Clinical Study Protocol

Cover Page

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Abstract:

Precision oncology demands robust methodologies to guide the selection of effective, personalized therapies. We developed **OPTin** (Organotypic Platform for Therapeutic Investigation), a tissue slice–based functional drug screening platform that integrates ex vivo tissue slicing, optical clearing, and high-resolution confocal imaging to evaluate drug-induced apoptosis at spatial resolution. In a patient with advanced hypopharyngeal carcinoma, OPTin screening identified venetoclax as an effective agent against the primary tumor, although the metastatic lesion remained resistant. A second OPTin analysis using metastatic tissue identified lenvatinib as a sensitizing agent. The combination therapy of venetoclax and lenvatinib led to a near-complete clinical remission and a marked reduction in serum AFP levels from 1077 to 371 ng/mL. These findings highlight the potential of OPTin to provide spatially resolved, functional assessment of drug responses, enabling tailored treatment decisions in complex oncologic scenarios. Based on this proof-of-concept, we aim to systematically evaluate OPTin in a prospective clinical trial across diverse cancer types.

Key Words:

Tissue slice culture, drug screening, cell apoptosis.

Introduction:

Precision medicine is transforming cancer treatment by replacing uniform protocols with therapies tailored to the genetic, molecular, and environmental profiles of individual patients and their tumors.[1,2] Tumor heterogeneity arises from genetically diverse subclones that coexist and evolve within a single tumor, contributing to varied therapeutic responses.[3] To address this complexity, precision tools such as multi-region sequencing and biomarker testing reveal actionable mutations and predictive markers, enabling molecularly guided therapies with improved efficacy over conventional chemotherapy.[4] By aligning treatment strategies with tumor-specific biology, precision medicine intends to enhance survival outcomes, reduces treatment-related burden, and offers the potential for durable disease control.

Three-dimensional culture systems better replicate the in vivo tumor microenvironment than traditional 2D models by preserving cell–cell and cell–matrix interactions, biochemical gradients, and tissue-specific barriers to drug delivery.[5] Among these, organotypic tissue slice cultures offer a physiologically relevant, cost-effective platform that retains native extracellular matrix and immune components.[6] Originally used to evaluate chemotherapeutic efficacy[7], this approach has been extended to immune checkpoint[8] and CAR-T[9] therapies. While the approach holds promise for ex vivo drug testing, its clinical application in guiding patient-specific treatment decisions remains limited due to current technological constraints in drug screening and reproducibility.[6] In particular, the conventional analytical method–relying on slicing tissue sections into 3–5 μm thin sections for immunohistochemical (IHC) analysis–may introduce sampling bias, contributing to poor reproducibility across experiments.

To address the need for physiologically relevant, patient-specific drug testing, we developed **OPTin** (Organotypic Platform for Therapeutic Investigation), a screening system that integrates organotypic tissue slice cultures, optical clearing, and high-resolution fluorescence imaging. This platform enables spatially resolved assessment of drug-induced apoptosis within preserved tumor microenvironments and supports rapid evaluation of multiple agents for screening, including combination therapies. In our previous study, OPTin was applied to head and neck cancer specimens to assess the efficacy of chemotherapeutics, targeted drugs, and immune checkpoint inhibitors, with the goal of guiding compassionate treatment in late-stage disease.

Methods:

Clinical background of the patient

A 54-year-old male presented with persistent hoarseness and was eventually diagnosed with poorly differentiated carcinoma of the hypopharynx, exhibiting yolk sac tumor features. He initially deferred standard treatment and re-presented two months later with acute airway obstruction, necessitating emergency endotracheal intubation followed by tracheostomy. He received the bleomycin/etoposide/cisplatin (BEP) chemotherapy from November 2023 to February 2024, resulting in a partial response with decreased serum AFP levels. However, disease progression ensued, marked by rising AFP levels and new cervical lymph node metastases with extranodal extension. Re-biopsy of the cervical lymph node revealed a carcinoma with concurrent germ-cell-like and squamous differentiations. A second-line regimen of gemcitabine and oxaliplatin (GemOx), followed by pembrolizumab plus cisplatin/5-FU (PF), failed to control disease progression. Due to progressive airway compromise, a second tracheostomy was performed in December 2024, followed by concurrent chemoradiotherapy administered from December 2024 to January 2025. The patient's therapeutic timeline, including the administration of treatment regimens, is depicted in Figure 1A.

