quantify.

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qPCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from NPC cell lines. A NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to assess the quantity and quality of total RNA. RNA was reverse transcribed into cDNA using the HiScript III 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co., Ltd, Nanjing, China). PCR was performed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd, Nanjing, China). Primers were synthesized by Shanghai Sangon Biotech Company (Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as the internal standard. The subsequent primers were utilized: *GAPDH* forward primer, 5'-CTTAGCACCCCTGGCCAAG-3'; *GAPDH* reverse primer, 5'-GATGTTCTGGAGAGCCCG-3'; *EGFR* forward primer, 5'-CCCACTCATGCTCTACAACCC-3'; *EGFR* reverse primer, 5'-TCGCACTTCTTACACTTGCGG-3'; *P63* forward primer, 5'-GGACCAGCAGATTCAGAACGG-3'; *P63* reverse primer, 5'-AGGACACGTCGAAACTGTGC-3'. The relative expression was calculated using the $2^{-\Delta\Delta C_t}$ technique, and each experiment was carried out in triplicate.

Western blotting

Protein extracts from nasopharyngeal cancer cells were subjected to a lysis buffer including phosphatase and protease inhibitors. A bicinchoninic acid kit (Thermo Fisher Scientific) was used to measure the protein content. On a 10% sodium dodecyl sulfate-polyacrylamide gel, equal amounts of protein were electrophoresed before being transferred to a polyvinylidene fluoride membrane. Membranes were first blocked with 5% skim milk for two hours at room temperature, followed by a primary antibody inoculation overnight at four degrees celsius. Membranes were cleaned three times with Tris-buffered saline and Tween-20 before being incubated for two hours at room temperature with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit or anti-mouse; Abcam). A suitable quantity of ECL chemiluminescence solution (Thermo Fisher Scientific) was added dropwise after washing with TBST, and images were taken using multipurpose gel imaging equipment (Syngene, Cambridge, UK). Following the experiment, the relative protein content of each set of cells was determined by calculating the intensity of bands in each group using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA).

Cell Counting Kit-8 (CCK-8) assay

In a 96-well plate, cells were seeded at a density of 5×10^3 cells per well. A 10 μ l CCK-8 detection reagent (Solarbio Science & Technology Co., Ltd., Beijing, China) was applied to each well without creating air

bubbles, and the plate was then left to incubate in the dark. Each group received five wells. Each well's absorbance (OD value) was evaluated at 450 nm, and the capacity of the cells to proliferate was assessed after 0, 24, 48, 72, and 96 hours.

Cell migration assay

On the back of a 6-well plate, three horizontal lines were made using a marker to represent the position. Each well of a 6-well plate got an inoculation of 2.5×10^5 cells overall. A 200 μ l pipette tip was used to scratch vertical lines on the plate surface when the culture had reached 95% confluence. The images were taken at 0 and 24 hours. The distance migrated was examined using the ImageJ program.

Cell invasion assay

To test invasion ability, dilute Matrigel matrix gel (BD Biosciences, Franklin Lakes, NJ, USA) and serum-free 1640 medium in a 1:8-1:10 ratio. After the Matrigel had set, 600 μ l of full RPMI-1640 with 20% serum was added to the bottom layer of the chamber, and 200 μ l of cell suspension (8×10^4 cells) was put in the top layer. It was kept in a cell culture incubator for 18-24 hours, after which it was fixed for 20 minutes in 4% paraformaldehyde, stained for 20 minutes in 0.1% crystal violet solution, rinsed for 3 minutes in tap water, and the inner wall was wiped dry with a cotton swab. After air drying, a microscope was used to note five fields of view as needed in each chamber. The number of cells going through the chamber was examined using the ImageJ program. All procedures were carried out on ice with experimental consumables pre-cooled at -20°C .

Statistical analysis

Data were examined using SPSS23.0 software (IBM, Chicago, IL, USA). The LSD t-test was used to compare the differences between the two groups. For the purpose of comparing count data and determining the association between each index and clinicopathological characteristics, Fisher's exact probability method or the χ^2 test was utilized. The test level $\alpha=0.05$ and $P<0.05$ was regarded as statistically significant.

Results

EGFR was expressed at low levels in NSNPC tissues

We used immunohistochemistry to identify the expression of each pathway-related protein in two groups of nasopharyngeal carcinoma tissue samples, and scored by two pathologists for statistical analysis. Our goal was to investigate the molecular mechanism of the low expression of p63 in NSNPC. The results showed that compared with the classic nasopharyngeal carcinoma group, EGFR, PI3K, p-AKT and p-mTOR

proteins were lowly expressed in NSNPC ($P<0.05$); there was no significant difference in the protein expressions of AKT, mTOR, Notch, STAT3 and p-STAT3 between the two groups of nasopharyngeal carcinoma ($P>0.05$) (Fig. 1). This shows that EGFR/PI3K/AKT/mTOR pathway activity inhibition may be the cause of the lower p63 expression in NSNPC tissue samples compared to classical NPC.

