



Establishment of a patient-derived organoid model and living biobank for nasopharyngeal carcinoma

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Background: Recurrent nasopharyngeal carcinoma (NPC) remains a major challenge for clinicians and scientists. Tumor organoid is a revelational disease model that highly resembled the heterogeneity and histopathological characteristics of original tumors. This study aimed to optimize the modeling process of patient-derived NPC organoids (NPCOs), and establish a living-biobank of NPCOs to study the mechanism and explore the more effective treatment of the disease.

Methods: Sixty-two fresh NPC tissue samples and 15 normal mucosa samples were collected for 3-dimensional (3D) organoid culture. The organoids were confirmed using hematoxylin and eosin assays. The expression levels of *CD133*, *CD44*, *BMI-1*, and Epstein-Barr virus (EBV)-encoded small RNAs (EBERs) were detected by immunohistochemistry (IHC) and in situ hybridization (ISH). Recurrent NPCOs were frozen in liquid nitrogen for 6 months and then resuscitated and passaged.

Results: We identified a novel two-step enzymatic strategy for the treatment of NPC and nasal mucosa specimens and an optimal medium for culturing NPCOs and nasal mucosa organoids (NMOs). Organoid cultures were generated from 34 primary NPC samples, 28 recurrent NPC samples, and 15 normal mucosa samples. The success rates for primary NPCO, recurrent NPCO, and NMO formation were 47.06%, 81.25%, and 86.5%, respectively. All the NPCOs were *EBER* positive and *CK7* negative. Recurrent NPCOs had higher expressions of stem cell markers, including *BMI-1*, *CD44*, and *CD133*. Additionally, recurrent NPCOs could be cultured to passage 4 and frozen and revived repeatedly, while primary NPCOs were challenging to culture.

Conclusions: In summary, we successfully established a living biobank using the NPCO model, which has enormous potential in basic and clinical research on NPC.

Keywords: Nasopharyngeal carcinoma (NPC); organoid; 3-dimensional culture; biobank

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Introduction

Nasopharyngeal carcinoma (NPC) originates from nasopharynx epithelial cells and is common in southern China and Southeast Asia (1-3). NPC is causally linked to Epstein-Barr virus (EBV) infections (4,5). The 5-year overall survival rate for NPC is above 80% (6,7). However, recurrence occurs in 10% to 15% of patients with NPC, and the 5-year overall survival rate for patients with recurrent NPC is only 13.2% to 38% (8,9). The high rate of recurrence is responsible for the majority of failed NPC treatments with unfavorable prognosis (10), and tumor heterogeneity might be a cause of tumor recurrence (11,12).

Currently, NPC cell lines are the most commonly used model of NPC study. However, many NPC cell lines were reported to be contaminated by HeLa cell (13). Moreover, EBV viral genome was observed to be lost during long-term culture in most NPC cells (14,15). Most importantly, cell lines do not reflect the molecular heterogeneity of clinical NPC tissues (16). As a revelational disease model, 3-dimensional (3D) organoids are based on high hopes in oncology studies. Organoids maintain phenotypic and molecular heterogeneity of their parental tissue, be for with minor genetic aberration or transformation extended periods cultured *in vitro* (17,18). The lack of a living model is a factor limiting the study of relapsed and refractory NPC. Therefore, the establishment of a recurrent NPC biobank using the patient-derived organoid model is of significance to clinical and experimental studies.

In this study, we optimized technological process of patient-derived NPC organoids (NPCOs) modeling, and raised the success rate to more than 80%. For the first time, we established a living biobank/database of, which will provide powerful tools for high throughput research and drug development of recurrent nasopharyngeal carcinoma. We present the following article in accordance with the MDAR reporting checklist (available at <https://atm.amegroups.com/article/view/10.21037/atm-22-1076/rc>).