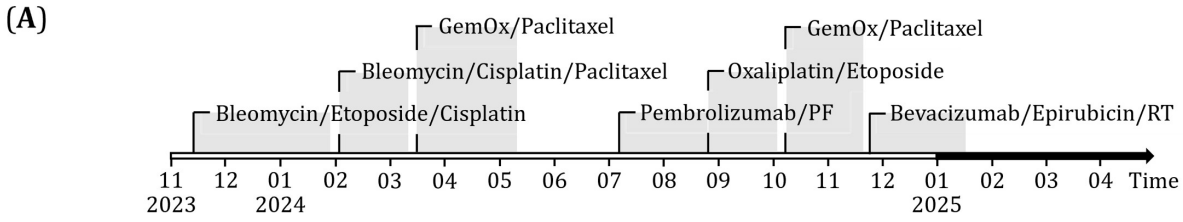


Figure 1. Clinical course and histological and immunohistochemical analysis.
(A) Timeline summarizing the treatment history from November 2023 to April 2025.

OPTin analysis

The tissue specimen (approximately $(0.5\ cm)^3$) was sectioned into 500 μm slices with a tissue matrix within 2 hours after surgical removal from China Medical University Hospital. Tissue slices were placed on SPONGOSTAN™ sponges (Ethicon, #MS0005) and cultured in RPMI medium (Gibco, #31800105) supplemented with 20% fetal bovine serum (Corning, #35-010-CV) and antibiotic–antimycotic solution (Corning, #30-004-CI) in a humidified incubator at 37°C with 5% CO_2. A total of 15 agents, including single and combination therapies, were evaluated in this study. The selection of investigational drugs was guided by institutional approval and constrained by the patient's financial accessibility. Each drug was administered at a concentration of 10 μM[10], and tissue slices were incubated for two days to allow drug exposure. Following this, tissue slices underwent fixation for one day, permeabilization, and subsequent immunostaining, which spanned a total of ten days. Apoptosis was visualized using cleaved caspase-3 antibody staining, while cell nuclei were identified by DAPI staining. In groups treated with immune checkpoint inhibitors, additional

staining for IFN-γ was performed to assess immune cell activation. After immunostaining, the samples were then processed using SeeDB tissue clearing protocol[11], a technique that enhances optical transparency for deeper imaging, over a five-day period. Cleared tissues were imaged using Dragonfly confocal microscopy with optical sections acquired at 5-μm intervals. For tissue slices measuring 500 μm in thickness, a total of 100 sequential layers were scanned and digitally reconstructed into a composite three-dimensional image, enabling detailed visualization and analysis of drug responses at both the cellular and subcellular levels. Thus, this procedure takes total 18 days to identify which drugs might be positive.

Preliminary Results:

Histopathological and immunohistochemical analysis

Despite multiple lines of treatment, the therapeutic response of the patient with the head and neck cancer remained limited (Fig. 1A and Method), prompting a re-evaluation of the tumor's pathological and molecular features through discussion in a multidisciplinary molecular tumor board. Histopathological analysis of the primary and metastatic tumor tissues revealed a squamous cell carcinoma with germ cell-like differentiation (Fig. 1B). Immunohisto-chemically, sal-like protein 4 (SALL4) and alpha-fetoprotein (AFP) are germ cell markers, cytokeratin 7 (CK7) is a non-keratinizing epithelial marker and p40 is a squamous differentiated marker. The initial biopsy of primary hypopharyngeal tumor revealed sheeting basophilic, round tumor cells exhibiting co-expression of CK7 and SALL4, but lack of p40 and AFP expression in the solid and glandular (yolk sac–like) patterns (Fig. 1C), suggestive of germ cell–like differentiation. Notably, a repeat biopsy of the primary hypopharyngeal tumor demonstrated strong AFP expression in the germ cell–like tumor cells, supporting germ cell-like tumor cells with a yolk sac differentiation (Fig. 1E-G). The metastatic carcinoma within the cervical lymph node shared partial histological similarity to the primary lesion, including the presence of germ cell–like differentiated components. However, regions of well-differentiated squamous cell carcinoma with prominent keratinization were also identified (Fig. 1D). Immunohistochemical stain demonstrated diffuse p40 positivity in the squamous components, while germ cell–like regions remained p40-negative, indicating divergent differentiations within the same lesion (Fig. 1H-I).

