Commentary

An Emerging Role for Cytopathology in Precision Oncology

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Precision medicine is an emerging field in medicine for disease prevention and treatment that takes into account the individual variability in genes, environment, and lifestyle for each individual patient. The authors have developed a special program as part of the Englander Institute for Precision Medicine to grow patient-derived, 3-dimensional tumor organoids for tumor-specific drug testing, tailoring treatment strategies, and as models for studying drug resistance. Routine cytology preparations represent a cost-effective and powerful tool to aid in performing molecular testing in the age of personalized medicine. In this commentary, the platforms used for the characterization and validation of patient-derived, 3-dimensional tumor organoids are outlined and discussed, and the role of cytology as a cost-effective and powerful quality-control measure is illustrated. ***Cancer Cytopathol* 2016;124:167-73.** *© 2015 American Cancer Society.*

KEY WORDS: 3-dimensional (3D) tumor organoid; cytology; cytology smear; in vitro models; next-generation sequencing; organoids; precision medicine.

INTRODUCTION

Precision oncology is a therapeutic approach, and its objective is to tailor treatment strategies for various types of malignancies based on the individual genetic profile of each patient's cancer.[1] This approach uses molecular information from the individual's cancer to determine diagnosis and prognosis. At the Englander Institute for Precision Medicine at Weill Cornell Medicine and New York-Presbyterian Hospital, we established a clinical program for whole-exome sequencing and development of patient-derived, 3-dimensional (3D) tumor organoids to be used for functional studies, such as pharmacologic screenings and drug resistance in tumors.[2,3] 3D in vitro models have been used in cancer research as an intermediate model between in vitro cancer cell line cultures and xenografts.[4–6] One of the limiting factors in growing primary tumor samples is the overgrowth and contamination of these samples by benign cells from adjoining tissues and the tumor microenvironment. To detect such contamination and to monitor tumor cell enrichment for patient-derived primary cultures, cytology appears to play a crucial role.

3D TUMOR ORGANOIDS

Over the past few decades, we have learned that cell-line models do not recapitulate the exact genetic make-up and tumor microenvironment of individual tumors and thus, greatly limit preclinical evaluation of newly targeted therapeutic agents.[7,8] Furthermore, high failure rates have been attributed in part to the use of 2-dimensional (2D)

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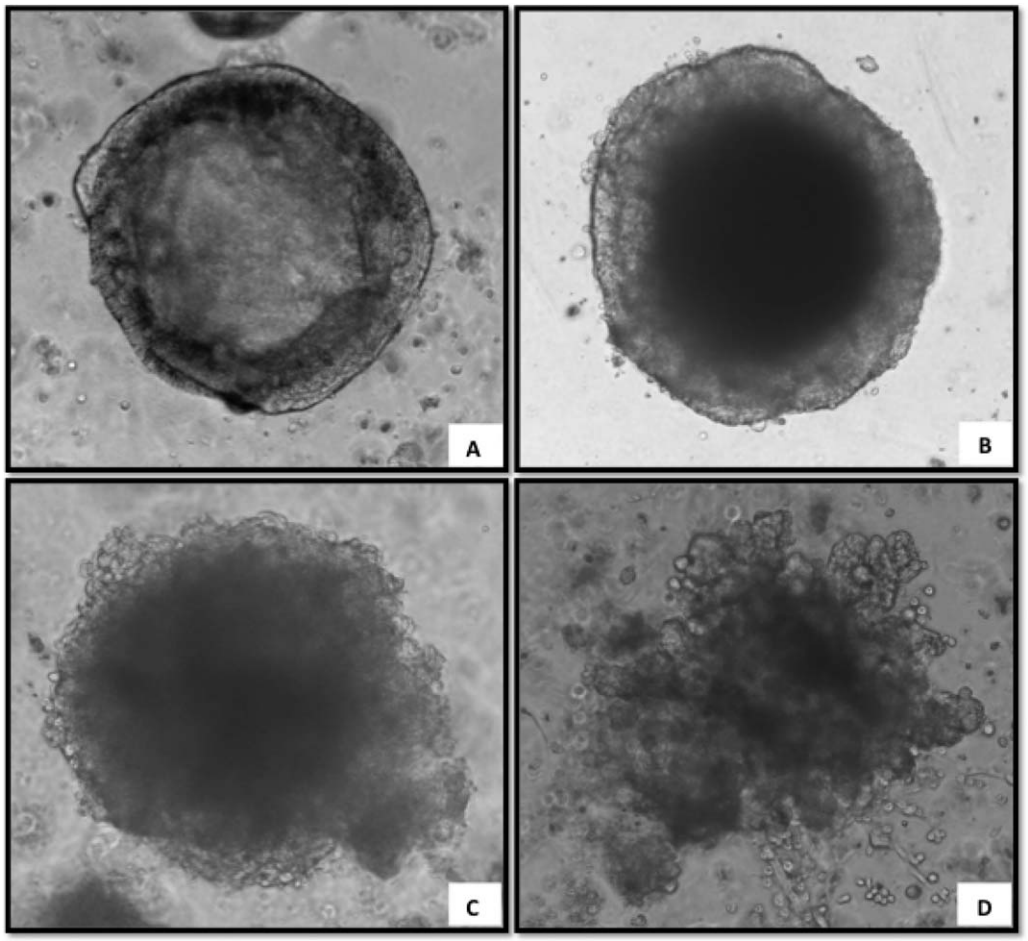