Methods

Ethical approval and sample collection

All the tissue samples were obtained from the Nanfang Hospital of the Southern Medical University (Guangzhou, China). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Biopsy and surgical resection samples were collected after written informed consent forms were obtained from the patients,

and all related procedures were performed with the approval of the Research Ethics Committee at the Nanfang Hospital of the Southern Medical University (approval No. TCHIRB-10701103-E). Half of the NPC samples prepared for organoid generation were used for pathological experiments. After being washed with phosphate-buffered saline (PBS) containing 3% penicillin, streptomycin, and amphotericin B (Sigma), the NPC tissues used to generate the NPCOs were blotted dry and weighed. Each NPCO and NPC tissue was embedded in paraffin and sectioned at 4 μ m for evaluation by hematoxylin and eosin (H&E), immunofluorescence (IF), immunohistochemistry (IHC), and EBV-encoded small RNA (*EBER*) in situ hybridization (ISH). Each section was independently evaluated by two pathologists.

Organoid culture, cryopreservation, and resuscitation

After thoroughly washing the NPC tissues with PBS, they were cut with scissors into 3 mm \times 3 mm \times 3 mm pieces and then digested in digestion buffer I (500 ng/mL hydrocortisone, 300 U/mL collagenase II, 100 U/mL hyaluronidase) at 37 $^{\circ}$ C and 50 rpm for 1.5 to 2 hours. This was followed by a second round of digestion within digestion buffer II (5 mg/mL Dispase II, 0.1 mg/mL DNase I). Large clumps and cell debris were removed using a 100- μ m filter, after which the filtered cells were washed 3 times with ice-cold PBS. The NPC cells were resuspended using Matrigel at a density of 6×10^4 cells/mL. The cells were seeded into 6-well plates at 3 droplets per well and placed in an incubator at 37 $^{\circ}$ C. The organoid culture medium was changed every 3 days. The Matrigel was disrupted by pipetting to passage the organoids. The organoids were digested using TrypLE (Life Technologies, cat. no. 12605-010) at 37 $^{\circ}$ C for 3 to 5 minutes. After centrifugation at 300 to 400 \times g for 2 minutes, the cell pellet was resuspended in Matrigel supplemented with organoid culture medium and plated in 30- μ L droplets in a 6-well plate. When the Matrigel was polymerized, the organoids were cultured in the organoid culture medium at 37 $^{\circ}$ C.

H&E staining

To validate whether NPCOs could reliably recapitulate the histopathological characteristics of NPC tissues, we performed an H&E staining assay using NPC tissues, NPCOs, and nasopharyngeal mucosa organoids (NMOs). Formalin-fixed paraffin-embedded (FFPE) slides from

the tissues or organoids were subjected to routine H&E staining. The H&E-stained slides were independently assessed by two pathologists.

Immunohistochemistry

Paraffin-embedded sections were cut into 4- μ m slices with a microtome (Leica RM2125 RTF). The slides were stained with primary antibodies against *CK7* (1:200, Zhongshan Golden Bridge Biotechnology, Beijing China), *BMI-1* (1:500, Zhongshan Golden Bridge Biotechnology), and negative control antibodies. Subsequently, the tissue sections were stained with DAKO (DAKO, K346711-2, Copenhagen, Denmark) liquid 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes, counterstained with 10% Mayer's hematoxylin, dehydrated, and mounted. Each section was independently evaluated by 2 pathologists.

Immunofluorescence

Organoids were fixed with 10% formalin, permeabilized in Triton X-100 (Sigma-Aldrich, cat. no. 9036-19-5), and blocked with 5% bovine serum albumin (BSA) in PBS. The slides were incubated with primary antibodies against *CD44* and *CD133* (1:200, Affinity, Colorado, United States,) overnight, washed 3 times with PBS, and stained with the appropriate secondary antibody along with phalloidin (Thermo Fisher Scientific, Delaware, United States). The nuclei were counterstained using Hoechst (Thermo Fisher Scientific). All the samples were mounted using ProLong Gold (Thermo Fisher Scientific). Fluorescent cells were recorded and photographed using a Leica fluorescence microscope.

In situ hybridization

To determine EBV status, paraffin-embedded tissues underwent ISH using oligonucleotides complementary to *EBER* transcripts. An *EBER ISH* kit was used in accordance with the manufacturer's instructions (Zhongshan Golden Bridge Biotechnology, Beijing, China). Appropriate negative and positive controls were used.