Upregulation of EGFR upregulated p63 expression in nasopharyngeal carcinoma cells through the PI3K/AKT/mTOR pathway

We transfected EGFR overexpression plasmid into nasopharyngeal carcinoma cell lines, resulting in up-regulation of EGFR expression in CNE-2 and 5-8F cells. The qPCR experiment was used to detect the expression of EGFR and p63, and the results demonstrated that this expression increased (Fig. 2A). To ascertain if p63 was impacted by the PI3K/AKT/mTOR signaling system, we then used western blotting to analyze the protein expression of each component of the pathway, including PI3K, AKT, p-AKT, mTOR, p-mTOR, and p63. Notably, after EGFR overexpression in CNE-2 and 5-8F cells, the levels of PI3K, p-AKT and p-mTOR were increased and p63 expression was upregulated (Fig. 2B). Consequently, EGFR activated the PI3K/AKT/mTOR pathway to increase the expression of p63 in nasopharyngeal carcinoma cells.

Knockdown of EGFR inhibited p63 expression in nasopharyngeal carcinoma cells through the PI3K/AKT/mTOR pathway

We transfected EGFR shRNAs into nasopharyngeal carcinoma cell lines, resulting in the down-regulation of EGFR expression in CNE-2 and 5-8F cells. QPCR test detected the expression of EGFR and p63, and the results showed that the expression of EGFR and p63 decreased (Fig. 3A). To find out if p63 is impacted by the PI3K/AKT/mTOR signaling pathway, we then used Western blotting to assess the protein expression of PI3K, AKT, p-AKT, mTOR, p-mTOR, and p63. It is worth noting that after EGFR was knocked down in CNE-2 and 5-8F cells, the levels of PI3K, p-AKT and p-mTOR decreased, while p63 expression was downregulated (Fig. 3B). Therefore, down-regulation of EGFR inhibited p63 expression in nasopharyngeal carcinoma cells through the PI3K/AKT/mTOR pathway.

Overexpression of EGFR upregulated p63 expression and promoted the proliferation, migration, and invasion in NPC cells

After transfecting 5-8F and CNE-2 cells with empty vector plasmid and EGFR overexpression plasmid, the expression of EGFR and p63 increased in the EGFR overexpression (oeEGFR) group. As seen in Fig. 4A, cell proliferation was considerably increased in the

oeEGFR group following overexpression of p63 in comparison to the negative control (NC) group ($P<0.05$). Similar to this, once p63 was overexpressed, migration in the oeEGFR group was considerably higher than in the negative control (NC) group ($P<0.05$; Fig. 4B). The Transwell assay was performed to measure the invasive ability in order to more thoroughly assess the impact of p63 expression on invasion. Overexpressing p63 in CNE-2 and 5-8F cells led to an increase in the number of cells moving through the chamber in the oeEGFR group, as can be shown in Figure 4C. Hence, these findings suggested that p63 had an impact on cell progression.

Knockdown of EGFR downregulated p63 expression and inhibited the proliferation, migration, and invasion in NPC cells

After transfecting 5-8F and CNE-2 cells with empty vector plasmid and EGFR shRNAs, the expression of EGFR and p63 in the shEGFR group decreased. According to Figure 5A, cell proliferation in the shEGFR group was significantly lower than in the negative control (NC) group following the down-regulation of p63 ($P<0.05$). Similar to this, migration was markedly reduced in the shEGFR group following down expression of p63 in comparison to those in the negative control (NC) group ($P<0.05$; Fig. 5B). The Transwell assay was performed to evaluate the invasive ability in order to more fully assess the impact of p63 expression on invasion. As can be seen in Figure 5C, the shEGFR group's cell crossing rate decreased as a result of the downregulation of p63 in CNE-2 and 5-8F cells. Hence, these findings suggested that p63 had an effect on cell progression.

Discussion

The incidence of nasopharyngeal cancer is low compared to other cancers. In the past few decades, the incidence of nasopharyngeal carcinoma has gradually decreased worldwide due to lifestyle and environmental changes, but its incidence is still high in southern China (Tang et al., 2016; Wei et al., 2017). Immunohistochemistry showed that most NPC had a squamous phenotype and expressed p63 and p40 (Badoual, 2022), but in the NSNPC cases first proposed in this study, both p63 and p40 were lowly expressed. It caused trouble to our pathological diagnosis and classification, so we explored the clinicopathological characteristics of NSNPC through preliminary experiments, observed the morphological characteristics of nasopharyngeal carcinoma in the two groups under a light microscope and electron microscope, and detected nasopharyngeal carcinoma in the two groups by immunohistochemistry. Pharyngeal carcinoma-related tumor suppressor gene expression and etiological analysis were performed. Finally, a retrospective analysis of the two groups of nasopharyngeal carcinoma patients was conducted. The results showed that it still belongs to the undifferentiated