Figure 1. Images of patient-derived tumor organoids in a Matrigel scaffold (Corning Inc, Corning, NY) include tumor organoids from (A) ductal adenocarcinoma of the pancreas, (B) malignant mixed Mullerian tumor/carcinosarcoma of the uterus, (C) endometrial adenocarcinoma, and (D) urothelial carcinoma.

monolayer cultures for drug screening and resistance studies, which tend to produce inaccurate results, and thus fail to predict chemoresistance.[9] These limitations can be reduced by the use of 3D-cultured tumor cells derived from individual patient's tumors to recapitulate a tumor-like microenvironment.[10]

3D cell cultures create an environment in which the cells can grow and interact with their surroundings in all 3 dimensions, more accurately imitating the in vivo tumor environment compared with monolayer cells grown on plastic.[10] At the Englander Institute for Precision Medicine, we grow patient-derived, 3D tumor organoids for pharmacologic testing (Fig. 1). All patient-derived tissue samples are collected with the patient's informed consent according to Institutional Review Board-approved protocols. Fresh tissue biopsies are washed with antimicrobial Primocin (Invivogen USA, San Diego, Calif) and culture media enriched with Rho-associated protein kinase inhibitor (Selleckchem.com, Houston, Tex) and are either mechanically dissociated or enzymatically digested with collagenase Type IV (Life Technologies/Thermo Fisher Scientific, Waltham, Mass) and trypsin-ethylene diamine tetraacetic acid (Invitrogen USA) to obtain single cells or small cell clusters. Single cells and/or cell clusters are mixed with growth factor reduced Matrigel (Corning Inc, Corning, NY) and cultured with primary culture media. 3D cultures are passaged and expanded weekly or every other week, depending on the growth rate. Frequent and regular viability assessments are performed using confocal microscopy (Zeiss LSM510; Carl Zeiss Microscopy GmbH, Oberkochen, Germany) and a Live/Dead Viability Assay (Promega, Madison, Wis) (Fig. 2). We developed different platforms as part of the work process to further characterize and validate the 3D tumor organoids.