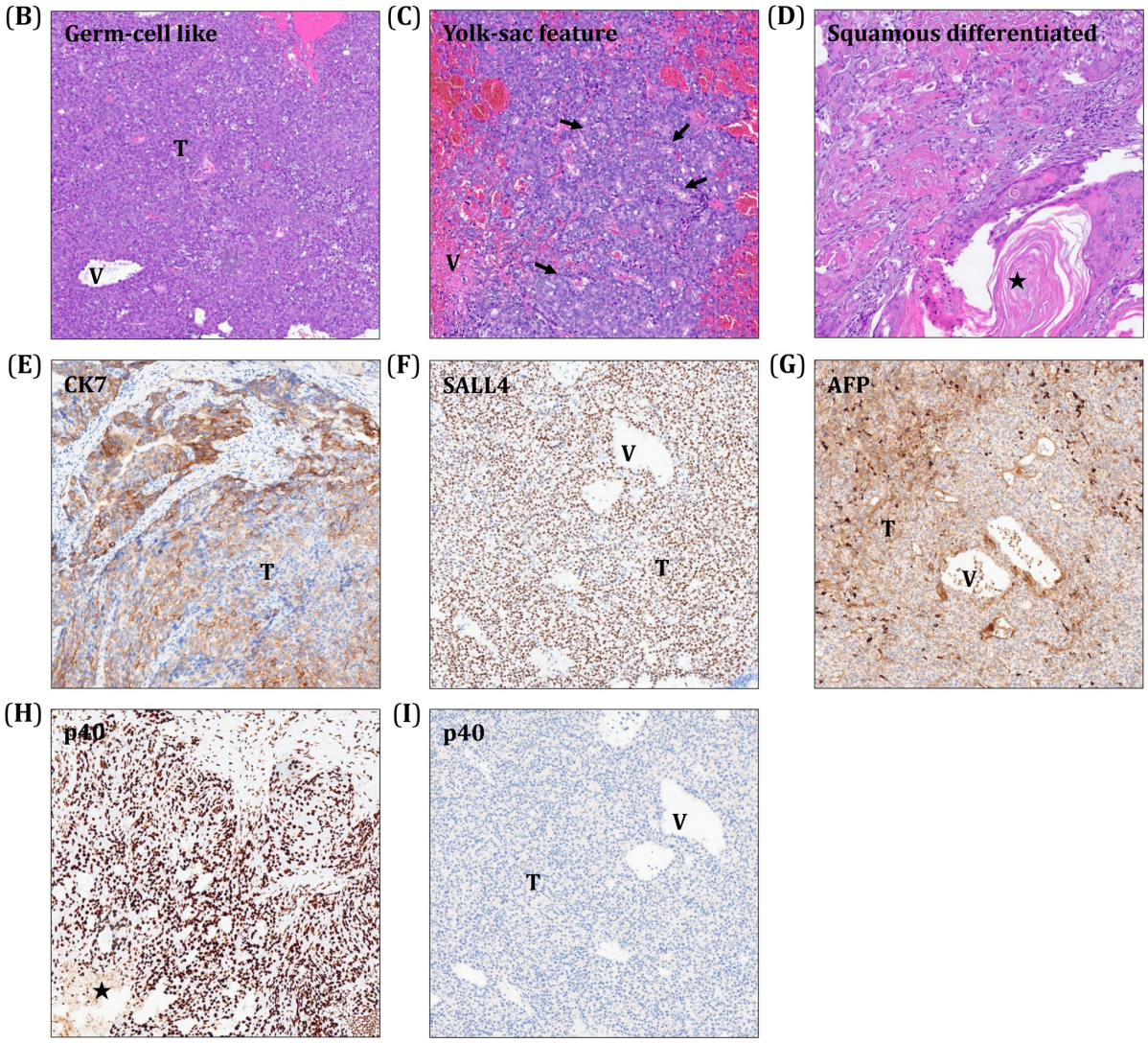


Figure 1. Clinical course and histological and immunohistochemical analysis.