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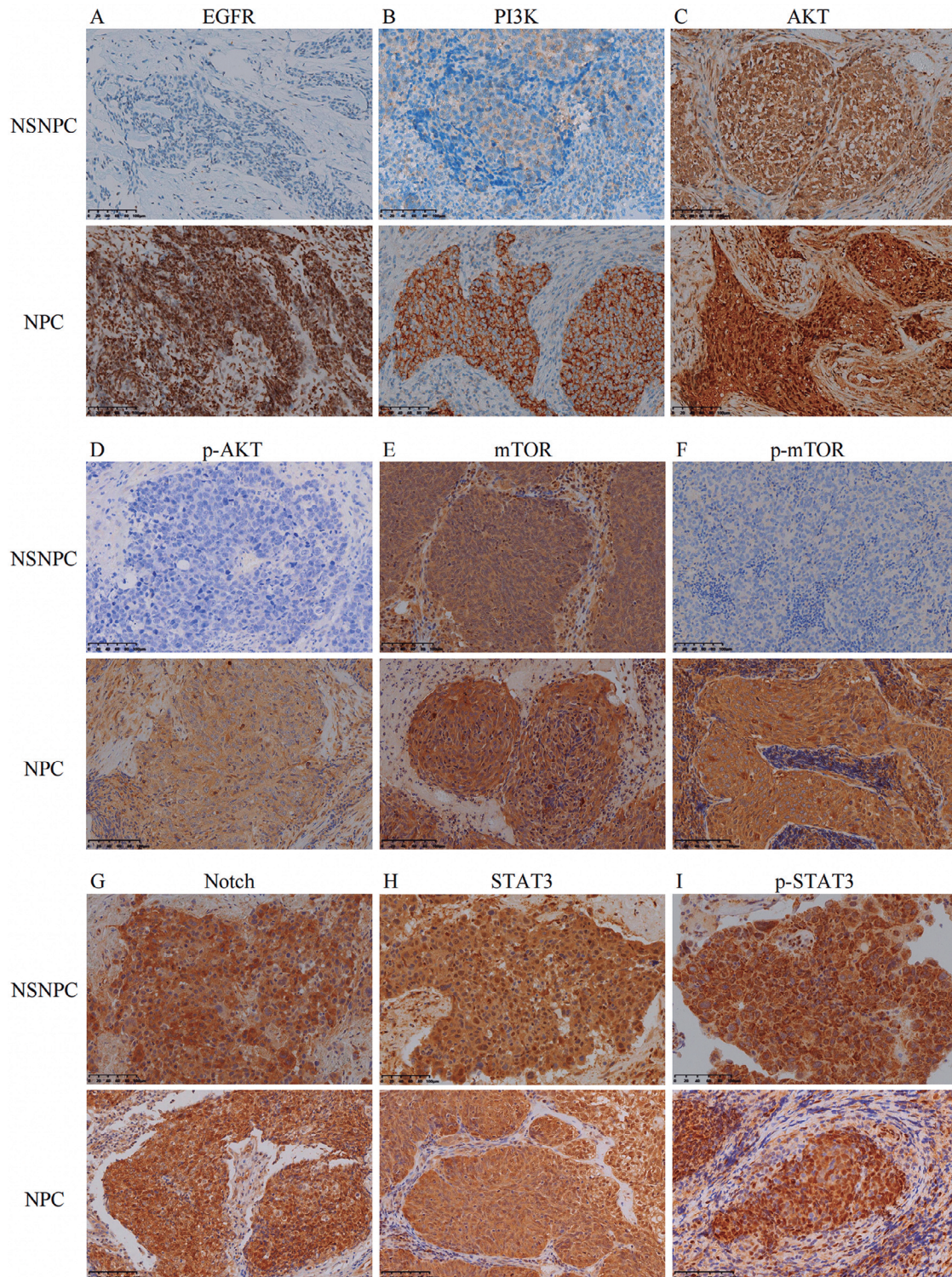


Fig. 1. Immunohistochemical images showing the differences in EGFR/PI3K/AKT/mTOR pathway, Notch pathway, and STAT3 pathway-related molecular proteins in NSNPC and NPC tissue samples. **A.** Image showing EGFR staining. EGFR expression in NSNPC is lower than that in NPC tissues. **B.** Image showing PI3K staining. PI3K expression in NSNPC is lower than that in NPC tissues. **C.** Image showing AKT staining. AKT expression in NSNPC is not different from that in NPC tissues. **D.** Image showing p-AKT staining. p-AKT expression in NSNPC is lower than that in NPC tissues. **E.** Image showing mTOR staining. mTOR expression in NSNPC is not different from that in NPC tissues. **F.** Image showing p-mTOR staining. p-mTOR expression in NSNPC is lower than that in NPC tissues. **G.** Image showing Notch staining. Notch expression in NSNPC is not different from that in NPC tissues. **H.** Image showing STAT3 staining. STAT3 expression in NSNPC is not different from that in NPC tissues. **I.** Image showing p-STAT3 staining. p-STAT3 expression in NSNPC is not different from that in NPC tissues. SP method. Scale bars: 100 μ m.

