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Functional Characterization of p53 in NPC C666-1 cells by CRISPR-based Gene Knockout and Transcriptome Sequencing

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Abstract: *Introduction*: Nasopharyngeal carcinoma (NPC) is a head and neck cancer associated with Epstein-Barr virus (EBV) in Southern China and Southeast Asia. Although mutation of the p53 tumor suppressor gene is a rare event in NPC, NPC has a high frequency of over-expressed/accumulated p53 protein, which is considered to be a dysfunction or inactivation by EBV in NPC. However, the activity and role of p53 in the NPC remain controversial. *Methods*: The CRISPR-Cas9 gene editing system was used to establish NPC C666-1 cell lines with p53 knockout (KO), and transcriptome sequencing was performed to identify differentially expressed mRNAs and miRNAs in the p53 KO and control C666-1 cell lines. EdU incorporation assay, soft agar colony formation assay, and plate colony formation assay were performed to detect cell proliferation, and flow cytometry was used to analyze cell cycle and apoptosis. *Results*: 766 differentially expressed genes (DEGs) and 46 differentially expressed miRNAs were identified. The KEGG pathway enrichment analysis of both the DEGs and miRNAs target genes showed that the p53 signaling pathway was significantly enriched. In the enriched p53 signaling pathway, genes involved in cell cycle arrest and apoptosis induction were significantly downregulated by p53 KO, and re-expression of exogenous p53 rescued their expression in the C666-1 cells. Moreover, p53 KO promoted cell proliferation, decreased cell apoptosis, and accelerated G1/S phase progression in the C666-1 cells, and re-expression of exogenous p53 rescued these phenotypes in the C666-1 cells. *Conclusions*: Our data indicate that p53 retains tumor suppressor function in NPC C666-1 cells.

Keywords: Nasopharyngeal carcinoma; p53; Epstein-Barr virus; CRISPR-based gene knockout; Transcriptome sequencing

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1. Introduction

Nasopharyngeal carcinoma (NPC) that originates from the nasopharyngeal mucosal epithelium is one of the most common cancers in Southern China and Southeast Asia, where the observed incidence rates range from

15 to 50 per 100,000 persons [1]. This distinctive racial and geographic distribution implies that both genetic susceptibility and environmental factors contribute to the development of NPC. NPC is strongly associated with Epstein-Barr virus (EBV) infection and molecular genetic alterations [2–4].

p53, a tumor suppressor protein, regulates gene expressions involved in G1 and G2/M cell cycle checkpoint regulation, DNA damage recognition and repair, apoptosis, and cell death regulation [5]. It has been termed as the "guardian of the genome", or "cellular gatekeeper of growth and division" due to its important biological roles in protecting cells from becoming cancerous [6,7]. Mutations in the p53 gene damage its tumor suppressor function, thus contributing to cancer initiation and development [8–10]. The p53 gene is the most frequently mutated in epithelial malignancies, and its mutations often result in the accumulation of the mutant p53 protein, which either loses tumor suppressor function or gains oncogenic activity [11]. Unlike other human tumors, p53 mutations are rare in NPC, and point mutations in hot spots of exons 5, 7, and 8 are in < 10% of NPC cases [4,12,13]. Moreover, numerous studies showed that more than 95% NPC have an accumulation of p53 protein [14,15]. However, the activity and role of p53 in the NPC remain unclear and controversial [16–19].

EBV was first discovered as an oncogenic gamma-1 herpesvirus, and infects more than 90% of the worldwide adult population. NPC is an EBV-associated tumor [2]. EBV was detected in all the NPC samples, and consistent elevation of EBV DNA or antibody is a well-established risk factor of development of NPC, which strongly supports a role for EBV in the pathogenesis of NPC [20,21]. The interplay between EBV and p53 in EBV-associated malignancies has been observed [22]. For example, EBV-encoded oncoproteins interact with p53 and alter its expression, EBV latent protein EBNA3C inhibits the transcriptional activity of p53 by inhibiting the DNA binding activity of p53, and EBV oncoprotein latent membrane protein 1 represses p53-mediated DNA repair and apoptosis [18,22–24]. These studies indicate that p53 may be functionally inactivated by EBV in EBV-associated NPC.

