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Original Article

Patient-Derived Organoids from Multiple Sites of a Single Tumor Recapitulates Intratumoral Heterogeneity in Patients with Gastric Cancer

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Background/Aims: Patient-derived organoids (PDOs) are promising preclinical models that replicate critical tumor features. However, intratumoral heterogeneity challenges the clinical utility of PDOs, especially in capturing diverse tumor cell subpopulations.

Methods: Single-cell transcriptomics was used to analyze PDOs from distinct sites within a single gastric cancer tumor, aiming to assess their ability to reflect intratumoral heterogeneity.

Results: The PDOs displayed similarities in gene expression but also exhibited distinct profiles. Single-cell analysis of PDOs revealed upregulation of markers for neuroendocrine tumors, which was validated via immunohistochemistry staining of neuron-specific enolase in the primary tumor. Notably, heat shock proteins showed significant variability among the PDOs, impacting immune responses. Tumors with abundant heat shock proteins are reported to have increased cytotoxic T cell activity.

Conclusions: Intratumoral heterogeneity poses challenges for PDO-based models, highlighting the need for comprehensive assessment. Despite their limitations, PDOs offer valuable insights into precision medicine for patients with gastric cancer, aiding in the development of therapeutic strategies. **(Gut Liver, 2026;20:245-253)**

Key Words: Stomach neoplasms; Organoids; Tumor heterogeneity

INTRODUCTION

Patient-derived organoids (PDOs) are promising preclinical models due to their ability to replicate key characteristics of the original tumor. However, the challenge of intratumoral heterogeneity raises concerns about their representativeness. This versatile tool has significantly contributed to the advancement of precision medicine, enabling researchers to gain critical insights into the unique features of individual patients' tumors and to tailor therapeutic strategies accordingly.[1,2]

However, the clinical utility of PDOs can be compromised when dealing with tumors characterized by intratumoral heterogeneity. This phenomenon refers to the presence of distinct tumor cell subpopulations within a single tumor mass, each harboring unique genetic and phenotyp-

ic traits. Intratumoral heterogeneity poses a considerable challenge when attempting to generate PDOs, particularly when working with limited regions of the tumor for organoid culture. In such cases, there is a genuine concern that PDOs may fail to accurately recapitulate the full spectrum of tumor cell subpopulations present within the primary tumor.

In tumors exhibiting a high degree of intratumoral heterogeneity,[3,4] it becomes imperative to critically assess the extent to which PDOs can faithfully reproduce the diversity of cellular components within the tumor microenvironment. To address this challenge, we embarked on a comprehensive and in-depth analysis of the transcriptomic landscape at the single-cell level. Specifically, we focused our investigation on three distinct PDOs, each derived from different spatial locations within a single tumor mass.

The primary objective of our research was to elucidate the degree to which these PDOs, originating from discrete regions of the same gastric cancer (GC) tumor, accurately recapitulate the complex intratumoral heterogeneity observed in this malignancy. GC is a highly aggressive malignancy with well-documented biological complexity. Notably, GC frequently exhibits histological and molecular diversity even within a single tumor, reflecting a high degree of intratumoral heterogeneity.[5] This heterogeneity is thought to underlie variable therapeutic responses and disease progression in GC. Here, we aimed to provide a detailed and nuanced understanding of the extent to which PDOs can faithfully mirror the intricate molecular and cellular landscape of the GC tumor, which is known for its high degree of intertumoral heterogeneity.[2,6]

MATERIALS AND METHODS

1. Sample collection

GC specimen was collected via surgical resection. The resected stomach specimen was laterally unfolded to expose the cancer lesion. Each tumor fragment was obtained from four sites of a GC surgical specimen, and then marked as t1-t4. The minimum lateral distance between two collection points was 10 mm. The size of the tissue fragment for extracting RNA was set to 0.5–1.0 cm^3. The resected cancer tissue was placed in Advanced DMEM/F12 supplemented with 2% antibiotic-antimycotic (Cat#12634028; Gibco, Grand Island, NY, USA) and transported from the operating room to the laboratory in conical tubes. Detailed pathologic information about the tumor is summarized in Table 1. The study was approved by the Institutional Review Board of Gangnam Severance Hospital (IRB number: 3-2018-0209). Subject provided informed consent before enrollment in the study. The study was performed in accordance with the Declaration of Helsinki.