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type of non-keratinizing squamous cell carcinoma. The retrospective analysis showed that NSNPC had a low degree of malignancy and a better prognosis. However, the reason why it does not express p63 and p40 is not clear, so this study further explored the related molecular mechanism of NSNPC.

P63 is an essential regulator of epidermal growth (Soares and Zhou, 2018). This has been shown in several animal models and human diseases linked to p63 mutations. For instance, in mice, complete deletion of p63 caused loss of the epidermis and appendages associated with it, as well as defects in other tissues related to the epithelium (Yang et al., 1999). According to studies, p63 regulates numerous target genes that are involved in cell proliferation, differentiation and

adhesion, as well as embryonic epidermal development and epidermal keratinocyte proliferation and differentiation (Carroll et al., 2006). P63 contains two protein isoforms with T/A or Δ /N domains, while the Δ Np63 variant (p40) is the predominant isoform expressed in squamous epithelium, and overexpression of the Δ Np63 (p40) gene promotes squamous carcinogenesis and growth of cancer cells (Tilson and Bishop, 2014; Moses et al., 2019).

Several studies have shown that the expression of p63 is regulated by multiple signaling pathways. Studies have shown that inhibition of EGFR signaling in head and neck cancer cells can reduce Δ Np63 α expression (Matheny et al., 2003). Subsequent studies found that treatment of keratinocytes with EGF resulted in