C666-1 is a human NPC cell line established from a xenograft of NPC in athymic nude mice (Xeno-666), which consistently carries EBV and expresses the EBV-encoded RNAs (EBERs), EBV nuclear antigen-1(EBNA1), latent membrane protein-1 (LMP1), and LMP2 in long-term *in vitro* culture [25]. Moreover, the C666-1 cell line retains wild-type p53 characteristics along with the polymorphism at codon 72 of exon 4 that is commonly found in the human wild-type p53 gene [4,26]. Therefore, C666-1 is a human NPC cell line suitable for studying the function of p53 in NPC.

In the present study, we established a p53 knockout (KO) C666-1 cell line using the CRISPR-Cas9 gene editing system, followed by mRNA and miRNA sequencing for the p53-KO and control C666-1 cell lines. Bioinformatics analysis revealed that the p53 signaling pathway, mismatch repair, and transcriptional misregulation in cancer were significantly affected by p53 KO in the C666-1 cells. We also observed that p53 KO promoted cell proliferation, accelerated G1/S phase progression, and decreased cell apoptosis, whereas re-expression of exogenous p53 rescued these phenotypes in the C666-1 cells. Our data indicate that p53 functions as a tumor suppressor gene in NPC C666-1 cells.

2. Materials and methods

2.1. Knockout of p53 in C666-1 cells using the CRISPR-Cas9 gene editing system

C666-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (ThermoScientific, USA) at 37 ℃. To knock out p53, the guide RNA (gRNA) sequence was designed using

the website software from the Massachusetts Institute of Technology (USA) (http://crispr.mit.edu/). The guide RNA (gRNA) sequence was GCAGTCACAGCACATGACGG. Editing efficiency determined by DNA sequencing and immunoblot analysis was used to determine editing efficiency.

2.2. Western blot

Cells were washed with PBS and lysed using RIPA buffer, and then proteins were separated using SDS-PAGE, followed by transferring onto PVDF membranes (Millipore, USA). Blots were incubated overnight with antibodies against p53 (DO-1) (#sc-126, Santa Cruz) at 4 ℃. Blots were then incubated with HRP-conjugated secondary antibody for 2 hours at room temperature. The signal was captured by using enhanced chemiluminescence detection reagent (Millipore, USA).

2.3. RNA isolation and sequencing

Total RNA was extracted from cells with TRIzol Reagent (Invitrogen, USA). RNA integrity and concentration were measured with the Agilent 2100 Bioanalyzer. RNA purity was determined with the Nanodrop spectrophotometer (ThermoScientific). mRNA and Small RNA library preparation and sequencing were implemented using NEBNextR Ultra™ RNA Library Prep Kit for IlluminaR (#E7530L, NEB, USA) and Small RNA Sample Preparation Kit (Illumina, RS-200-0048) following the manufacturer's recommendation at Annoroad Gene Technology Co., Ltd (Beijing, China).

2.4. QRT-PCR

Total RNA was extracted from NPC cells by using Trizol reagent (Invitrogen). For miRNA qRT-PCR, 2 μg total RNA were reverse transcribed with a reverse transcription (RT) kit (Vazyme Biotech, China) and miRNA-specific primer (BulgeLoop miRNA qPCR primer). Real-time PCR was implemented by using the miRNA Universal SYBR qPCR Master Mix kit (Vazyme Biotech, China). For mRNA qRT-PCR, 1 μg total RNA was reverse transcribed to cDNA with an RT kit and Oligo dT primer (Vazyme Biotech, China), respectively. The RT products were amplified with the SYBR qPCR Master Mix kit (Vazyme Biotech, China). The products were quantified using a 2-DDCt method against GAPDH or U6 for normalization.

2.5. 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay

The edU incorporation assay was implemented to analyze cell proliferation as previously described by us [27–30]. All assays were performed in triplicate.