Statistical analysis

All data were represented as mean \pm the standard deviation calculated from 3 independent experiments. P values <0.05 were considered statistically significant.

Results

Generation of NPCOs and NMOs

In January 2021, Professor Sasidharan Swarnalatha Lucky published an article saying that PDX-derived nasopharyngeal carcinoma organoids were established for the study of nasopharyngeal carcinoma radiotherapy (19). In June of the same year, Professor Deng publicized the establishment of nasopharyngeal carcinoma organoids for the study of the molecular mechanism of nasopharyngeal carcinoma (20). However, both organoids have the defects of high culture cost, complicated operation process and low success rate. In this study, we developed a improved protocol to generate NPCOs. NPC tissues were minced into small pieces with ophthalmic scissors. The pieces were digested and subsequently passed through a cell strainer. We found that Matrigel could help to generate NPCOs (Figure S1A). Previous studies had found that several cytokines, including Wnt3a, R-spondin-1 (Rspo-1), EGF, Y27632, Noggin, and FGF2, were important for organoid generation (21-24). To develop an optimized culture medium for NPCO, we detected the growth of NPCOs in conditioned media with different concentration gradients of each cytokine. We found that the optimal concentrations of Wnt3a, Rspo-1, EGF, Y27632, Noggin, and FGF2 were 250, 500, 5, 10, 500, and 5 ng/mL, respectively (Figure S1B,S1C).

After the NPC cells had been cultured for 24 hours, NPCOs were generated (Figure S2). We used 62 NPC samples, including 34 primary and 28 recurrent NPC tissue samples, to generate the NPCOs. Information regarding these NPC samples, including the method of tissue obtention, patient sex and age, clinical stage, disease characteristics, lesion location, tissue mass, and tissue *ex vivo* time, is summarized in Table 1. A total of 39 NPCOs were successfully generated with an overall efficiency of 62.9%. Of these 39 NPCOs, 16 were derived from primary NPC patients with no chemotherapy history, and 23 were derived from patients with recurrent NPC (Table 1). The success rates of primary and recurrent NPCO formation were 47.06% and 82.14%, respectively (P=0.004). For the 28 recurrent NPC samples, the success rates of organoid generation from primary and metastatic lesions were 87.5% and 50%, respectively (Table 1). The biopsy mass of the NPC tissues also affected the NPCO generation, which might have been due to the NPC cell numbers (P=0.009). The success rate of the NPCO generation was not affected by age or sex (P=0.916 and 0.114, respectively), but it was significantly influenced by the method used to obtain the tissue, disease characteristics,

Table 1 Relationship between the success rate of the NPCO culture, clinical characteristics, and tissue characteristics

Variables	Grouping	Cases	No. of successful cases	Success rate (%)	P value
Method of tissue obtention	Biopsy	31	13	40	0.0006***
	Surgery	31	26	83.87	
Sex	Male	35	25	71.43	0.114
	Female	27	14	51.85	
Age, years	≤45	30	18	60	0.916
	45–59	23	15	65.22	
	>59	9	6	66.67	
Clinical stage	III	32	20	62.5	0.944
	IV	30	19	63.33	
Primary/recurrent NPC	Primary	34	16	47.06	0.004**
	Recurrent	28	23	82.14	
Lesion location	Primary focus	58	27	46.55	0.784
	Metastasis	4	2	50	
Tissue mass	≥50 mg	49	38	77.08	0.001**
	<50 mg	13	1	3.22	
Tissue <i>ex vivo</i> time	≤12 h	35	25	71.43	0.023*
	12–24 h	18	12	66.67	
	24–48 h	9	2	22.22	

Among them, 30 of 31 biopsy samples were obtained by endoscopy and only 1 case was from pleural metastases of nasopharyngeal carcinoma metastases. *, P<0.05; **, P<0.01; ***, P<0.001. NPCO, nasopharyngeal carcinoma organoid.

tissue mass, and tissue *ex vivo* time (P=0.0006, 0.004, 0.001, and 0.023, respectively) (Table 1).