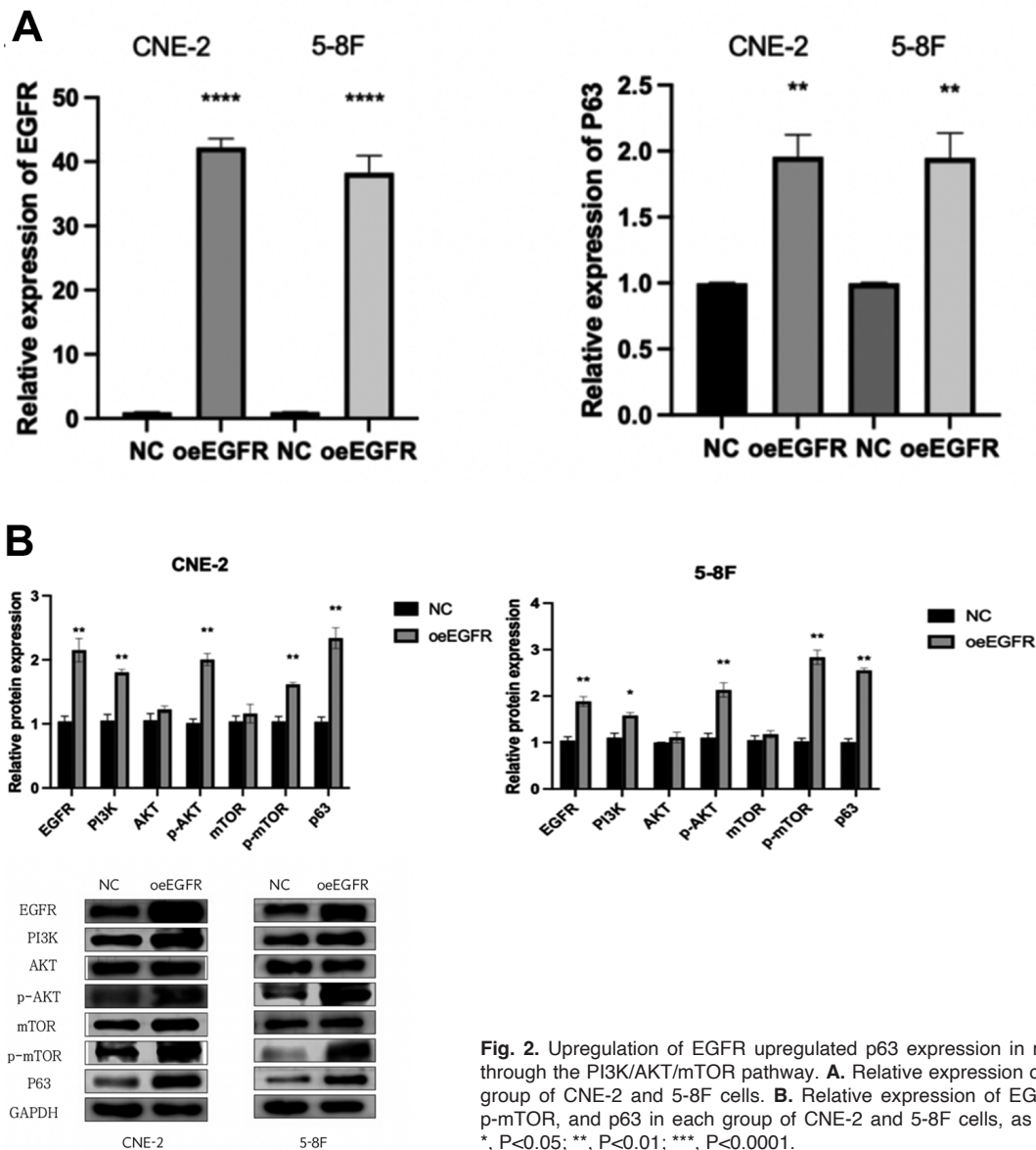


Fig. 2. Upregulation of EGFR upregulated p63 expression in nasopharyngeal carcinoma cells through the PI3K/AKT/mTOR pathway. **A.** Relative expression of EGFR and p63 mRNA in each group of CNE-2 and 5-8F cells. **B.** Relative expression of EGFR, PI3K, AKT, p-AKT, mTOR, p-mTOR, and p63 in each group of CNE-2 and 5-8F cells, as determined by western blotting. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

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increased Δ Np63 α expression, an effect that could be eliminated by inhibiting PI3K signaling, suggesting that the regulation of Δ Np63 α expression by EGFR through the PI3K pathway may play a key role in the survival and differentiation of squamous epithelial cells (Barbieri et al., 2003). Cancer is linked to the occurrence and development of the Notch pathway, which can affect cell differentiation, proliferation, and apoptosis (Meurette and Mehlen, 2018). According to some studies, Notch

activation can reduce the expression of p63 in keratinocytes, ectodermal precursor cells, and mammary epithelial cells. In keratinocytes, this reduction is caused by the transcription factors IRF3 and IRF7 (Nguyen et al., 2006). Signal transducer and activator of transcription 3 (STAT3) is a transcription factor with important functions in normal and transformed cell biology (Yu et al., 2014). STAT3, which is a key regulator of cell survival and proliferation, plays an