2.6. Flow cytometric analysis of cell cycle and apoptosis

Analysis of cell cycle and apoptosis was implemented by flow cytometry as previously described by us. All assays were implemented three times in triplicate.

2.7. Statistical analysis

All the quantified data were represented as an average of three times. Data are shown as mean ± SD. Two-tailed student's *t*-test was used for comparisons between the groups. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Generation of p53 knockout C666-1 cell lines by CRISPR/Cas9 gene editing system

To explore the role of p53 in C666-1 cells, we knocked out the p53 gene of C666-1 cell lines by using the CRISPR/Cas9 system and established p53 KO C666-1 cell lines. Single-guide RNA was constructed to target exon 5 of the p53 gene (**Figure 1a**).

Sanger sequencing was performed to identify the cell lines in which both alleles of p53 were deleted, and it was found that the p53 gene was knocked out in both alleles (**Figure 1b**). Western blot analysis demonstrated that there was no detectable p53 protein in the p53 KO cells (**Figure 1C**). The results indicate p53 KO C666-1 cell lines were established.

3.2. Differentially expressed genes in the p53 KO and control C666-1 cells

mRNA sequencing analysis showed that a total of 766 differentially expressed genes (DEGs) (|log2 Fold change |≥ 1.5 and $p < 0.05$) were identified in the p53 KO and control C666-1 cells (**Figure 1d,e**). Among them, 276 DEGs were downregulated, and 489 DEGs were upregulated by p53 in C666-1cells. To validate the mRNA sequencing results, a qRT-PCR assay was implemented to detect the mRNA levels of 5 genes (DDB2, BAX, BTG2, LDHA, and IPO5). The results showed that the relative expression patterns of the five genes were consistent with mRNA sequencing data (**Figure 1f**), with a correlation coefficient of 0.93 between qRT-PCR and mRNA sequencing results (**Figure 1g**).

3.3. Gene ontology and KEGG pathways enrichment analysis of DEGs in the p53 KO and control C666-1 cells

The 766 DEGs were used to query the GO database. The results displayed that the DEGs were categorized into three major groups: molecular function, biological process, and cellular component domains, as well as 52 functional groups ($q < 0.05$). In the molecular function, biological process, and cellular components, 12, 25, and 15 functional groups were annotated, respectively, which are involved in antioxidant and metabolic processes, cell proliferation, and cell adhesion, *etc*. (**Figure 1h**).

The 766 DEGs were mapped to KEGG database for pathway enrichment analysis. The results displayed that 14 signaling pathways ($q < 0.05$) were statistically enriched, consisting of “p53 signaling pathway”, “transcriptional misregulation in cancer”, “PI3K-Akt signaling pathway”, “focal adhesion”, and “pathways in cancer”, etc (**Figure 1i**).