We then used nasopharyngeal mucosa specimens to establish NMOs. From 15 attempts, 13 NMOs were successfully generated. In contrast to the NPCOs, the NMOs presented 3 morphology types: regular vacuoles, irregular vacuoles, and irregular solid shapes (Figure 1A). The vacuolated NMOs were similar to the lumen of the nasopharyngeal mucosa gland, and their diameters ranged from 200 to 250 μm (Figure 1A). However, the NPCOs showed a regular spherical shape with a diameter of 150 to 200 μm (Figure 1A). H&E staining showed that the NMO cell morphology was consistent with that of its corresponding parent nasopharyngeal mucosa tissue (Figure 1B). NMOs, like nasopharyngeal mucosa tissues, were well-differentiated, and their cell and nucleus sizes were uniform (Figure 1B). However, the NPCO and NPC tissues were poorly differentiated in H&E staining (Figure 1C). Furthermore, IHC showed the NMOs to be CK7 positive,

while the NPCOs were CK7 negative, consistent with the IHC results for NPC tissue (Figure 1D).

Almost 100% of NPC cases are associated with EBV infection. We performed EBER ISH assays and found that each NPCO and its parental NPC tissue were EBER positive (Figure 1E).

Overall, we successfully generated NMOs and NPCOs and found that the NPCOs could maintain the features of NPC tumors, including EBV infection status and clinical characteristics.

Composition of stem cell-derived NPCOs

Previous studies have suggested that organoids be derived from stem cells and generated via special differentiation pluripotent stem cells (PSCs) (24,25). Most NPC tissues are poorly differentiated. We performed IF assays to detect the expression of typical stem cell markers, including *CD44* and *CD133*. The IF results showed that *CD44* and *CD133*