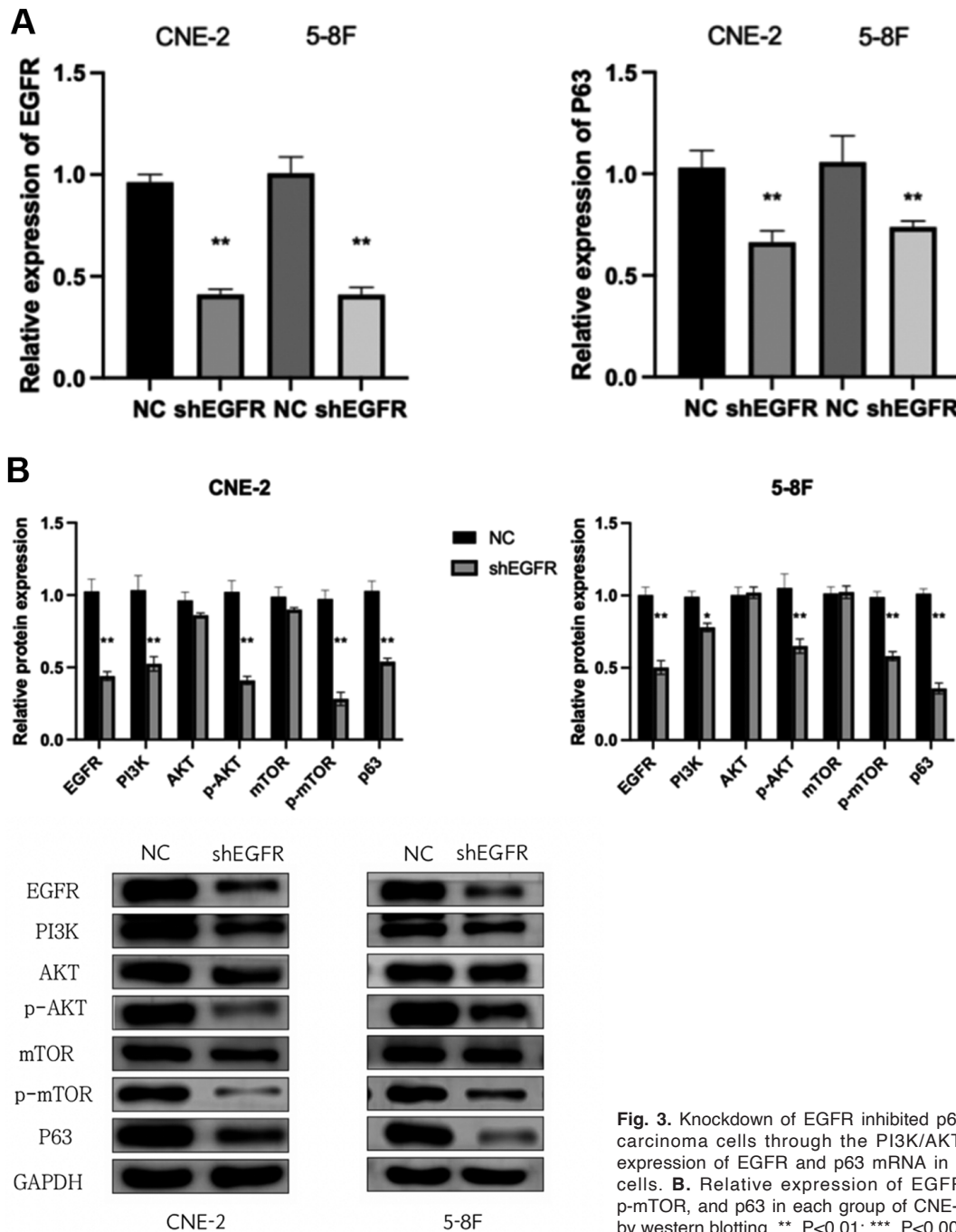


Fig. 3. Knockdown of EGFR inhibited p63 expression in nasopharyngeal carcinoma cells through the PI3K/AKT/mTOR pathway. **A.** Relative expression of EGFR and p63 mRNA in each group of CNE-2 and 5-8F cells. **B.** Relative expression of EGFR, PI3K, AKT, p-AKT, mTOR, p-mTOR, and p63 in each group of CNE-2 and 5-8F cells, as determined by western blotting. **, $P < 0.01$; ***, $P < 0.0001$.