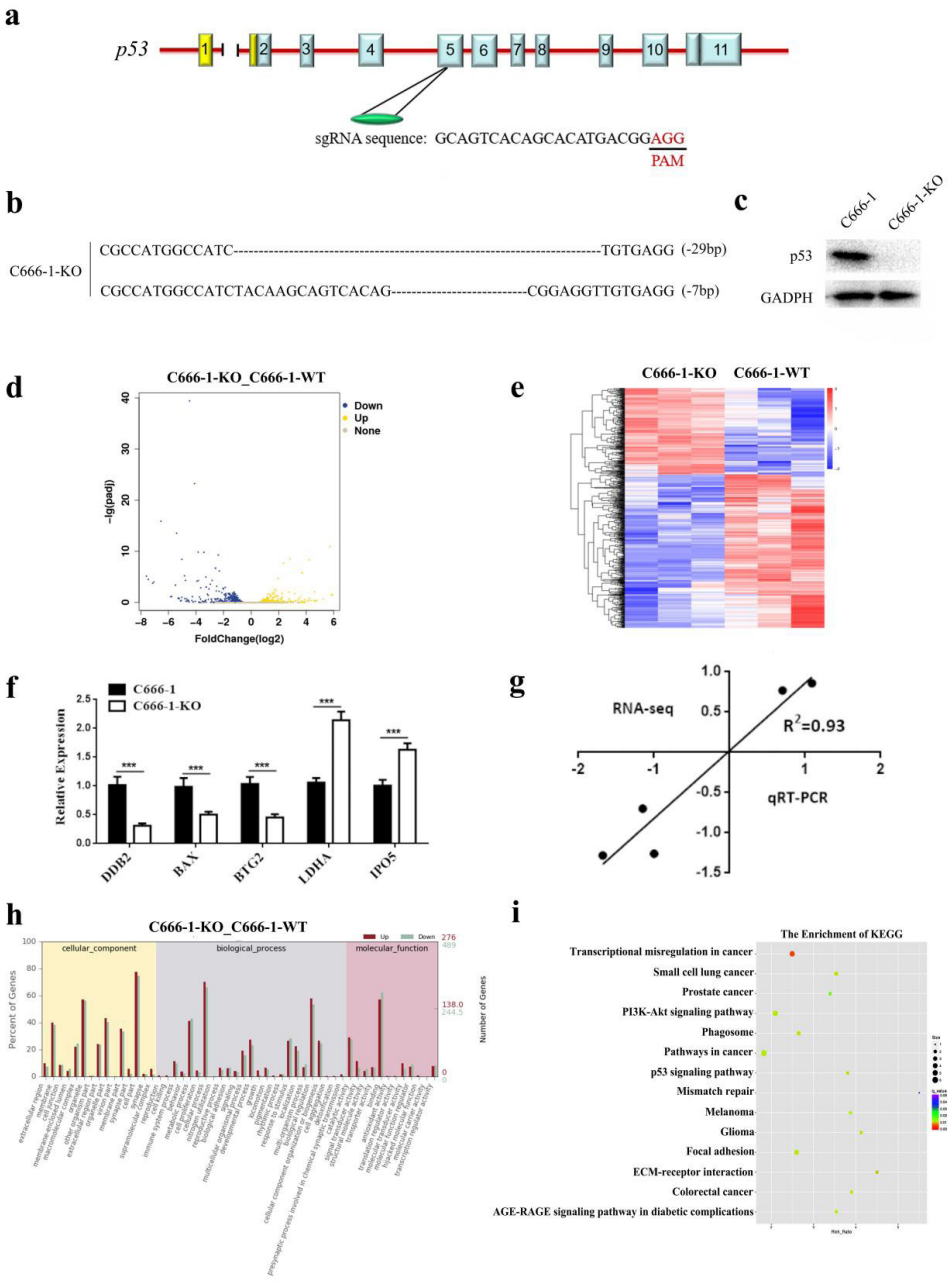

Figure 1. Establishment of p53 knockout C666-1 cell lines and mRNA sequencing in the p53 KO and control C666-1 cells. **(a)** The human gene structure of p53 in the genome (top) and sgRNA target sequence in the p53 loci (bottom) are displayed. PAM: protospacer adjacent motif (black bar). **(b)** Analysis of the sequences of the p53 gene in the KO C666-1 cells. The black characters "-" indicate the deleted bases of the p53 gene. **(c)** Analysis of p53 protein expression levels in the p53 KO and control C666-1 cells. **(d)** Volcano plot of differentially expressed genes (DEGs) in the p53 KO and control C666-1 cells. **(e)** Hierarchical clustering of DEGs in the p53 KO and control C666-1 cells. **(f)** QRT-PCR showing the expression levels of the five DEGs. **(g)** Correlation between Log2 fold change obtained from mRNA-sequencing and the Log2 fold change derived from qRT-PCR. **(h)** GO enrichment analysis of DEGs in the p53 KO and control C666-1 cells. **(i)** KEGG enrichment analysis of DEGs in the p53 KO and control C666-1 cells. ***$p < 0.001$; KO, p53 knockout. Normal, normal nasopharyngeal mucosal tissues.