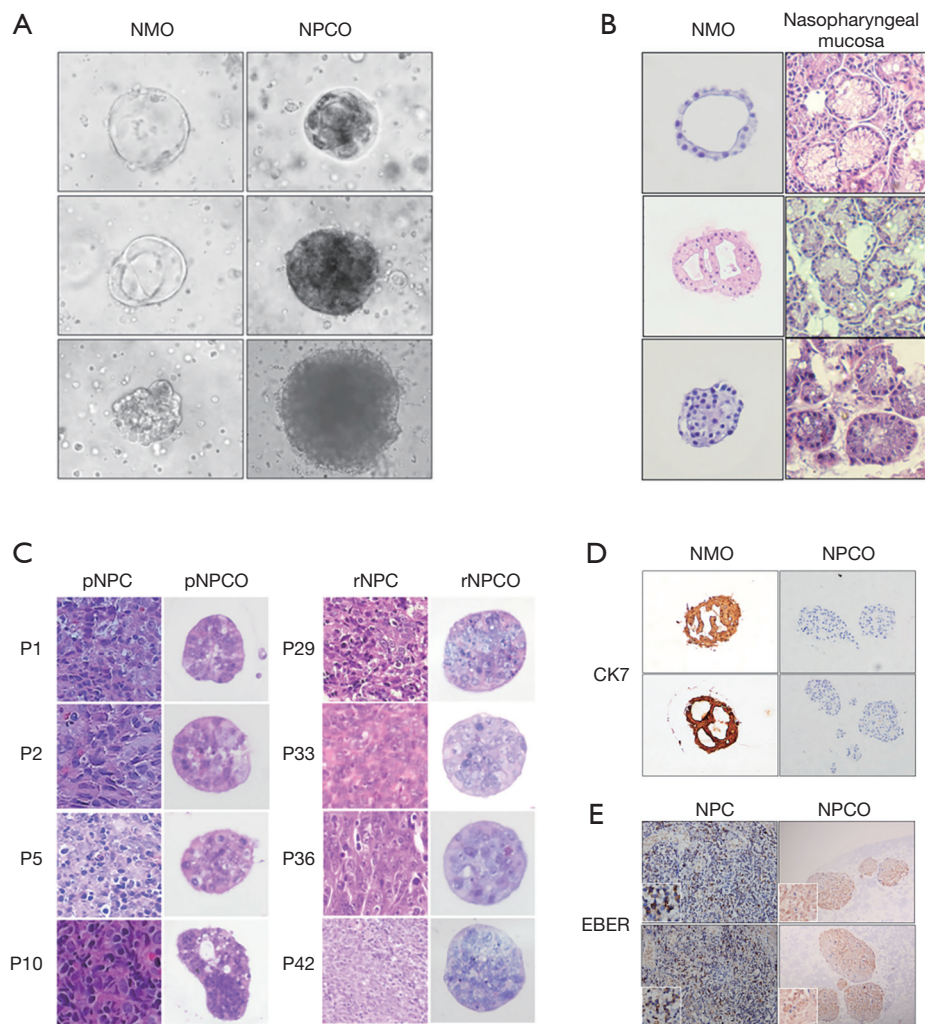


Figure 1 Morphological and histopathological profiles of NMOs and NPCOs. (A) The morphology of NMOs and NPCOs under light microscopy; (B) H&E staining results for NMOs and their corresponding tissues; (C) H&E staining results for NPCOs and their corresponding NPC tissues; (D) the IHC results for CK7 in NMOs and NPCOs; (E) EBER ISH results for NPCOs and their corresponding NPC tissues. 40× magnification used for organoid models and 20× for tissues. NMO, nasal mucosa organoid; NPCO, nasopharyngeal carcinoma organoid; H&E, hematoxylin and eosin; pNPC, primary NPC; pNPCO, primary NPCO; rNPC, recurrent NPC; rNPCO, recurrent NPCO; IHC, immunohistochemistry; EBER, Epstein-Barr virus (EBV)-encoded small RNAs; ISH, in situ hybridization.

were widely distributed throughout the NPCOs, which indicated that the NPCOs were stem-like cells (*Figure 2A*). Previous data have indicated that recurrent NPCOs are more easily generated than primary NPCOs. To determine the difference between primary and recurrent NPCOs, we analyzed the expression levels of stem cell markers. Our results showed that the percentage of *BMI-1*-expressing cells was higher (>80%) in the recurrent NPCOs than in the primary NPCOs (*Figure 2B*). We speculated that a higher expression of *BMI-1* might aid in the generation

of NPCOs, leading to the higher success rate of recurrent versus primary NPCO generation.

Challenges and expectations for the establishment of a recurrent NPCO biobank

We attempted to cryopreserve 39 NPCOs in liquid nitrogen for 6 months. Of these, 32 organoids were successfully revived. The NPCOs maintained good cell viability after long-term cryopreservation (*Figure 3A*). These 32 NPCOs