2. Generation of PDOs

GC surgical specimens were extracted from the lesion of a stomach cancer patient. All materials were coated with 0.1% bovine serum albumin to protection against cell loss. Every tissue was washed three times with phosphate-buffered saline and minced about into 1 mm^3 pieces. Pieces were transferred into a 15-mL conical tube and incubated in 8 mL of basal medium containing 0.5 mg/mL collagenase type I (Sigma-Aldrich, St. Louis, MO, USA) at 37℃ on a roller shaker for 1.5 hours. The digested pellets were passed through 70 μm mesh strainer, and undigested pellets were separated by pressing with a plastic stick. The cell suspension was washed two times with phosphate-buffered saline. After the final wash, the pellet was cooled on ice for a while, and an appropriate volume of Matrigel (Corning, Corning, NY, USA) was added. The carefully resuspended pellet was plated at 25 μL per well in a 48-well plate. We were already using the widely known complete medium for organoid growth. Cells were incubated at 37℃ for 1–2 weeks. The medium was changed every 2 to 3 days. The complete medium used contained 50% Wnt-3a conditional medium, 10% R-spondin conditional medium, 1 mM N-Acetylcysteine (Sigma-Aldrich), 1 nM [Leu]-Gastrin I (Sigma-Aldrich), 50 ng/mL mEGF (Invitrogen, Carlsbad, CA, USA), 100 ng/mL Noggin (PeproTech, Rocky Hill, NJ, USA), 200 ng/mL FGF10 (PeproTech), 10 mM nicotinamide (Invitrogen), 10 μM RHOKi Y-27632 (Wako, Osaka, Japan), and 1X (100 μg/mL) Primocin (Invitrogen). Organoids were cultured in 25-μL Matrigel droplets in 48-well plates and subcultured every 1 to 2 weeks. According to the experimental purpose, grown organoids were dissociated into small pieces or single cells and used.

Table 1. Clinicopathological Characteristics of the Patient

Characteristic	Value
Sex/age	M/87
Tumor size, cm	10.2×9.5
Pathologic stage (AJCC 8th)	T4aN3aM0
Location	Lower third
Histologic type	Tubular adenocarcinoma, poorly differentiated
Histologic type by Lauren	Intestinal
Immunohistochemical stain results	
p53	Focal positive
HER2	Negative
EBV *in situ* hybridization	Negative in tumor cells

AJCC, American Joint Committee on Cancer; HER2, human epidermal growth factor receptor 2; EBV, Epstein-Barr virus.

3. Single-nucleus RNA sequencing analysis

After the frozen tissue of the organoid was homogenized and nuclei were counted, the nuclei were isolated using flow cytometry. We utilized the 10X Genomics Chromium Instrument and cDNA synthesis kit (10x Genomics: Chromium Next GEM Single Cell 3′ Library and Gel Bead Kit v3.1; Pleasanton, CA, USA) to generate a barcoded cDNA library for single-nucleus RNA sequencing from sorted nuclei. cDNA library quality was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Using this library, we ran two paired-end 200bp flow cells on an Illumina NovaSeq 5000/6000 S1 Reagent Kit v1.5 (200 cycles) (read lengths: 28 bp Read1, 10 bp I7 Index, 10 bp I5 Index, and 90 bp Read2; Illumina, San Diego, CA, USA).

4. Immunohistochemistry and microsatellite instability status evaluation of tumor tissue

Immunohistochemistry (IHC) was performed using a Ventana XT automated staining instrument (Ventana Medical Systems, Tucson, AZ, USA). The following antibodies were used in accordance with the manufacturer's instructions: HER-2 (HercepTest Kit; Dako, Santa Clara, CA, USA), MutL homolog 1 (MLH1; clone M1; Roche, Indianapolis, IN, USA), MutS protein homolog 2 (MSH2; clone G219-1129; Roche), MutS homolog 6 (MSH6; clone 44; Cell Marque, Rocklin, CA, USA), and post-meiotic segregation increased 2 (PMS2; clone MRQ28; Cell Marque). Microsatellite analysis was performed using DNA extracted from tumor tissues, as previously described.[7] Tumors with microsatellite instability-high/mismatch repair-deficient were defined as those with the presence of at least two of the five instability markers in the Bethesda microsatellite panel (BAT25, BAT26, MFD15, D2S123, and D5S346) or the absence of one or more proteins (MLH1, MSH2, MSH6, and PMS2) based on IHC staining.

5. Transcriptome analysis

RNA sequencing data and clinicopathological variables on the Cancer Genome Atlas (TCGA) stomach adenocarcinoma (n=440) were downloaded from cBioPortal (www.cbioportal.org). The mRNA expression level was compared according to the $\log_2$ RSEM (Batch normalized from Illumina HiSeq_RNASeqV2) value.

6. Bioinformatic analysis

The FASTQ files from single-cell 5' profiling were analyzed using the 10X Genomics Cell Ranger software (v5.0.0), adhering to the recommended protocols. The GRCh38 reference genome was used for alignment. The R package Seurat was used to establish the object and detect cell clusters based on gene expression and to identify markers enriched in each cluster. Cells detected with fewer than 500 and more than 6,000 features, and those with mitochondrial genes higher than 5% were excluded from the analysis. Finding variable genes was computed using the "vst" method with 2000 features. Cell clustering was performed with 0.5 resolution and the results were visualized by uniform manifold approximation projection (UMAP). For differential genes, the FindMarkers function was used with the following options, only.pos=TRUE, min.pct=0.25, logfc.threshold=0.25.