3.4. The effects of p53 on C666-1 cell proliferation, cell cycle and apoptosis

The KEGG pathway enrichment analyses of both the DEGs and 490 upregulated target genes showed that "p53 signaling pathway" was significantly enriched (**Figure 2a**). In this enriched pathway, genes involved in apoptosis induction and cell cycle arrest were significantly downregulated by p53 KO in the C666-1 cells (**Figure 2a**). To validate the regulation of these genes by p53, we transfected a plasmid expressing wild-type p53 into the C666-1 cells with endogenous p53 KO, and established C666-1 cell lines with stable expression of exogenous p53 (**Figure 2b**), then detected the mRNA levels of CDKN1A (encoding p21), BBC3 (encoding PUMA), BAX, and MDM2 by qRT-PCR. The results showed that their expression levels significantly decreased in the p53 KO C666-1 cells relative to control C666-1 cells, and re-expression of wild-type p53 rescued their expression (**Figure 2c**).

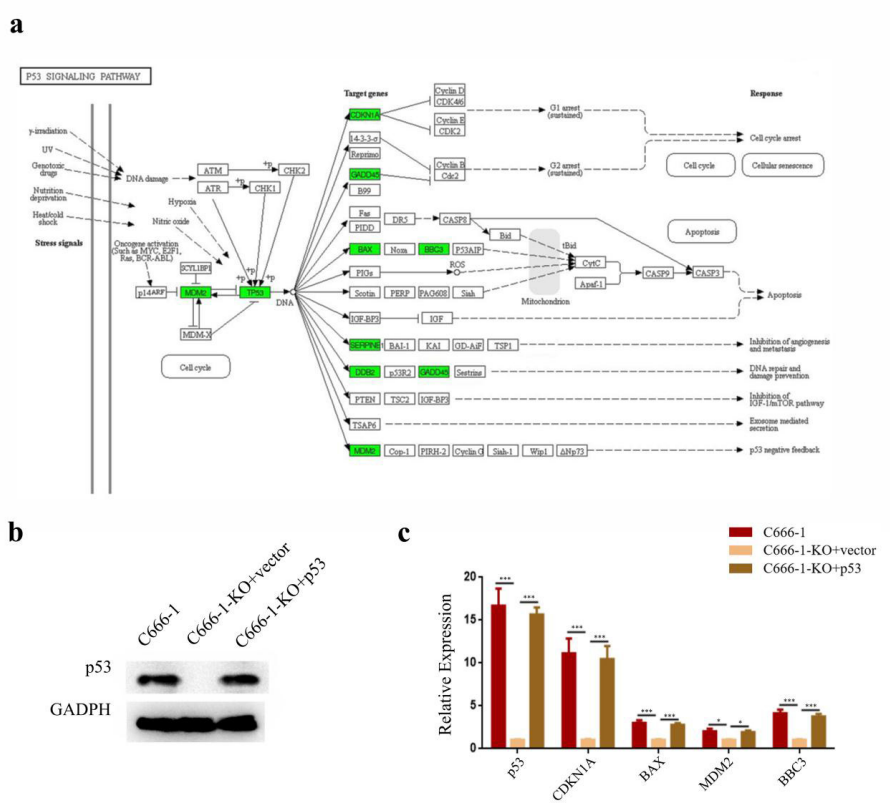

Figure 2. The effects of p53 on the p53 signaling pathway and its relative genes in the C666-1 cells. **(a)** KEGG pathways enrichment analysis of DEGs and upregulated target genes showed that "p53 signaling pathway" is significantly enriched, and the genes involved in apoptosis induction and cell cycle arrest were significantly downregulated by p53 KO in the C666-1 cells. **(b)** Western blot analysis showing p53 levels in the C666-1, p53 KO C666-1, and p53 KO C666-1 cells stably transfected with exogenous p53. **(c)** QRT-PCR displaying the expression levels of CDKN1A, BBC3, BAX, and MDM2 in the C666-1, p53 KO C666-1, and p53 KO C666-1 cells stably transfected with exogenous p53. *$p < 0.05$; ***$p < 0.001$; KO, p53 knockout.