(B and C) Representative H&E-stained sections demonstrating the primary hypopharyngeal carcinoma exhibiting round to polygonal tumor cells with germ cell-like differentiation [T] and yolk sac features (arrow) in a vascular-rich [V] stroma. (D) H&E-stained sections demonstrating a metastatic carcinoma in a lymph node with coexisting germ cell-like differentiation as the same as primary lesion, and squamous differentiation with keratin formation [★]. B to D, original magnification, 200x. (E to I) Immunohistochemically, germ cell-like tumor cells showing positive reactivity to CK7 (E), SALL4 (F) and AFP (G), confirming the germ cell-like nature. The p40 showing a diffuse nuclear positivity in squamous differentiated components (H) and a negative staining in germ cell-like components (I). Brown color as a positive staining. E to I, original magnification, 100x.

Whole exome sequencing (WES) analysis

To identify potential therapeutic targets, WES[12] was performed on both the primary and metastatic tumors of the patient with poorly differentiated hypopharyngeal adenocarcinoma

exhibiting yolk sac features. Shared mutations were identified in *TP53*, *NOTCH1*, *FAT1*, and *CSF1R*, while the metastatic lesion also harbored *PIK3CA* and *NFE2L2* alterations. These findings suggest potential avenues for targeted therapy, including PI3K/AKT/mTOR and CSF1R-mediated pathways. While *PIK3CA* (p.E542K) is a well-characterized activating mutation and may confer sensitivity to PI3K inhibitors such as alpelisib, its role in head and neck cancer has only recently been evaluated in clinical trials.[13] However, similar to the *CSF1R* mutation (p.V784M), which was detected at a low variant allele frequency, the *PIK3CA* mutation may represent a passenger alteration rather than a true driver event, thereby limiting its therapeutic relevance. Together, the co-occurrence of multiple mutations and the genomic divergence between primary and metastatic sites highlight the complexity of this tumor and the limitations of genomics alone in guiding treatment selection, underscoring the need for integrated functional platforms in precision oncology.

OPTin analysis

To address these therapeutic challenges, we developed OPTin (Fig. 2A), a functional drug screening platform that integrates ex vivo organotypic tissue slice culture with tissue clearing and high-resolution confocal imaging to assess patient-specific drug responses within a physiologically relevant microenvironment. Application of OPTin to the patient's primary hypopharyngeal tumor tissue revealed the most extensive apoptosis following venetoclax treatment, indicating its potential as an effective therapeutic agent (Fig. 2B). The average fluorescence intensity (aFI) of cleaved caspase-3, a marker of apoptosis, was displayed in the lower left corner of each fluorescence image. Notably, the administration of venetoclax as a compassionate therapy (IRB identifier: CMUH110-REC2-097 by the Ethics Committee of China Medical University Hospital, Taichung, Taiwan) in this case led to complete regression of the hypopharyngeal tumor (Fig. 2C).

Although venetoclax demonstrated significant inhibitory activity against the primary hypopharyngeal tumor, it showed no apparent efficacy in the metastatic cervical lymph node tissue, which exhibited no signs of regression (Data not shown). To further investigate this discrepancy, we re-evaluated the apoptotic effects of the previously tested drugs on the metastatic lesion. Confocal fluorescence imaging revealed a strikingly differential drug response between the primary and metastatic tumors (Fig. 2B vs. 3A), highlighting the presence of intratumor heterogeneity, consistent to the WES analysis. In line with the observed lack of clinical response, the metastatic tumor showed resistance to venetoclax despite its efficacy in the primary site.