7. Statistical analyses

Statistical analysis was conducted using the Student t test for comparisons between two groups. For multiple group comparisons, analysis of variance with *post hoc* tests was used. The p-values were adjusted with the Benjamini-Hochberg false discovery rate method in analyzing TCGA tumor data. A p-value <0.05 was considered statistically significant.

RESULTS

1. Patient background and tumor characteristics

GC is well-known for high degree of intratumoral heterogeneity.[4] An 87-year-old male underwent total gastrectomy after diagnosed with GC. The tumor was poorly differentiated adenocarcinoma staged T4aN3aM0 (Table 1). The primary tumor showed microsatellite instability without HER2 overexpression (Fig. 1A-F). Cancerous cells were collected from four different sites of the surgical specimen (Fig. 1G). PDOs were successfully generated from three sites among four collected sites (Fig. 1G). Hematoxylin and eosin staining of the primary tumor confirmed the existence of cancerous cells from four different sites of the specimen prepared for cell collection (Fig. 1H-K).

2. Single-nucleus RNA sequencing of PDOs

Next, we performed single-nucleus RNA sequencing analysis on GC organoids originated from different locations (t2, t3, and t4) of the single tumor. Single-nucleus sequencing of three PDOs resulted in transcriptomic information for a total of 13,041 cells (Fig. 2A). As in the primary tumor, mRNA expression of ERBB2 was not amplified (Fig. 2B). CD44, a marker of cancer stem cells, also showed uniform distribution across the PDOs (Fig. 2C). Interestingly, genes highly elevated in the PDOs included neuropeptide S receptor 1 and enolase 2, which are markers for neuroendocrine tumors (Fig. 2D and E).[8,9] To validate these findings from single-cell transcriptomic analysis, we