This study finally analyzed the effects of p53 on the proliferation, apoptosis, and cell cycle of C666-1 cells. Soft agar colony formation assay, EdU incorporation, and plate colony formation assay showed that p53 KO significantly promoted C666-1 cell proliferation, whereas re-expression of exogenous p53 inhibited C666-1 cell proliferation (**Figure 3a-c**). Flow cytometric analysis displayed that p53 KO decreased cell

apoptosis and accelerated G1/S phase progression, whereas re-expression of exogenous p53 increased cell apoptosis and blocked G1/S phase progression (**Figure 3d,e**).

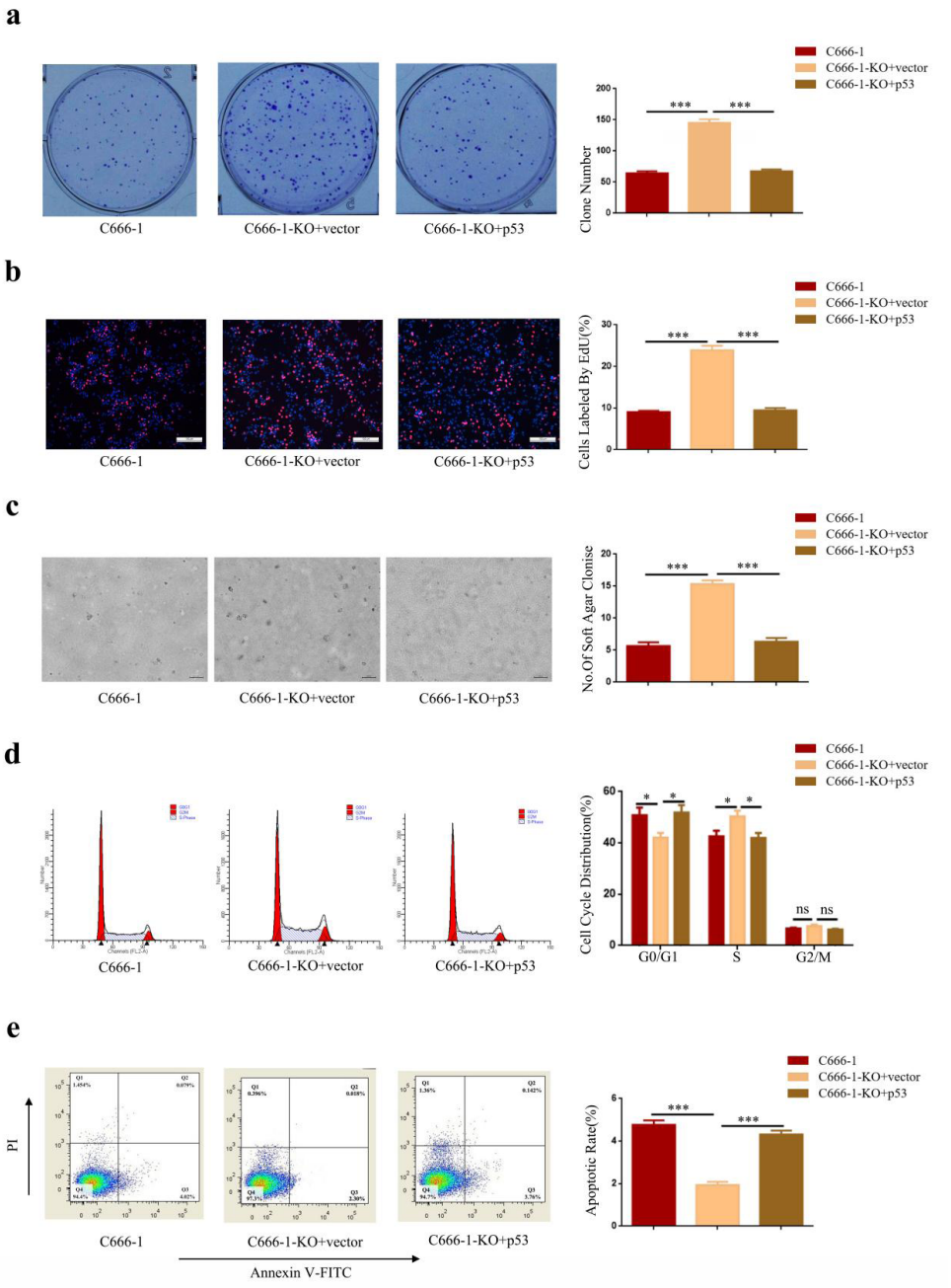

Figure 3. The effects of p53 on C666-1 cell proliferation, cell cycle and apoptosis. Representative results (*left*) and statistical analyses (*right*) of cell proliferation detected by plate clone formation (**a**), EdU incorporation (**b**), and soft agar colony formation (**c**) assay in the C666-1, p53 KO C666-1, and p53 KO C666-1 cells stably transfected with exogenous p53. **(d)** Representative results (l*eft*) and statistical analyses (*right*) of cell cycle distribution analyzed by flow cytometry in the C666-1, p53 KO C666-1, and p53 KO C666-1 cells stably transfected with exogenous p53. **(e)** Representative results (*left*) and statistical analyses (*right*) of cell apoptosis analyzed by flow cytometry in the C666-1, p53 KO C666-1, and p53 KO C666-1 cells stably transfected with exogenous p53. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, no significance. KO, p53 knockout.

4. Discussion

p53 gene is the most frequently mutated gene in epithelial malignancies, but p53 gene mutations are rare in NPC, and p53 protein is accumulated in the most of NPC [4,11–15]. NPC is an EBV-associated tumor, and p53 may be functionally inactivated by EBV in EBV-associated malignancies [2,21–24]. However, the activity and role of p53 in the NPC remain unclear.

In this study, C666-1, a human EBV-positive NPC cell line carrying the wild-type p53 gene, was used to explore the function of p53 in NPC. A p53 knockout (KO) C666-1 cell line was established, and transcriptome sequencing was performed to compare the differentially expressed mRNA and miRNA in the p53 KO and control C666-1 cells. The KEGG pathway enrichment analysis of both the differentially expressed genes (DEGs) and