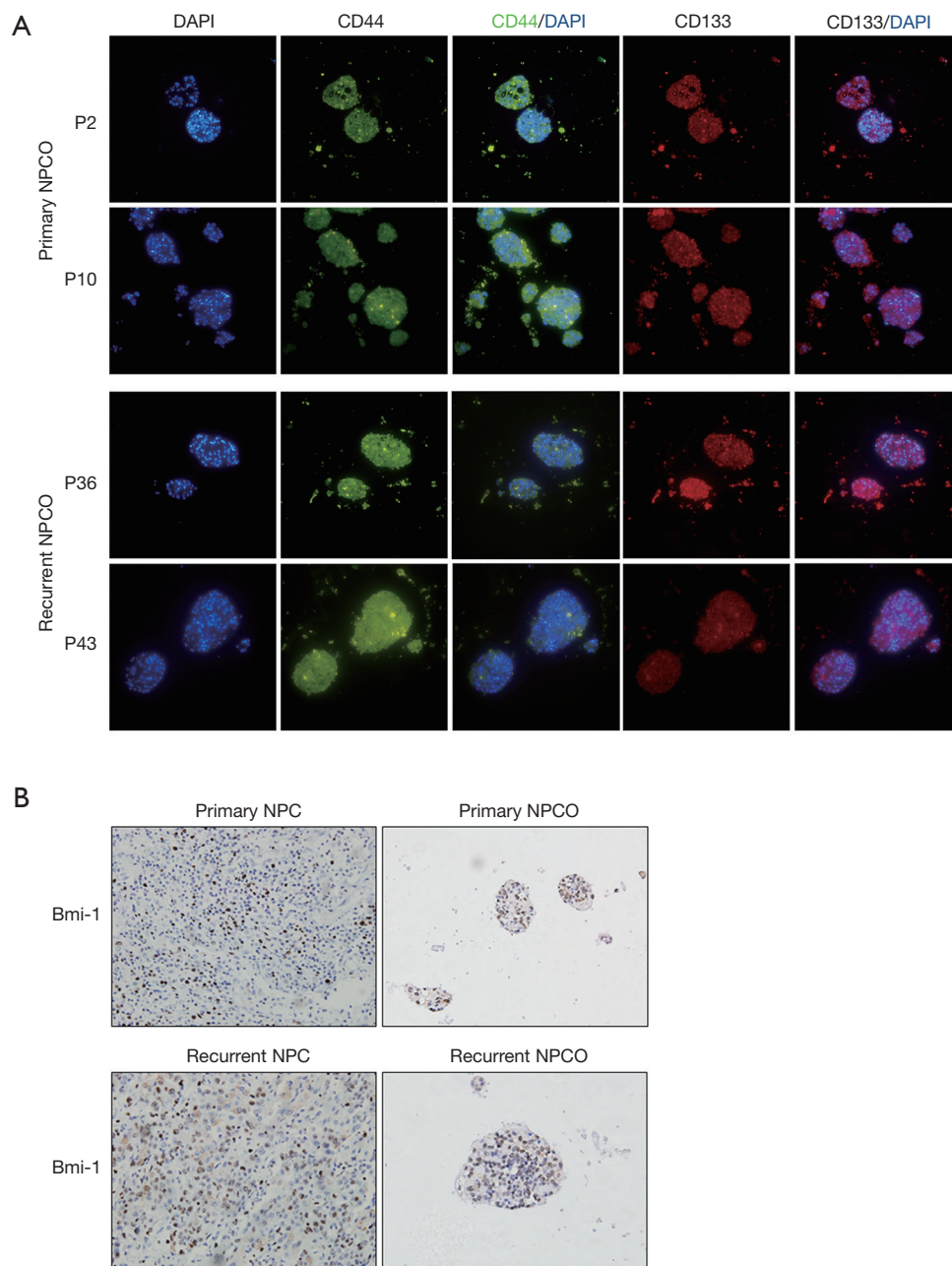


Figure 2 Detection of the stemness of NPCOs. (A) IF results for the stem cell markers CD44 and CD133 in the primary and recurrent NPCOs; (B) IHC results of BMI-1 in the primary and recurrent NPCOs and NPC tissues. 40× magnification used for organoid models and 20× for tissues. IF, immunofluorescence; NPCO, nasopharyngeal carcinoma organoid; IHC, immunohistochemistry.

were passed for four generations while maintaining NPC cell viability. All the NPCOs had good cell viability within two generations (*Figure 3A*). After 4 generations, some of the NPCOs, like NPCO-P42 and NPCO-P52, grew more slowly, became blurry, and disintegrated, and some NPCOs, including NPCO-P44, did not meet the experimental

criteria due to the small number and diameter of the organoid spheroids. In some NPCOs, like NPCO-P48, fibroblasts were activated and became dominant, significantly inhibiting organoid growth in 1 instance (*Figure 3A*). The fibroblasts used the cytokines necessary for organoid growth and enclosed the whole organoid, restricting its growth