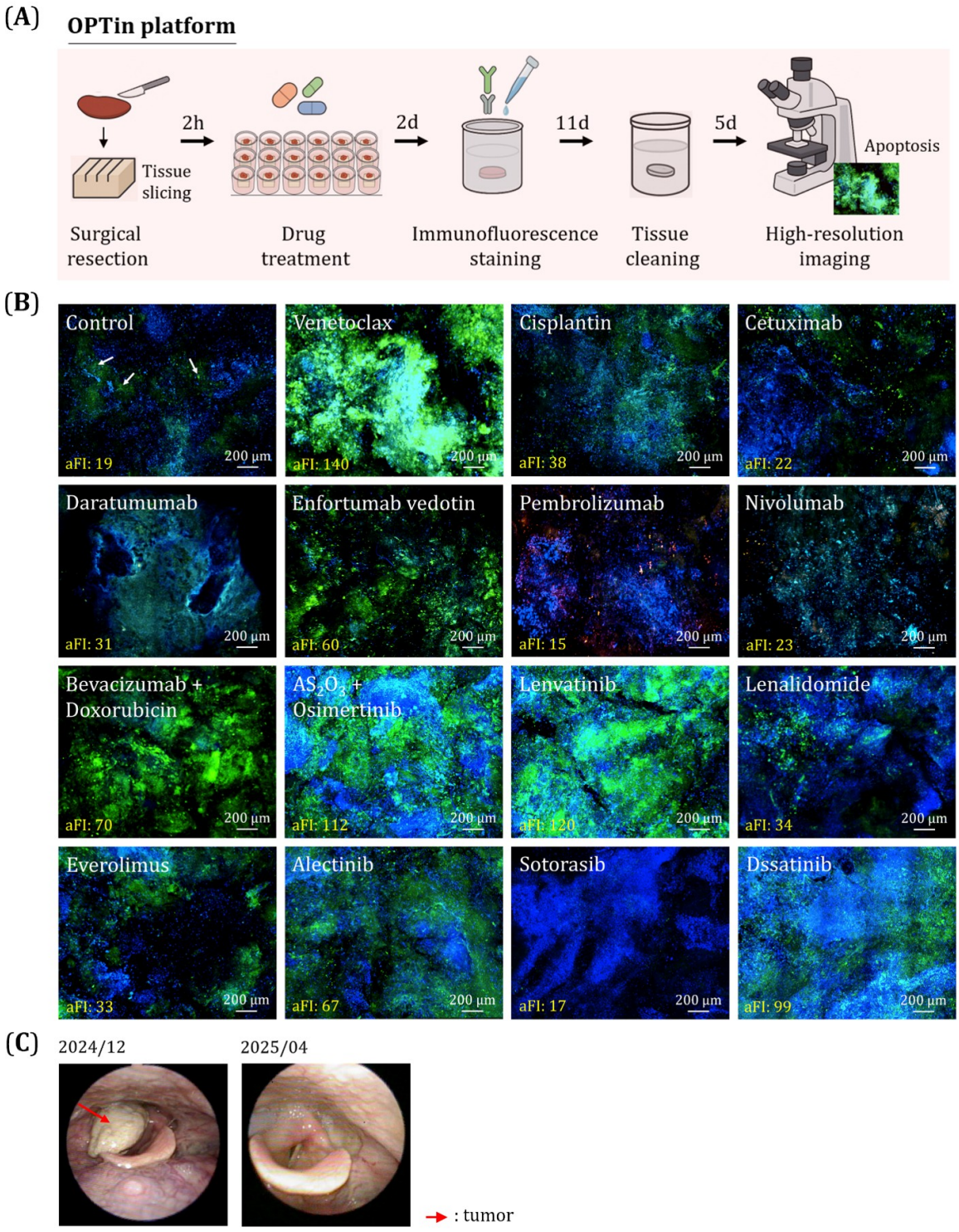


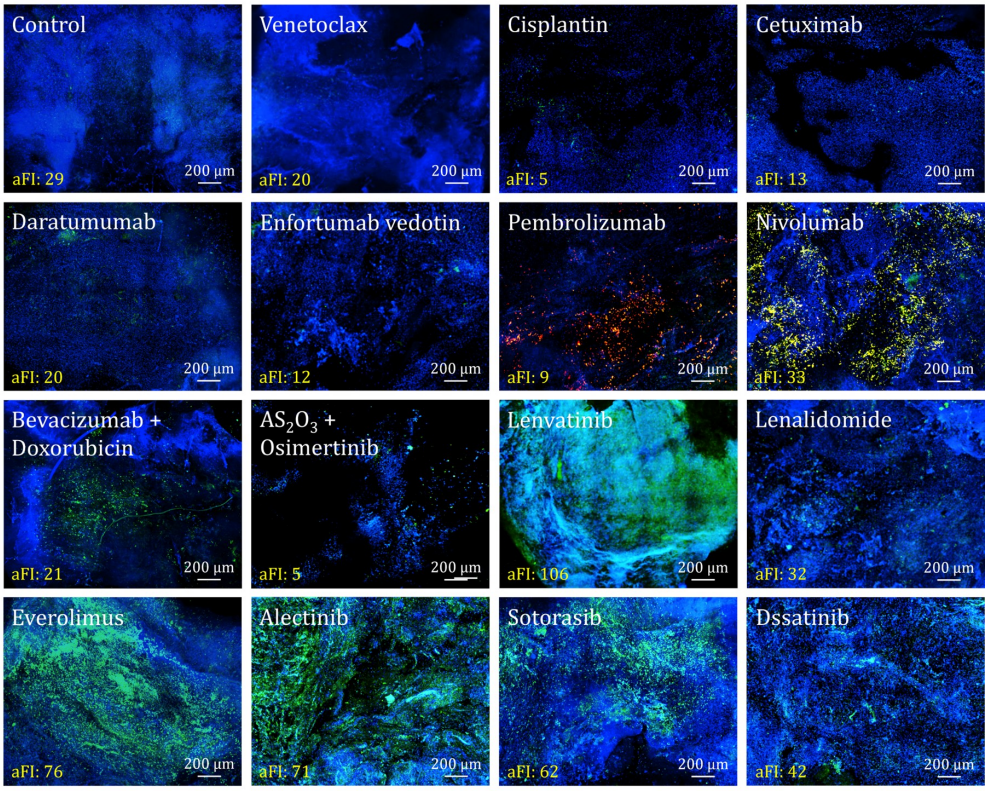
Figure 2. Drug-induced apoptosis profiling in primary hypopharyngeal carcinoma tissues using the OPTin platform.

(A) Schematic overview of the OPTin platform for ex vivo drug screening. The workflow integrates freshly resected tumor tissue slicing, drug treatment on sponge-supported culture, tissue clearing, immunofluorescence staining, and high-resolution confocal imaging to evaluate patient-specific therapeutic responses in a physiologically relevant

microenvironment. (B) Ex vivo tissue slices from the primary hypopharyngeal tumor were treated with the indicated therapeutic agents. Fluorescence imaging was performed using Dragonfly confocal microscopy. Green indicates cleaved caspase-3; DAPI stains nuclear DNA. The average fluorescence intensity (aFI; integrated density/area) was analyzed using ImageJ 1.54g software. Scale bars, 200 μm. (C) Representative fiberoptic nasopharyngolaryngoscopy images obtained before and after venetoclax treatment, showing complete regression of the hypopharyngeal tumor. Red arrow, tumor.

Among the agents tested, lenvatinib demonstrated the greatest apoptotic effect on the metastatic tumor tissue (Fig. 3A). Based on this finding, we initiated a second compassionate therapy combining lenvatinib with venetoclax. Following the combination treatment, the cervical lymph node metastasis decreased in size even 3 weeks post treatment. Computed tomography (CT) analysis revealed partial regression of the metastatic lesion, with residual soft tissue density still present in the left cervical region post-treatment (Fig. 3B). Concurrently, serum AFP levels declined significantly from 1077 to 371 ng/mL (Fig. 3C). These results suggest that the organotypic tissue slice drug screening platform may effectively guide drug selection in the context of complex tumor heterogeneity, offering a promising strategy to achieve personalized and therapeutically meaningful outcomes.