upregulated target genes anti-correlated with miRNAs showed that the "p53 signaling pathway", "mismatch repair," and "focal adhesion", etc., were significantly enriched, indicating that the transcriptional activity of p53 in the NPC C666-1 cells is retained. Importantly, we also observed that p53 KO promoted cell proliferation, accelerated G1/S phase progression, and decreased cell apoptosis, whereas re-expression of exogenous p53 rescued these phenotypes in the C666-1 cells. Collectively, our data suggest that p53 functions as a tumor suppressor gene in NPC C666-1 cells.

Sequence analysis of the mRNA transcripts in the p53-KO and control C666-1 cells showed that most of the DEGs were regulated by p53, and KEGG pathway enrichment analysis showed that "p53 signaling pathway" was significantly enriched, and p53-related genes were involved in cell cycle arrest and apoptosis induction, such as MDM2, CDKN1A, GADD45, BAX, BBC3, DDB2, SERPINE1 and SERPINE5, were upregulated in the C666-1 cells, indicating that p53 gene retains the function for blocking cell cycle progression and inducing apoptosis in NPC C666-1 cells.

In the genome-wide CRISPR-based gene knockout screens, MDM2 was identified as an essential factor for NPC cell growth [31]. MDM2 is an E3 ubiquitin ligase for p53 and negatively regulates p53 expression through ubiquitination-mediated protein degradation. It has been reported that small molecule antagonists (Nultin-3, RG7388) inactivate MDM2 and reactivate the p53 tumor-suppressive capabilities in several human cancers, including NPC [32,33]. p53 functions as a tumor suppressor gene, and its mutations are rare in NPC, thus making MDM2 inhibitors a potential anti-NPC agent.

5. Conclusion

In summary, our data suggest that the p53 gene retains tumor suppressor function in the NPC C666-1 cell line. p53 knockout-associated DEGs and differentially expressed miRNAs provide valuable information to investigate the role and molecular mechanism of p53 in NPC C666-1 cells.

Data availability

The datasets of RNA-sequencing for this study can be found in the GEO: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138259.

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Disclosure statement

The authors declare no conflict of interest.

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