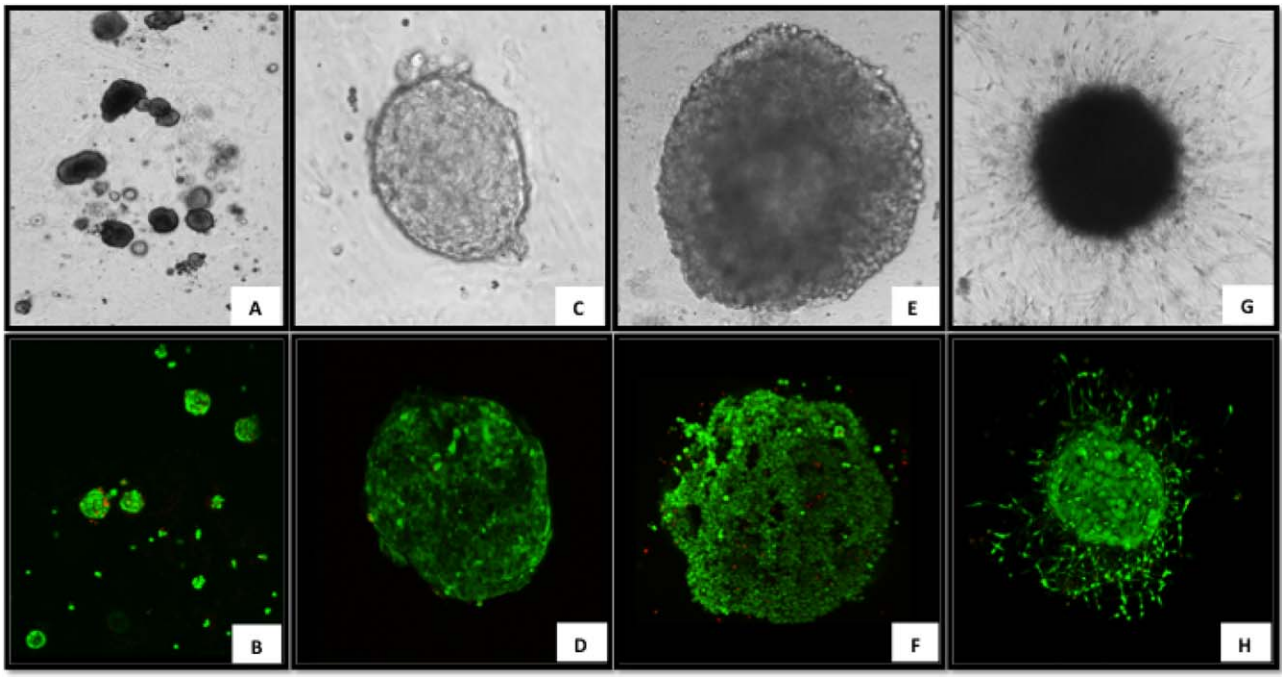

Figure 2. These images were obtained from a viability assessment of tumor organoids using the Live/Dead Viability Kit from Molecular Probes (Eugene, Ore). Tumor organoids were incubated with the dead-cell–permeable red fluorescent dye ethidium homodimer-1 and the live-cell–impermeable green fluorescent dye Calcein acetoxymethyl ester, which is a nonfluorescent, membrane-permeable probe that is hydrolyzed by cellular esterases to form a green fluorescent, membrane-impermeable compound. With this dye combination, dead cells are shown as red, and viable cells are shown as green. Imaging was performed with a Zeiss confocal microscope (LSM510; Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Images include tumor organoids from (A-D) a neuroendocrine tumor of the liver; (E,F) a metastatic, castration-resistant prostate cancer; and (G,H) an adenocarcinoma of the lung with surrounding tumor-associated fibroblasts.

PLATFORMS USED FOR THE CHARACTERIZATION AND VALIDATION OF PATIENT-DERIVED, 3D TUMOR ORGANOIDS

Cytology Smears

We evaluate smear preparations from the 3D cultures as the initial screening platform. Smears from the 3D cultures are prepared and screened to ensure that tumor cells are sampled from the cultures and not only from contaminants such as benign epithelial cells or fibroblasts.