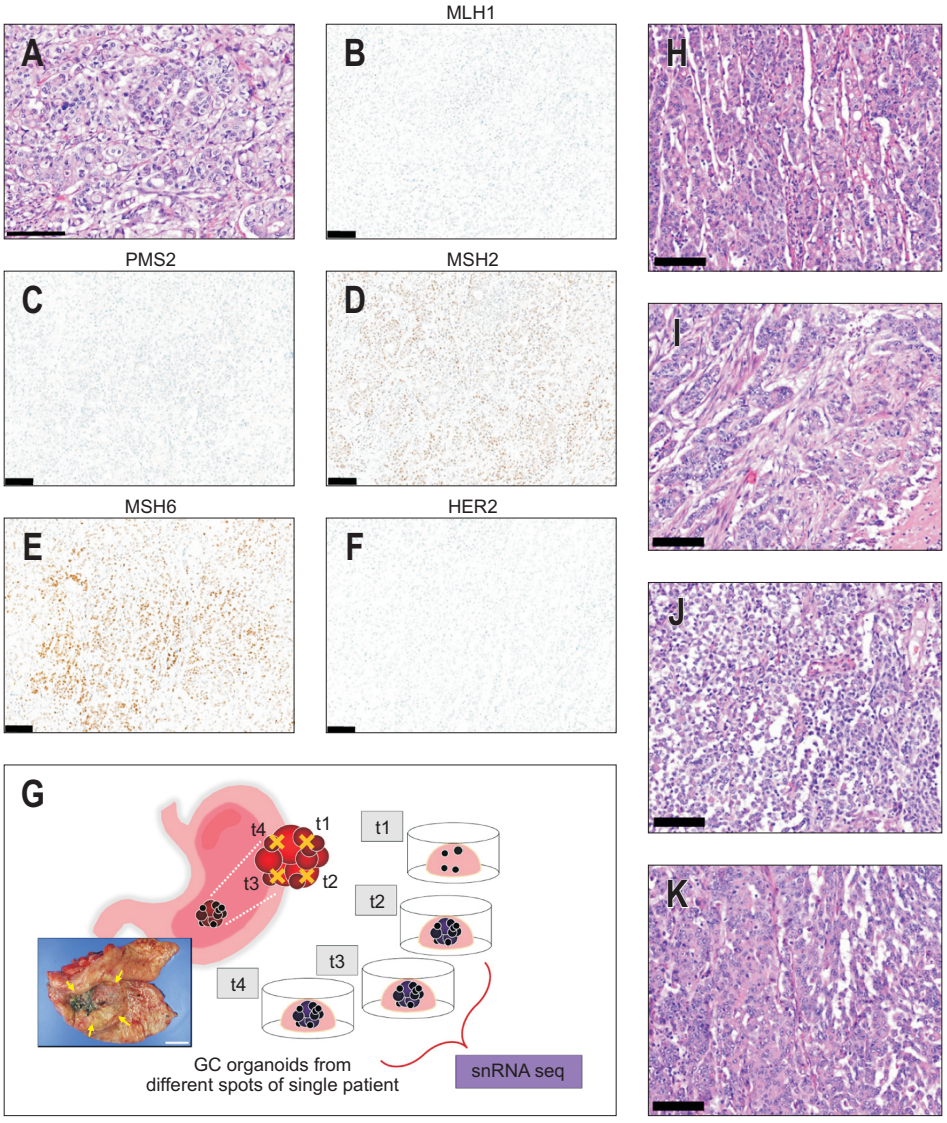

Fig. 1. Characterization of the primary tumor and schematics of the analysis. (A-F) Immunostaining of the primary tumor. Scale bar denotes 100 μm. (A) Hematoxylin and eosin staining of the tumor (×400). (B-F) MLH1, PMS2, MSH2, MSH6, and HER2 immunohistochemical staining of the tumor (×200), which shows the loss of staining of MLH1, PMS2, and HER2. (G) Schematic illustration of the generation of patient-derived organoids and further analysis. (H-K) Hematoxylin and eosin staining of the tumor (×400) collected from t1, t2, t3, and t4, respectively. Scale bar denotes 100 μm. GC, gastric cancer; snRNA seq, single-nucleus RNA sequencing.

performed IHC staining of neuron-specific enolase in the primary tumor, resulting in strong signals in a partial region (approximately 20% to 25%) as in the transcriptomic landscape of PDOs (Fig. 2F). The spatial distribution of neuron-specific enolase positive regions in the primary tumor corresponded to the single-nucleus RNA sequencing data from PDOs.

3. Transcriptomic variability across PDOs

Our analysis of PDOs from different regions of the same tumor revealed not only common characteristics but also noteworthy differences. Here, unsupervised clustering revealed ten cell clusters on UMAP across all three PDOs (Figs. 2A, 3A). Organoid cells from t2, t3, and t4 showed an overlap on UMAP yet with differences in cell distribution (Fig. 3B and C). Notably, more than 90% of cells in cluster 6 are derived from t3 PDO (Fig. 3D). Cluster 6 was characterized by significant upregulation of heat shock proteins (HSPs) including HSPA6, HSPA1B, HSPB1, and DNAJB1 (Fig. 4A). To validate these findings, we have conducted IHC staining with HSP70 on the primary tumor since HSPA1B and HSPA6 are encoding HSP70 member proteins. IHC staining with HSP70 showed positive results with variability in the degree of positivity due to intratumoral heterogeneity (Fig. 4B). PDOs generated from distinct regions of the primary tumor displayed distinct gene expression profiles, particularly regarding HSPs, which possibly affects therapy resistance and recurrence.[10]

4. Role of HSPs in anti-tumor immunity

HSPs are molecular chaperones expressed in response to exposure to stress. HSPs serve as immunomodulants in cancer via upregulation of immunogenicity by binding with antigenic peptides.[11] Extracellular HSP70 is also known to act as