(A)



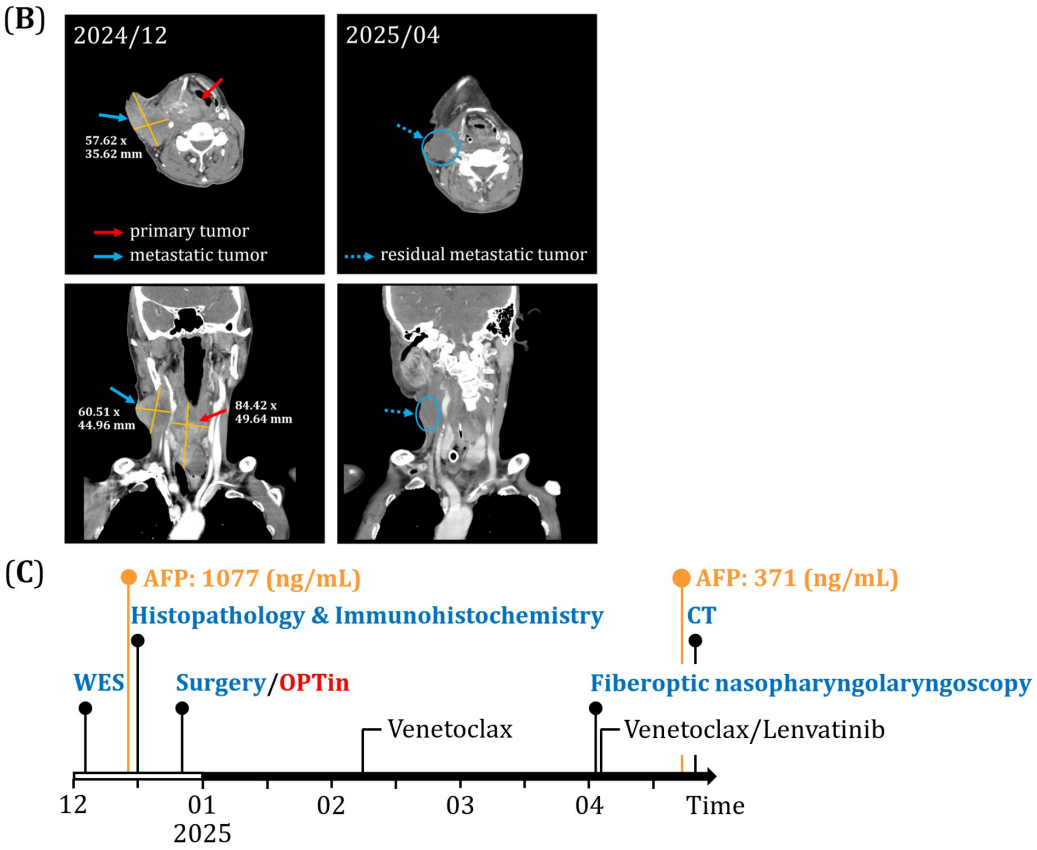


Figure 3. Drug-induced apoptosis profiling in metastatic lymph node carcinoma tissues using the OPTin platform.

(A) Ex vivo tissue slices from the metastatic lymph node tumor were treated with the indicated therapeutic agents. Fluorescence imaging was performed using Dragonfly confocal microscopy. Green indicates cleaved caspase-3; DAPI stains nuclear DNA. The average fluorescence intensity (aFI; integrated density/area) was analyzed using ImageJ 1.54g software. Scale bars, 200 μm. (B) Axial and coronal CT images obtained before and after combined venetoclax and lenvatinib treatment. In December 2024, both the primary tumor (red arrow) and metastatic cervical lymph node lesions (blue arrow) were visible. By April 2025, the primary tumor had regressed, consistent with the findings shown in Fig. 2C; however, residual metastatic tumor (indicated by the blue dotted arrow) had decreased in size but remained detectable. Yellow line, the largest dimension of tumors. (C) Timeline of clinical interventions, monitoring, and therapeutic strategies. The key procedures include WES, surgery, the OPTin assay, histopathological evaluation, imaging studies, and endoscopic assessment.