In the initial step to help differentiate between tumor growth and contaminant growth, we use the gross morphology of the tumor organoids as a guide. The gross morphology of the 3D cultures tend to vary between samples (intersample variation) and also within a single sample (intrasample variation). Intrasample variation has been noted frequently in samples that exhibit the growth of both tumor organoids and benign cells (Fig. 3).

Once a gross assessment of the 3D culture is made, up to 5 (morphology matched) individual organoids are collected using an Eppendorf Pipette under an inverted microscope and expelled onto a Super Frost Plus glass slide (Fisherbrand; Fisher Scientific/Thermo Fisher Scientific). A second slide is used as a spreader by placing it gently on top of the sample slide, either at a right angle or parallel to the sample slide. The top slide is then gently pulled across the sample (bottom) slide to thinly smear the sample. Air-dried smears are then stained with a Diff-Quik stain (Siemens Medical Solutions USA, Inc, Malvren Pa). The stained smears are then reviewed by a board-certified cytopathologist (Fig. 4). Only the organoids that contain tumor cells are individually harvested, further grown, and undergo molecular characterization according to the Precision Medicine Platform Standard Operation Procedures.

Histology

Formalin-fixed, paraffin-embedded (FFPE) tissue samples traditionally have been used for ancillary testing, such as

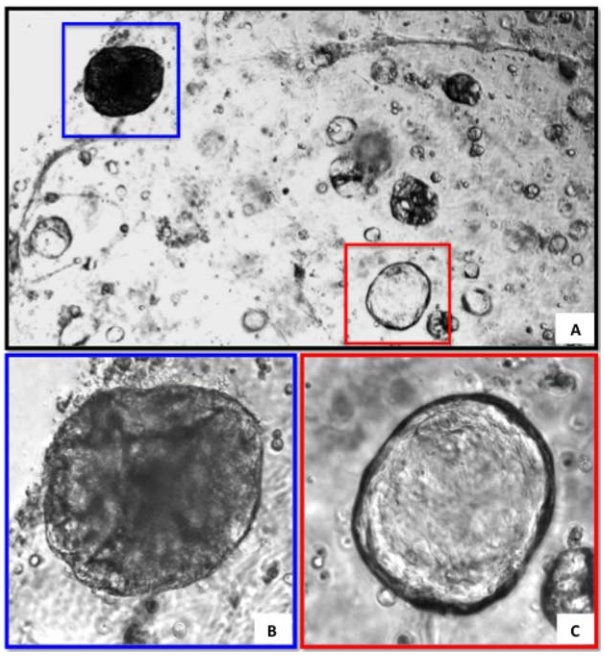

Figure 3. (A) 3-Dimensional culture in a Matrigel scaffold with intrasample variation in gross morphology, from patient derived lung cancer tissue. (B) Tumor organoid of an adenocarcinoma of the lung. (C) Benign epithelial cells "contaminants" form a sphere like structure that resembles a cystic structure.

immunohistochemistry (IHC) and/or fluorescence in situ hybridization (FISH). Similarly, we use FFPE samples prepared from the tumor organoids (cell blocks) for further ancillary testing and for other testing modalities to help characterize the tumor organoids.

We use a specific protocol to prepare cell blocks on grossly identifiable tumor organoids with an average greatest dimension of approximately 500 to 800 µM. Fresh tumor organoids are enzymatically dissolved (Dispase; Sigma-Aldrich Corporation, St. Louis, Mo) out of the Matrigel and mixed with fibrinogen and thrombin (dilution. 1:10 volume/volume fibrinogen; Sigma-Aldrich Corporation) to form a clot before fixation with 4% paraformaldehyde, pH 7.4, in phosphate-buffered saline (PBS) (Affymetrix Inc, Santa Clara, Calif) and embedding in paraffin. Smaller tumor organoids that measure <500 µM in greatest dimension are initially fixed within the Matrigel scaffold in 4% paraformaldehyde, pH 7.4, in PBS and then embedded in paraffin. These FFPE blocks are then used for routine histology and for ancillary testing, such as IHC and/or FISH (Fig. 5).