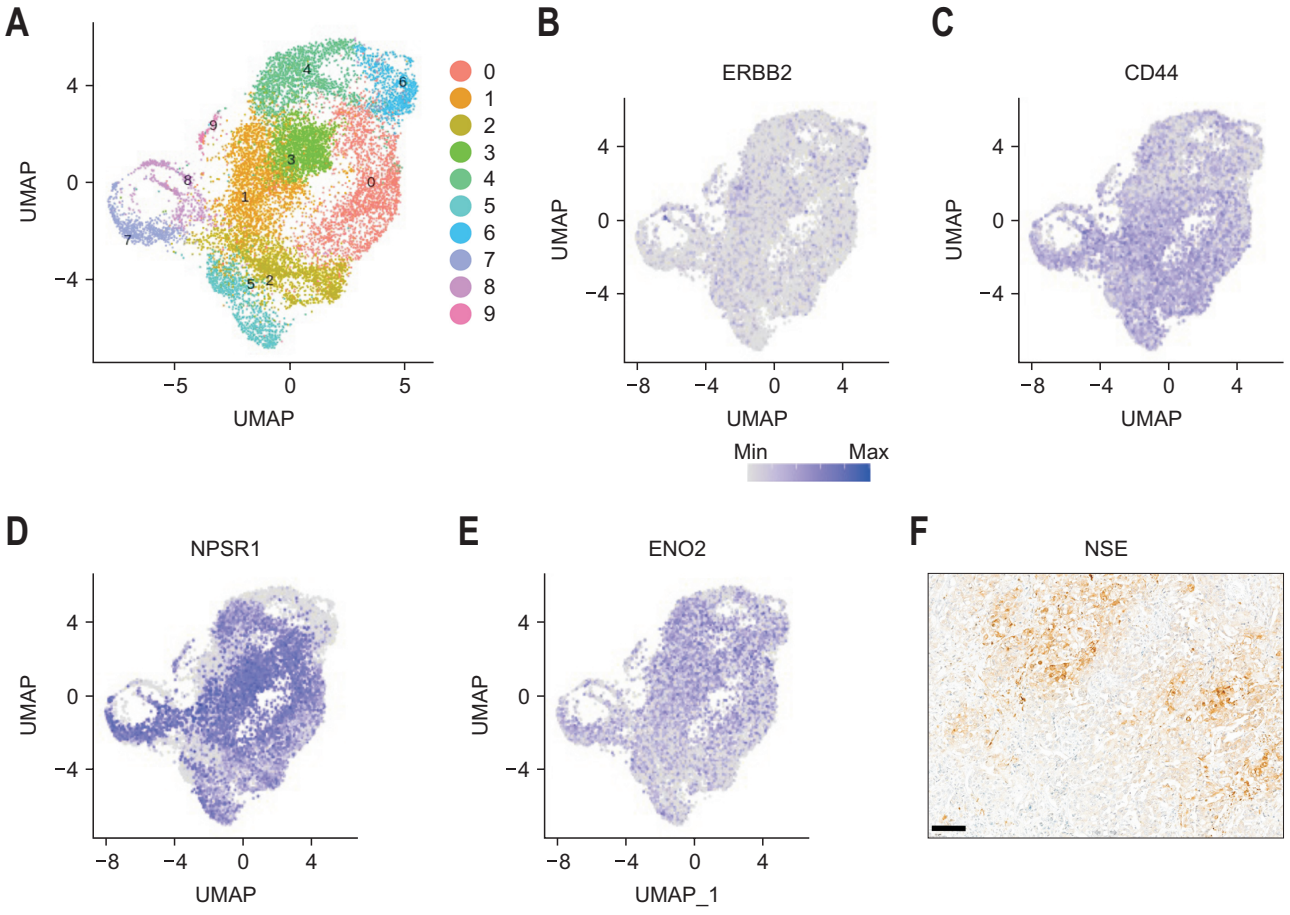

Fig. 2. General characteristics of the patient-derived organoids (PDOs) originated from three different locations of the primary tumor. (A) Single-nucleus RNA sequencing was performed on three PDOs from t2, t3, and t4. Uniform manifold approximation projection (UMAP) visualization of the cells (n=13,041) is shown after integration. (B-E) The expression levels of ERBB2, CD44, NPSR1 and ENO2 are marked in color on the corresponding UMAP. (F) Immunostaining of the primary tumor (×200) with neuron-specific enolase (NSE). Scale bar denotes 100 μm.

a danger signal that activates immune cells and promotes antigen presentation, bolstering anti-tumor immune response.[12] However, there have been no studies regarding the role of HSPs in inducing antitumoral immune response in GC. We compared the activity of cytotoxic T cells according to HSPA6 and HSPA1B levels by measuring the expression levels of IFNG and GZMB of the tumor in another independent GC cohort, The Cancer Genome Atlas-Stomach Adenocarcinoma (TCGA-STAD) (Fig. 4C). Interestingly, tumors with abundant HSPs showed high activity of cytotoxic T cells, indicating the role of HSPs in cancer cells as immune response activator in GC. Accordingly, transcriptomic profiling of the primary tumor also predicted high immune response (Fig. 4D).[6] HSP-rich profiles identified in PDOs correlated with immune-response markers in independent GC cohorts.

DISCUSSION

In this study, we demonstrate that PDOs established from multiple distinct regions of a single gastric tumor faithfully retain the intratumoral heterogeneity of the original cancer (Fig. 5). We successfully generated PDOs from different tumor sites and performed a thorough multi-level analysis including histological examination and genetic profiling to compare each PDO with its corresponding tumor region. Our results show that each organoid preserved region-specific characteristics, such as unique cellular morphologies and distinct genetic alterations, mirroring the diversity within the tumor. This finding confirms that PDOs can serve as an *ex vivo* microcosm of a patient's tumor, capturing subclonal differences that exist spatially within the same malignancy. By reflecting the tumor's complexity via single-nucleus RNA sequencing analysis, our organoid models provide evidence that even a single gastric tumor harbors multiple, divergent cell populations, which PDOs can recapitulate in culture. This is a critical advancement because it validates that organoid technology can be applied beyond interpatient variability to also model intra-tumor diversity, an aspect essential for under-