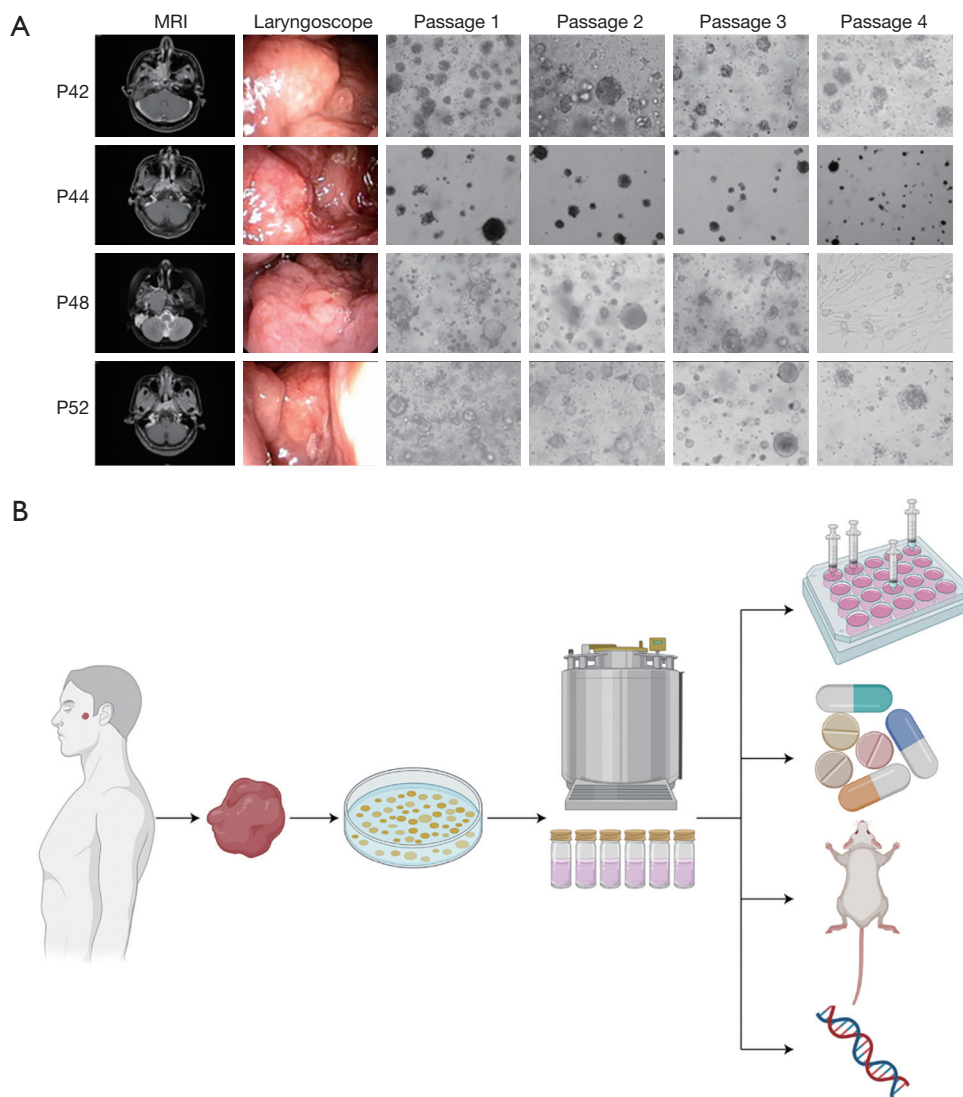


Figure 3 Challenges and expectations for the establishment of a recurrent NPCO biobank. (A) Morphological profiles of NPCOs passed for 4 generations, maintaining NPC cell viability after cryopreservation and recovery under C5 light microscopy; (B) potential workflow and application for a living biobank of NPCOs. 20× magnification used for organoid models. NPCO, nasopharyngeal carcinoma organoid.

(Figure 3A). Because of these issues, we determined that the continued cultivation of NPCOs is currently difficult but worth investigating in the future.

A biobank of recurrent NPC is still in its infancy. A tumor organoid biobank can recapitulate tumor heterogeneity and personalized genetic information (26–28), and a living biobank provides a rich resource and convenient platform for studying disease pathogenesis and enables the development of precision medicine (29). Here, we showed the potential workflow for establishing and applying a living biobank of recurrent NPCOs. The recurrent NPCOs

could be deposited in a living biobank with detailed clinical information, then used for precision medicine, the development of new pharmaceuticals, experimental studies, and the construction of patient-derived organoid xenografts (PDOXs) for further basic and clinical research on recurrent or refractory NPC (Figure 3B).

Discussion

Immortalized cell lines are the most commonly used model in NPC research. Almost 100% of NPC cases are associated

with EBV infection, but EBV genome were found lost in most of NPC cell lines (13,14). Additionally, several NPC cells have been found to be contaminated with HeLa cells. More and more scholars have raised questions: can cancer cell lines maintained the individual difference of the heterogeneity background and genetic characteristics after long-term *in vitro* culture and passage?