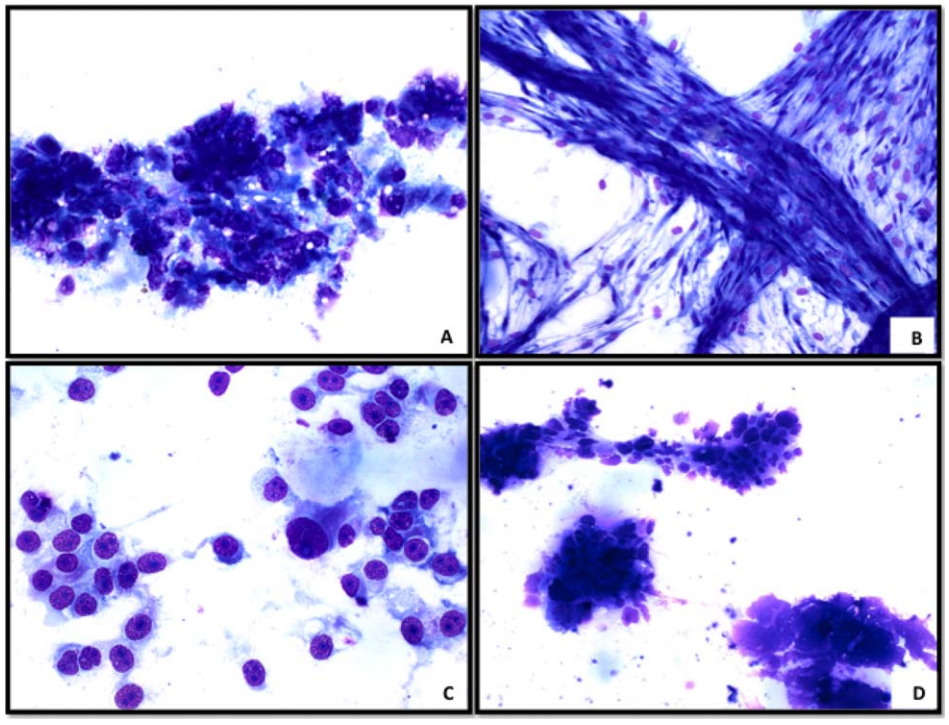

Figure 4. Images of Diff-Quik–stained, 3-dimensional culture smears of tumor organoids. (A) endometrial adenocarcinoma tumor organoids (×40 objective) and (B) benign fibroblastic cells cultured from adenocarcinoma of the lung that was overgrown by tumor-associated fibroblasts after a culture time of 3 months (×20 objective). Also shown are smears of (C) malignant melanoma tumor organoids (×40 objective) and (D) uterine carcinosarcoma tumor organoids (×40 objective).