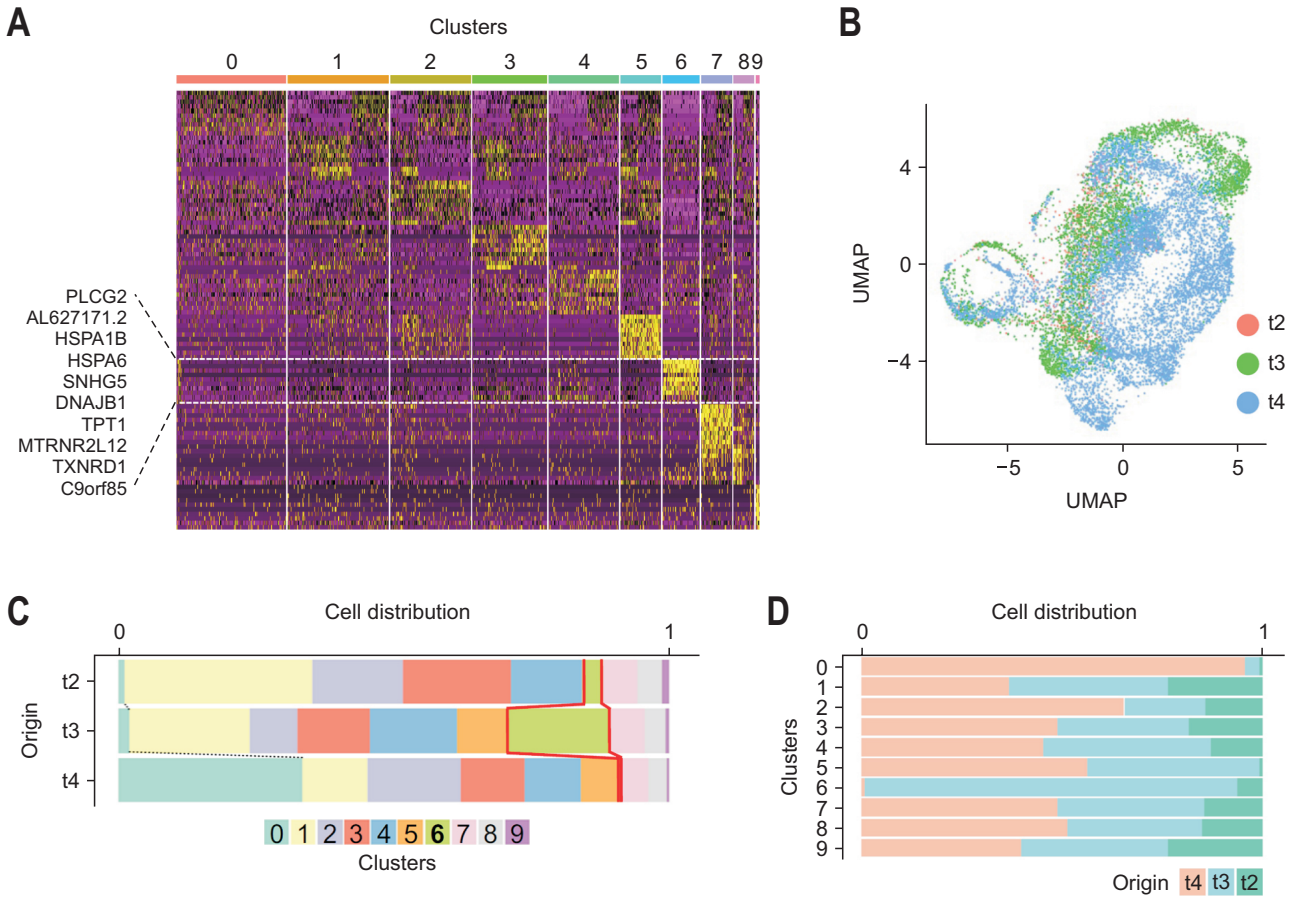

Fig. 3. Each patient-derived organoid (PDO) had a different cell distribution despite having the same origin. (A) Heatmap with the gene markers of each cluster. (B) Cancer cells from three PDOs are marked as a color according to their origin on the corresponding uniform manifold approximation projection (UMAP). (C) Bar chart of the distribution rate of each organoid from a different origin for each cell cluster. (D) Bar chart of the distribution rate of each cluster for each sample origin.

standing tumor behavior and treatment resistance.

Our study builds on prior evidence of GC heterogeneity and provides new insights by using multiple organoids from one tumor to model that variability. Previous research has established large GC organoid biobanks to represent differences between patients, but our approach highlights differences within a single patient's tumor.[13] For instance, while earlier studies showed that organoids maintain the overall characteristics of their parent tumors,[14] we showed that organoids from one tumor can each exhibit diverse features reflective of their specific site of origin. This underscores the extent of intratumoral diversity in GC, aligning with reports that spatially separated tumor samples often carry distinct genetic profiles and drug response patterns, a known driver of therapy failure.[5] Our findings are consistent with these reports and extend them by proving that even when grown in identical laboratory conditions, organoids derived from different regions maintain the divergent traits of their source locales. This retained heterogeneity in organoids suggests that the differences observed were inherent to the tumor subclones and not lost outside the human body, reinforcing the biological significance of intratumoral heterogeneity. Moreover, by comparing organoids from multiple sites, we observed how subclonal populations within the tumor with high level of HSPs[15] which is marker for high sensitivity to immunotherapy, an observation that would have been obscured if only a single biopsy-derived organoid was studied.