Study Design

This study aims to enroll **200 subjects** from whom fresh tumor tissue specimens will be collected for ex vivo analysis. The OPTin platform will be used to evaluate the efficacy of **FDA-approved anticancer drugs** by screening their ability to induce apoptosis in tumor tissues across multiple cancer types. Based on the results of the OPTin assay, potential

therapeutic drug options will be recommended for patients who no longer have standard treatment options available.

If the treating physician elects to adopt the drug recommendation generated by the OPTin platform, imaging-based clinical evaluations of the subject will be collected at each scheduled assessment. The therapeutic response to the selected treatment will be monitored for **up to one year** following initiation of therapy.

Study Procedures and Laboratory Analyses

Fresh tumor tissue specimens obtained during surgery must be **larger than 0.5 cm^3**. Immediately after surgical collection, the tissue will be sectioned into **500 μm slices** using a vibratome. Tissue slices will then be cultured and exposed to candidate drugs for **48 hours**.

Following drug treatment, the tissue slices will undergo **immunofluorescence staining** and **tissue clearing procedures**. High-resolution **confocal imaging** will subsequently be performed to quantify drug-induced apoptosis and evaluate features of the **tumor microenvironment**. The results of these analyses will be used to generate drug sensitivity profiles to assist clinicians in selecting potential therapeutic agents.

Inclusion Criteria

Subjects must meet **all** of the following criteria to be eligible for participation in the study:

1. Adults **≥ 18 years of age**.
2. Willing and able to provide **written informed consent** prior to participation in any study-related procedures.
3. Willing to undergo surgical collection of tumor tissue during the screening period, with a specimen size **greater than 0.5 cm^3**.
4. Willing to allow the use of their **previous clinical test results and residual tissue samples** for study-related analyses.

Exclusion Criteria

Subjects meeting any of the following criteria will be excluded from the study:

1. Inability to obtain sufficient tumor tissue through surgical procedures.
2. The treating physician decides not to adopt the drug recommendation generated by the study platform.
3. The principal investigator determines that the subject is not suitable for enrollment in the study.

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Research Ethics Committee
China Medical University & Hospital, Taichung, Taiwan

Clinical Trial/Human Research Approval

Date：Oct. 15, 2025

Protocol Title：Repurposing drugs guided by tissue slice screening in cancer patients without standard treatment
Protocol No. / CMUH REC No.： / CMUH114-REC1-159
Name of Principal Investigator：Ching-Yun Hsieh (Attending Physician , Medical Oncology)
Name of Institution：China Medical University Hospital
Date of Approval：Oct. 15, 2025
Date of Expiration：Oct. 14, 2026
Protocol：Version 1.1, Date: Oct. 03, 2025
Chinese Synopsis：Version 1.0, Date: Aug. 26, 2025
English Synopsis：Version 1.0, Date: Aug. 26, 2025
Informed Consent Form：Version 3.0, Date: Oct. 14, 2025
Frequency of Continuing Review：once per every 12 months

This is to certify that the above referenced research project has been reviewed by the 2025 10th meeting of the Research Ethics Committee (REC) I of the China Medical University and Hospital on Sep. 17, 2025. The REC is organized under, and operates in accordance with, the Good Clinical Practices guidelines and the governmental laws and regulations. The frequency of continuing review for the research project determined by the REC is mentioned above. Please submit a completed progress report at least two months before the time at which continuing review must occur.

All the amendments to the research project should be re-submitted and approved by the REC BEFORE implementation. Also, the principal investigator is required to report all serious adverse events and unanticipated problems involving risks to the subjects or others on time.

Martin M-T Fuh MD. DM Sci.
Chairman, Research Ethics Committee I
China Medical University & Hospital

The Committee is organized and operates in accordance with ICH6 GCP regulations and guideline.