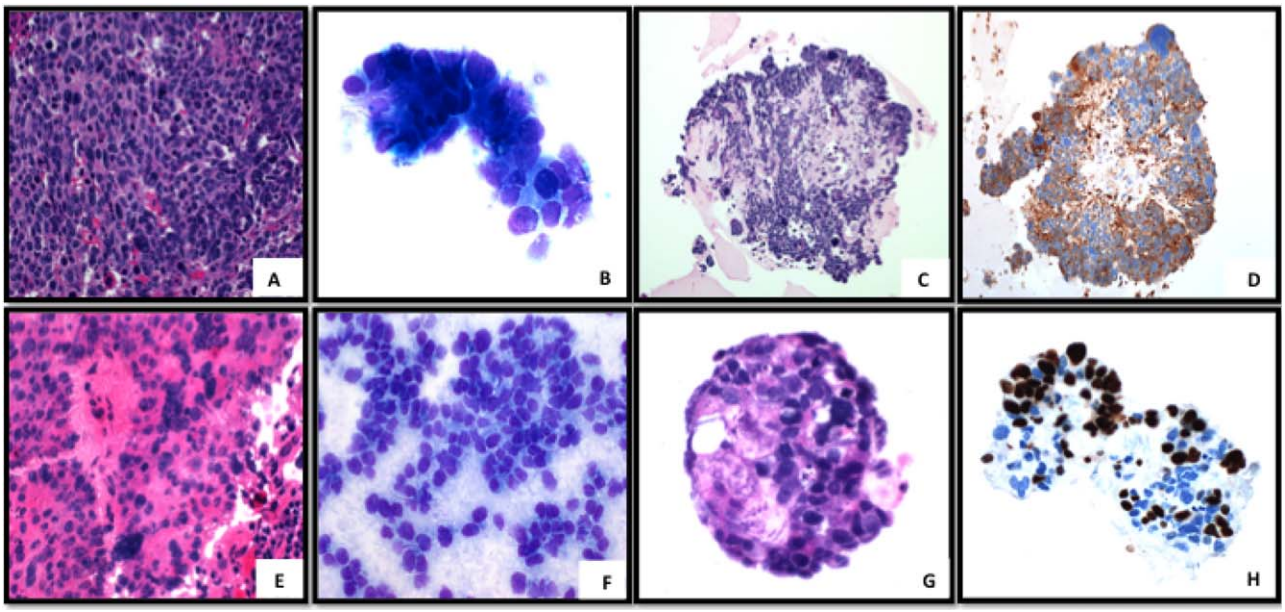

Figure 5. Histology of native tumor tissue in comparison to the matching tumor organoid smears and tumor organoid histology. Images include (A) a hematoxylin-and-eosin (H&E)-stained sample of neuroendocrine carcinoma of the liver (×20 objective), (B) a matching tumor organoid smear (Diff-Quik staining, × 40 objective), (C) the H&E-stained FFPE section of the tumor organoid (×20 objective), and (D) positive immunohistochemical staining for synaptophysin antibody (ab8049; Abcam, Cambridge, Mass; × 20 objective); and (E) an H&E-stained sample of uterine carcinosarcoma (×20 objective), (F) a matching tumor organoid smear (Diff-Quik staining, × 40 objective), (G) the H&E-stained FFPE section of the tumor organoid (×40 objective), and (H) increased proliferative activity of the tumor organoid cells on Ki67 immunostain. (ab8049; Abcam; × 40 objective).