Intratumoral heterogeneity is recognized as a major challenge in oncology because distinct subclones may respond variably to therapy, leading to partial treatment response or relapse.[16] Our results suggest that relying on a single tumor biopsy or a single organoid line could be insufficient for comprehensive treatment planning, as it might overlook resistant subpopulations.[17] By establishing PDOs from multiple tumor regions, clinicians could achieve a more comprehensive view of a patient's cancer.[13] In our study, we observed differentially expected drug sensitivities among the organoids derived from different sites of the tumor even though they originated from one

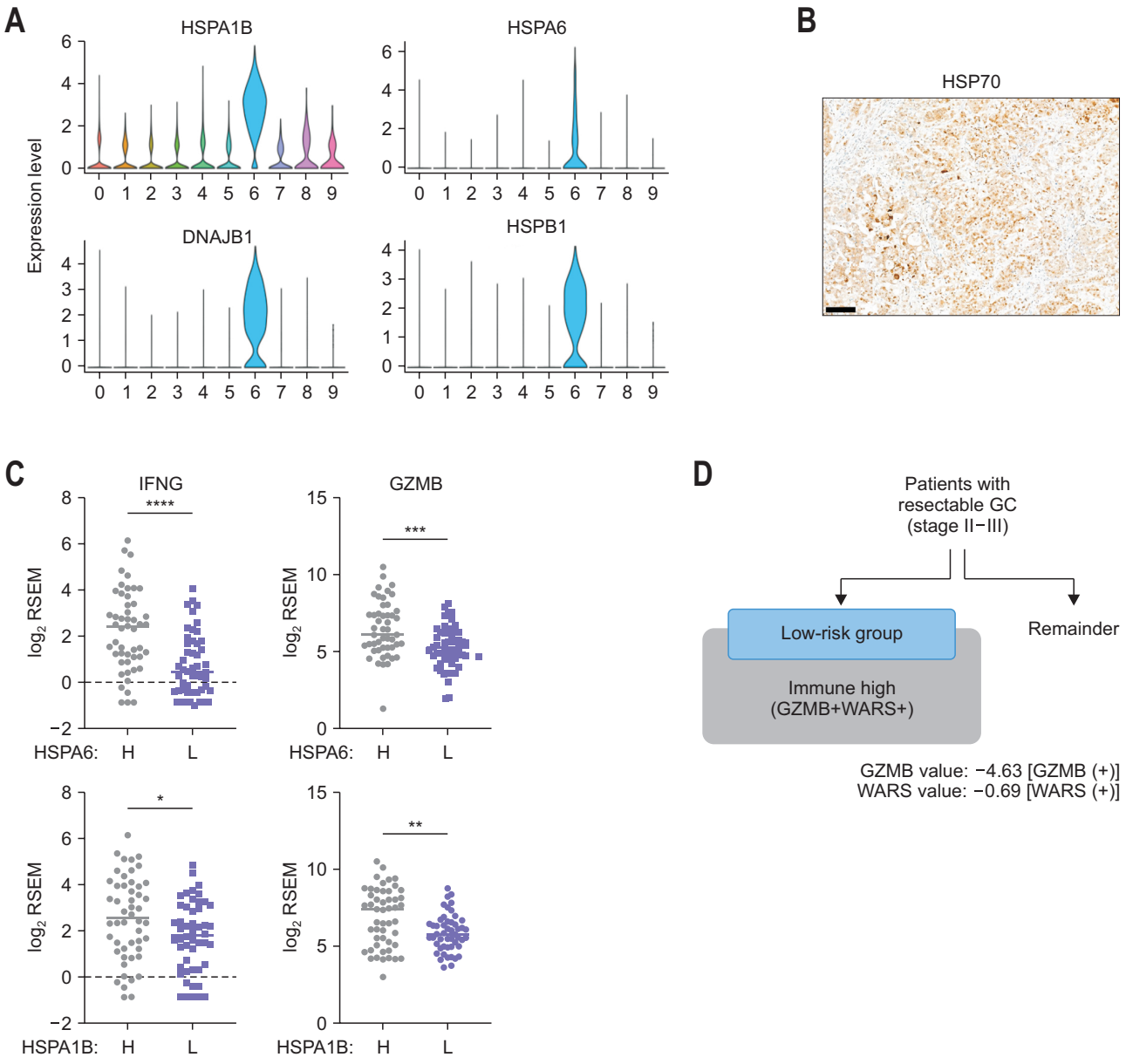

Fig. 4. Heat shock protein (HSP)-rich cell clusters, which are important for the antitumoral immune response, were not detected in all patient-derived organoids generated from a single tumor. (A) The expression levels of HSPA1B, HSPA6, DNAJB1, and HSPB1 in the identified cell clusters are shown as a violin plot. (B) HSP70 immunostaining highlights abundant HSP70 expression in the primary tumor (in brown, ×200). Scale bar denotes 100 μm. (C) The mRNA expression levels of IFNG and GZMB were compared between patients with high (n=49) and low (n=50) HSPA6 levels and patients with high (n=50) and low (n=50) HSPA1B levels in the Cancer Genome Atlas stomach adenocarcinoma cohort. Expression levels are marked as log2 RSEM normalized counts. (D) Detailed molecular characterization of the primary tumor via nProfiler.[2] IFNG, interferon gamma; GZMB, granzyme B; RSEM, RNA-Seq by expectation-maximization; WARS, tryptophanyl-tRNA synthetase; GC, gastric cancer. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

malignancy, highlighting that a therapeutic agent effective on one segment of the tumor could be less effective on another.[18] Therefore, a multi-organoid approach could enable testing of various drugs on each subclone, informing a combination therapy tailored to eradicate the full spectrum of tumor cells.[19] Ultimately, implementing such strategies in the clinic could improve outcomes: treatment regimens could be personalized not only to the patient but also to the intratumoral landscape of the disease.[20] Our work thus moves the field closer to truly precision medicine, where therapy is customized based on the complex architecture of an individual's tumor, rather than on a single tumor sample.[21]