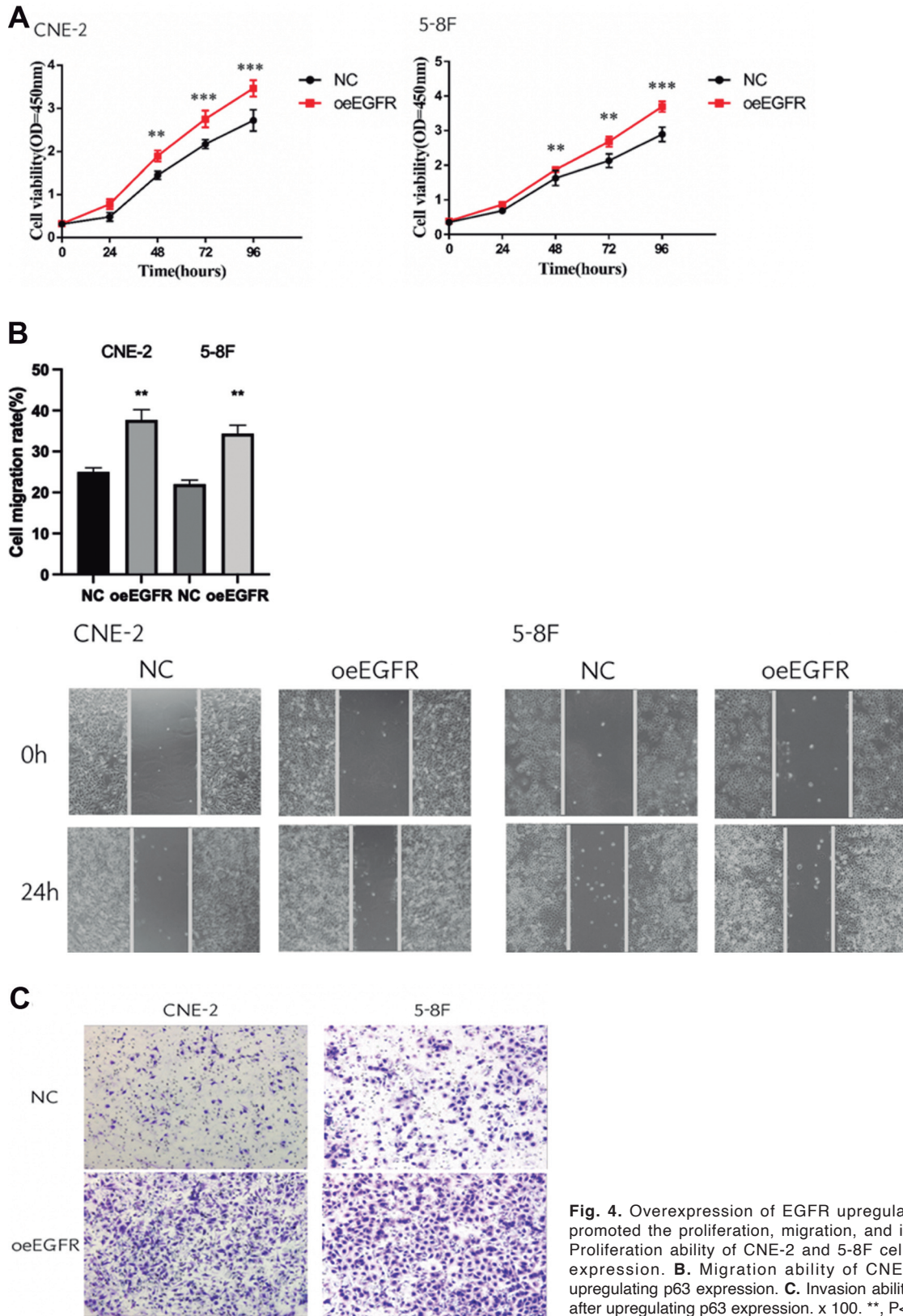


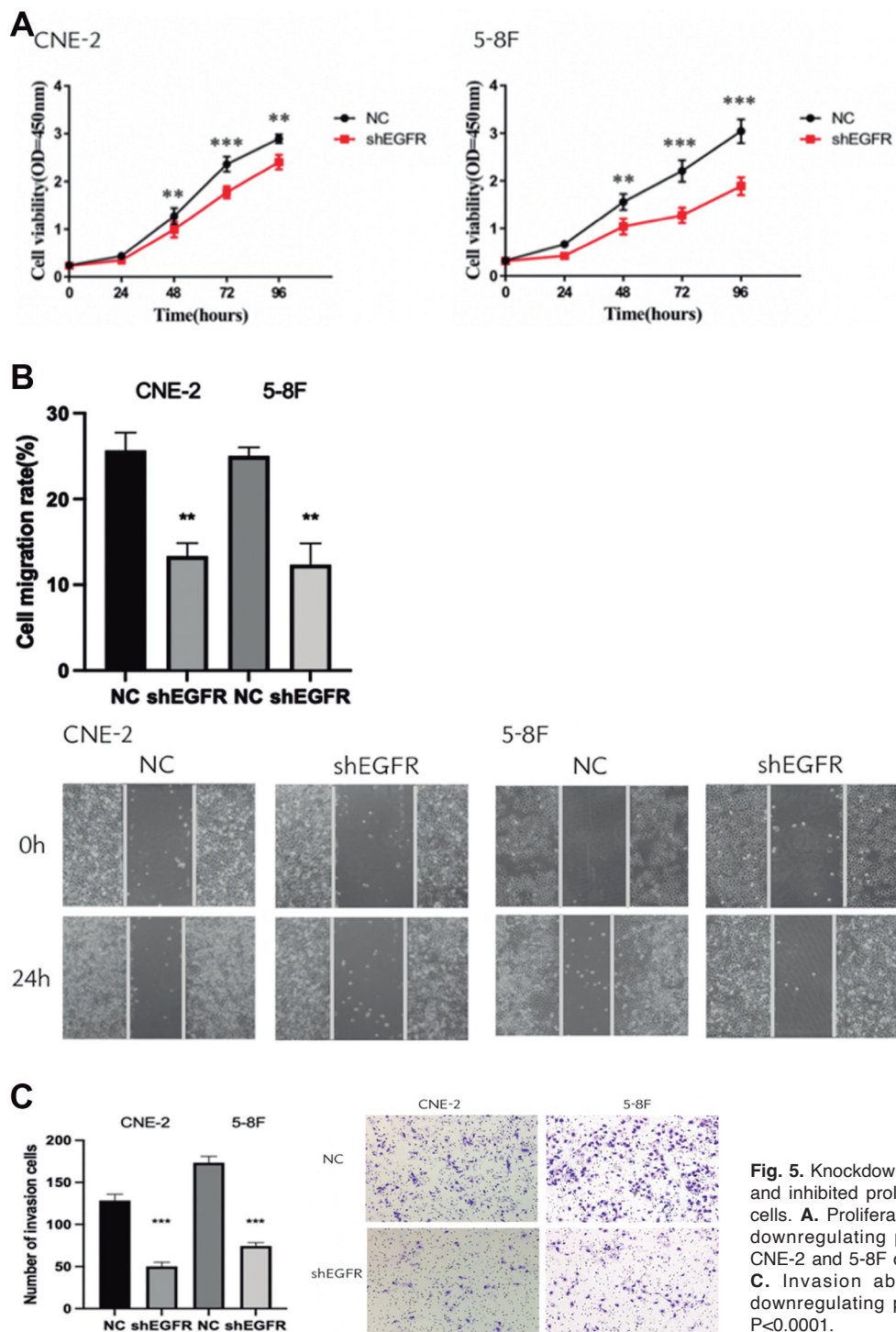
Fig. 4. Overexpression of EGFR upregulated p63 expression and promoted the proliferation, migration, and invasion in NPC cells. **A.** Proliferation ability of CNE-2 and 5-8F cells after upregulating p63 expression. **B.** Migration ability of CNE-2 and 5-8F cells after upregulating p63 expression. **C.** Invasion ability of CNE-2 and 5-8F cells after upregulating p63 expression. x 100. **, P<0.01; ***, P<0.0001.