Patient-derived tumor xenografts (PDXs) have been used to establish tumors for NPC studies. But the low success rate and high cost limits the large-scale application of PDX model (30,31). Here, we established a patient-derived organoid model to overcome the drawbacks of existing NPC disease models. Nasopharyngeal carcinoma organoids can be used to screen out sensitive drugs for nasopharyngeal cancer cells in patients, guide clinicians to provide individualized drug regimens for patients with refractory nasopharyngeal carcinoma, improve the cure rate of patients, and prolong the life of patients. To provide reliable drug susceptibility data for the promotion of precise treatment of nasopharyngeal cancer.

Nasopharyngeal carcinoma tissue is usually obtained by endoscopic biopsy. Small tissue volume, low content of tumor cells and poor cell activity makes it a great challenge for *in vitro* culture of NPC organoid. In this study, we developed an optimal culture medium for NPCO. Matrigel is a reconstituted extracellular matrix containing metalloenzymes, cytokines, and laminin, which can promote cellular proliferation (32,33). Our result showed collagen II can also provide the scaffold function required for 3D growth of NPCO, but Matrigel mimic the *in vivo* tumor microenvironment during long-term culture. We also optimized the concentrations of Wnt3a, Rspo-1, EGF, Y27632, Noggin, and FGF2 in the NPCO culture medium. The Wnt/ β -catenin signaling pathway is involved in NPC stem cell proliferation and self-renewal regulation in many tissues (13,34). Several studies have reported that *Wnt3a* can promote the differentiation of olfactory epithelium stem cells (32,33). EGF treatment activates *EGFR* signaling and promotes NPC cell proliferation and cell cycle progression (35). Similarly, FGF2 can promote stem cell division, proliferation, and apoptosis inhibition by activating the PI3K/PKB signaling pathway (36). Y27632 is known to be a selective inhibitor of the Rho-associated kinase p160-ROCK that reduces the loss of stem cells (21). The latest studies suggest that Y27632 promotes the establishment of NPC cell lines from NPC tissues (26,37). In this study, we generated NPCOs using our optimized NPCO culture medium.

We attempted to generate NPCOs using 62 NPC tissue

samples. A total of 39 NPCOs were generated successfully. The success rates for primary and recurrent NPCOs were 47.06% and 81.25%, respectively. Almost 100% of NPC cases are associated with *EBV* infection, and we found that all our NPCOs carried *EBV* and maintained viral expansion. The NPCOs were mainly composed of cancer stem cells that exhibited rapid proliferation in the organoid culture medium. Additionally, stem cell markers were more highly expressed in the recurrent NPCOs than in the primary NPCOs. We believe that a higher expression of stem cell markers increases the success rate of the recurrent NPCOs.

However, there were some challenges to generating NPCOs, including the overexpansion of fibroblasts. NPC tissues have many infiltrated lymphocytes (20). To date, it has been difficult to maintain a coculture of lymphocytes and NPC stem cells to form a tumor microenvironment. It has been reported that T and B cell lymphocytes can secrete some cytokines to control the growth of fibroblasts (38,39). Schäffer *et al.* found that after several passages, immune cells stopped growing and gradually died off in the NPCOs and that concentrations of *TGF- β* and *TNF- α* decreased and could not inhibit the growth of fibroblasts, leading to the expansion of fibroblasts (40). Therefore, we believe that inhibiting the growth of fibroblasts is important for extending the passage numbers of NPCOs and that this is worth further investigation.

In conclusion, we successfully established a stable and efficient NPCO model using NPC tissues. All our NPCOs were *EBV* positive. We found that the NPCOs could retain the heterogeneity of parental tumors and recapitulate their pathophysiological characteristics. A biobank of NPCOs generated using tissues from patients with recurrent and refractory NPC has the potential to help us develop new anticancer agents, investigate the tumorigenesis mechanism, and develop individual therapies.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Biopsy and surgical resection samples were collected after written informed consent forms were obtained from the patients, and all related procedures were performed with the approval of the Research Ethics Committee at the Nanfang Hospital of the Southern Medical University (approval no. TCHIRB-10701103-E).

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