We have also strengthened the logical understanding of tumor heterogeneity through this organoid model, though certain limitations should be acknowledged. First, our

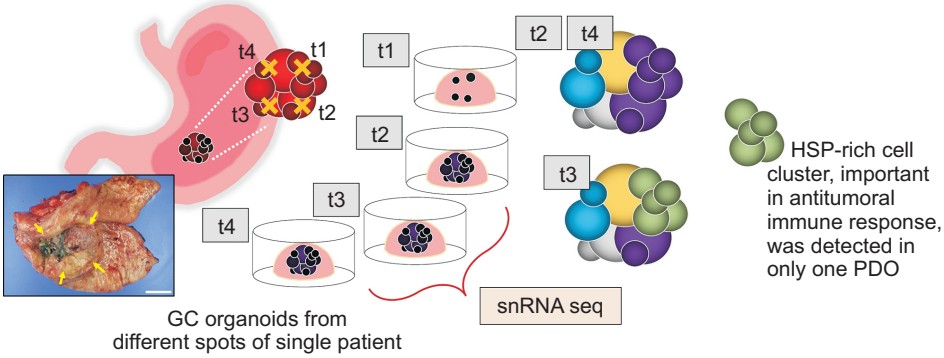

Fig. 5. Schematic diagram of the generation of patient-derived organoids (PDOs) from a single tumor and the difference in the characteristics. GC, gastric cancer; HSP, heat shock protein; snRNA seq, single-nucleus RNA sequencing.

study focused on one patient's tumor, and while this deep dive provides valuable insight, the generalizability of the findings should be confirmed in additional patients and tumor types. Expanding the approach to more cases would verify that the ability of PDOs to recapitulate intratumoral heterogeneity is broadly applicable in GC. Second, it is important to consider that generating multiple organoids per tumor increases the workload and complexity for clinical translation; therefore, developing efficient strategies for multi-site sampling and organoid testing will be crucial. Despite these limitations, our findings provide proof of concept that intratumoral differences can be effectively studied using organoids. In conclusion, we can gain deeper insights into GC biology and improve personalized therapeutic strategies. This work lays a foundation for future research to exploit intratumoral organoid models in guiding treatment and understanding resistance mechanisms in GC.

CONFLICTS OF INTEREST

J.H.K. is an editorial board member of the journal but was not involved in the peer reviewer selection, evaluation, or decision process of this article. No other potential conflicts of interest relevant to this article were reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: S.J.S., S.F., J.H.K. Data acquisition: Y.B., Y.J., S.J., Y.K., J.H.K. Data analysis and interpretation: B.K.Y., S.J., Y.K. Drafting of the manuscript: B.K.Y., Y.B., Y.J., S.J., Y.K. Critical revision of the manuscript for important intellectual content: S.J.S., S.F., J.H.K. Statistical analysis: B.K.Y. Obtained funding: J.H.K. Administrative, technical, or material support: Y.B., Y.J., S.J., Y.K. Study supervision: S.J.S., S.F. Approval of final manuscript: all authors.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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