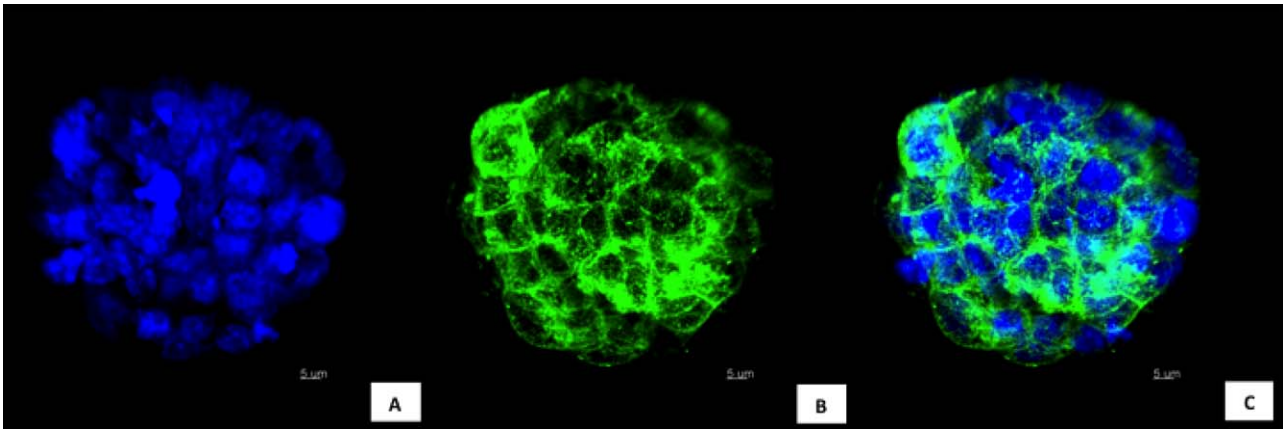

Figure 6. Direct immunofluorescence staining in a tumor organoid from a neuroendocrine carcinoma is observed in a Z-stack of × 40 objective images obtained using a Zeiss confocal microscope (LSM510; Carl Zeiss Microscopy GmbH). Samples were stained with (A) 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) nuclear stain and (B) synaptophysin antibody (ab8049; Abcam) coupled with Alexa-488 (Life Technologies Corporation, Carlsbad, Calif), which is seen as a green signal. C is a merged image of A and B.

Immunofluorescence

We use direct immunofluorescence staining to observe a specific protein or antigen in our tumor organoids by binding a specific antibody that is chemically conjugated with a fluorescent dye. Tumor organoids are dissolved out of the Matrigel (Corning Inc) and fixed in 4% paraformaldehyde, pH 7.4, before permeabilization (PBS and 1% Triton X-100) and blocking (PBS, 1% bovine serum

TABLE 1. Cancer Gene Mutation Panel-50 (Ion Torrent)

Native Tumor Tissue[a]	Gene	Genomic Alteration	Corresponding Tumor Organoid
Endometrial adenocarcinoma	CTNNB1	c.95A>G, p.D32G	Endometrial adenocarcinoma, tumor organoid
	PIK3CA	c.3140A>G, p.H1047R	
	PTEN	c.795delA, p.K267fs*9	
Metastatic neuroendocrine carcinoma to the liver	TP53	c.742C>T, p.R248W	Metastatic neuroendocrine carcinoma, tumor organoid
	TP53	c.463C>T, p.R155W	
MMMT/uterine carcinosarcoma	PIK3CA	c.1638G>T, p.Q546H	MMMT/uterine carcinosarcoma, tumor organoid
	PTEN	c.17_18delAA, p.K6fs*4	

Abbreviations: CTNNB1, catenin (cadherin-associated protein), β2; MMMT, malignant mixed Mullerian tumor; PIK3CA, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit α; PTEN, phosphatase and tensin homolog; TP53, tumor protein 53.
[a] Native tumor tissues and corresponding tumor organoids harbor the same cancer gene mutations.

albumin, 3% normal goat serum, and 0.2% Triton X-100). Incubation with the primary antibodies against the desired antigens is performed according to the manufacturer's suggestion or our specified Standard Operation Procedures. After secondary antibody incubation, tumor organoids are then imaged in a chamber slide (Nalge Nunc International, Rochester, NY) using a confocal microscope (Zeiss LSM510) (Fig. 6).

Molecular Analysis

After cytologic characterization of the 3D cultures, only tumor organoids that are positive for cancer cells are further characterized using next-generation sequencing. Our platform either uses the EXaCT-1 assay (HISeq2500; Illumina, Inc, San Diego, Calif) for whole-exome sequencing (data not shown) or the Cancer Gene Mutation Panel-50 (Ion Torrent), which interrogates 50 cancer-related genes in solid tumors. Good concordance in genomic alterations (copy numbers and mutations), have been demonstrated between the individual native tumors and the corresponding tumor organoids (Table 1).

Conclusion

Routine cytology smears can represent a cost-effective and powerful tool to aid molecular testing in the age of personalized medicine.[11] Cytopathology has become a very efficient and cost-effective screening tool to confirm tumor cell-rich 3D cultures. In our program, cytologic assessment has become an absolute requirement for the growth of tumor organoids to ensure good culture quality. Cell culture, molecular characterization, and tissue banking of benign epithelial cell or fibroblast cultures can cause unnecessary personnel and material expenses. In conclusion, we have developed a workflow with different platforms for the establishment and characterization of patient-derived tumor organoid cultures. Patient-derived cancer cultures represent a promising area in personalized medicine to test drug sensitivity or study the effects of drug resistance on tumors.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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