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essential role in the upkeep and differentiation of stem cells, and is involved in the oncogenic process of a variety of cells, and has been linked in several studies to p63 (Ripamonti et al., 2013). It has been established that STAT3 binds to the Δ Np63 promoter and regulates the proliferation and differentiation of rabbit limbal

epithelial cells through Δ Np63 (Hsueh et al., 2011).

At the tissue level, our study showed that EGFR was inhibited in the development of NSNPC, thereby reducing the expression of p63 by regulating the PI3K/AKT/mTOR pathway. The cellular level showed that overexpression of EGFR increased the relative



expression of p63; knockdown of EGFR reduced the relative expression of p63. Western Blot results showed that intracellular PI3K, p-AKT and p-mTOR were all affected. The protein expressions of PI3K, p-AKT, p-mTOR and p63 in EGFR-knockdown nasopharyngeal carcinoma cells were significantly inhibited, while overexpression of EGFR could significantly up-regulate the protein levels of PI3K, p-AKT, p-mTOR and p63.

In head and neck tumors, EGFR is overexpressed in more than 90% of tumor tissues, and research has revealed that epithelial growth factor (EGF) expression may be a poor prognostic indicator if it is higher than 25% in immunohistochemistry expression rather than being associated with the T and N stages or the occurrence of metastasis (Nicholson et al., 2001; Chua et al., 2004). Ye et al. showed that knockdown of p63 in esophageal squamous cell carcinoma cell lines inhibited cell proliferation (Ye et al., 2014). Loss of p63 expression reduces the proliferation rate of a variety of cancer cells. In this study, the expression of p63 was affected by overexpression or knockdown of EGFR in nasopharyngeal carcinoma cells. The results showed that the decrease of p63 expression inhibited the proliferation, migration, and invasion of NPC cells, while up-regulation of p63 expression promoted the proliferation, migration, and invasion ability of nasopharyngeal carcinoma cells. This also helps to explain why the clinical situation of NSNPC patients is better than that of classical nasopharyngeal carcinoma, which is consistent with the results of the previous follow-up survival analysis of our research group that NSNPC has a lower malignancy and a better prognosis.

Molecular targeted therapy can specifically block the signal transduction pathway in the growth process of tumor cells, preventing the growth of tumor cells to achieve the purpose of treatment (Kitamura et al., 2020). It is a brand-new tumor treatment mode. It is typically used in patients with cancer in intermediate to advanced stage or in combination with chemotherapy. Currently, the commonly used targeted drugs in nasopharyngeal carcinoma are EGFR monoclonal antibodies, including cetuximab and nimotuzumab (Cramer et al., 2019; Guo et al., 2021). A study divided patients with locally advanced HNSCC into two groups for comparison, namely the combined radiotherapy group (radiotherapy plus cetuximab) and the radiotherapy group alone. According to the findings, the combined therapy group's median local disease control period was 24.4 months, the radiotherapy alone group's median local disease control period was 14.9 months, and the median survival was 49.0 months and 29.3 months, respectively. It shows that EGFR monoclonal antibodies can help control disease progression and increase patient survival (Bonner et al., 2006). For the NSNPC patients in this study, whose EGFR expression is lower, whether they have a better response to the combination therapy is worthy of further research.

Our study verified that EGFR regulates the expression of p63 in NSNPC through the PI3K/

AKT/mTOR pathway at the tissue and cellular levels, and affects the occurrence and development of nasopharyngeal carcinoma. However, this study still has some limitations. The cases of NSNPC are very rare, the conventional treatment is radiation therapy, and the surgically removed tissue is relatively small. It is very difficult to obtain fresh tissue samples to extract primary cells for research. Currently, only classical nasopharyngeal cancer cell lines are supported. In the future, with the development of primary cell extraction technology, it is expected that primary cells will be extracted from NSNPC tissue, and the experiment will be further improved to conduct deeper research and provide a theoretical basis for research in related fields.

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Conflicts of interest. There are no conflicts of interest to declare.

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