ESTABLISHMENT AND CHARACTERIZATION OF CELL LINE OF UNDIFFERENTIATED PLEOMORPHIC SARCOMA

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Abstract

Background: Undifferentiated pleomorphic sarcoma (UPS) is an aggressive mesenchymal malignancy. Although patient-derived cell lines are invaluable tools for preclinical studies, there are only a few UPS cell lines available in public cell banks. In the present study, we established a cell line from the primary tumor tissue of a UPS patient. Methods: Primary UPS tumor tissues were sampled to establish cell lines. Morphological and proteomic analyses were performed and sensitivity to anti-cancer drugs was evaluated. Results: We established a novel UPS cell line, namely NCC-UPS1-C1 cells, and maintained the cells for over 100 passages. The characters of cells as morphology, growth rate, colony formation capacity, and immune-histochemical traits were confirmed. Mass spectrometric protein expression profiling revealed that the proteome of the original tumor tissue differed from that of the cell line. Sensitivity to 164 anti-cancer drugs was screened for their growth inhibitory effects. Conclusions: Patient-derived cell line in this study may be useful for understanding the molecular background of drug resistance in UPS. Furthermore, the use of the patient-derived cancer model will facilitate our understanding of molecular mechanisms underlying poor prognosis, and will contribute to novel therapeutic strategies.

Key Words: undifferentiated pleomorphic sarcoma, primary culture cells, proteome, drug resistance

Introduction

Undifferentiated pleomorphic sarcoma (UPS) is an aggressive sarcoma of soft tissue or bone. UPS is the fourth most common soft tissue sarcoma, with an increasing incidence. UPS is a pleomorphic variant of undifferentiated sarcomas, which have no identifiable line of differentiation. UPS shares a marked pleomorphism with spindle cells and multinucleated giant cells, and a highly variable morphology. The clinical outcome has room for improvement; the local recurrence rate is 19–31%, the metastatic rate, 31–35%, and the five-

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year survival rate, 65–70%\textsuperscript{2).} The therapeutic strategy for UPS is surgery with wide margins, with consideration of adjuvant treatment according to the surgical margins, location of tumor, and tumor-associated risk factors. There is no treatment optimized for patients with UPS.

Patient-derived cancer models aid basic and pre-clinical research. Investigation of cell lines may be crucial to elucidating the molecular backgrounds underlying the clinical and pathological diversity of UPS, facilitating our understanding of mechanisms of metastasis, recurrence, and resistance of UPS. Here, we report a novel UPS cell line, namely NCC-UPS1-C1 cells. The use of the NCC-UPS1-C1 cells may help elucidate the molecular mechanisms underlying the poor prognosis of patients with UPS. The NCC-UPS1-C1 cells will also be a pre-clinical model to develop novel therapeutic strategies for UPS.

Materials and Methods

This study was approved by the ethics committee of the National Cancer Center, and written informed consent was obtained from the patient.

Background of patient

An 83-year-old male was referred to the National Cancer Center Hospital, complaining of soft tissue mass (10 cm in diameter) that had been present in his scapular back for 2 months (Figures 1A–C). Histological diagnosis was performed by a certified pathologist (A. Y.), and pathological examination by needle biopsy was consistent with pleomorphic spindle cell sarcoma. Wide resection with pedicled latissimus dorsi flap operation was performed 4 weeks after the initial diagnosis. Postoperative pathological examination yielded a diagnosis of undifferentiated pleomorphic sarcoma (UPS). Four weeks after surgery, the patient developed multiple lung metastases with right pleural effusion. As it was difficult for this elderly patient to endure systemic chemotherapy, he decided to receive palliative care. The patient died of the cancer 6 weeks after the surgery.

Histological observation

The tissue staining was performed on 4-μm-thick sections from a representative paraffinized block of the tumor. The sections were deparaffinized and stained with hematoxylin and eosin (HE).

Primary tissue culture

The tumor tissue obtained at the time of surgical resection was subjected to experiments. The excised tumor tissue was minced with scissors, and seeded in a culture dish (24-well plate) in a humidified atmosphere containing 5% CO\textsubscript{2} at 37\textdegree C. The cells were maintained in RPMI 1640 medium supplemented with 5% (or 10%) fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 mg/mL streptomycin (Invitrogen Co., Carlsbad, CA). When the cultured cells reached confluence, they were dispersed with a 0.25% trypsin solution, and seeded in another dish (6-well plate). At the next passage, the cells were seeded in a larger dish (10 mm in diameter). The passage was serially performed until establishment of a cell line.

Chromosome analysis

Cells at the logarithmic phase were harvested in culture medium containing colcemid (10 ng/mL, Nacalai Tesque, Kyoto, Japan) overnight. The cells were suspended in a hypotonic solution (0.075 M KCl : 0.8% sodium citrate = 1:1), and subsequently treated in a fix solution (methanol: glacial acetic acid = 3:1). Chromosome specimens were stained with Giemsa solution, and the number of M phase chromosomes was counted by observation with a microscope (Carl Zeiss).

Mycoplasma contamination screening

To exclude the possible contamination with mycoplasma, DNA in the culture medium was examined to rule out the possibility of contamination by mycoplasma. According to the guide line from The International Cell Line Authentication Committee, we examined the tissue culture medium\textsuperscript{3).} We recovered DNA from the culture medium at 70–90% confluence. The recovered DNA was heated at 95\textdegree C for 10 min, and amplified using an e-Mycoplasm PCR Detection Kit (Intron biotechnology, Gyeonggi-do, Korea).
The amplified DNA was electrophoresed on a 1.5% agarose gel, stained with Midori Green Advanced (Nippon Genetics, Tokyo, Japan), and detected using an Amersham Imager 600 (GE Healthcare Biosciences).

Authentication of cell line

Genomic DNA was extracted from the original tumor tissue and tissue-cultured cells using DNeasy blood and tissue kits (Qiagen, Hilden, Germany) or AllPrep DNA/RNA mini kits (Qiagen). Extracted DNA concentrations were determined using a NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA). DNA samples were stored at –20ºC until use. For cell line authentication, we employed the analysis of short tandem repeats (STRs) using STR multiplex assays GenePrint 10 (Promega, Madison, WI), according to the manufacturers’ instructions. The analysis included amplification and detection of 10 loci (Supplementary Table 1). Genomic DNA (500 pg) from the original tissue and established cell lines was isolated and amplified. The amplified products were separated using an ABI 3130 or 3500xL Genetic Analyzer (Applied Biosystems, Waltham, MA). The resulting data were processed and evaluated using the GeneMapper 5.0 (Applied Biosystems) or PeakScanner (Applied Biosystems) programs. The STR profiles were compared with those from the ATCC, DSMZ, or JRCB databases for reference matching.

Cell growth and characteristics

The cells were seeded into 96-well culture plates at various cell numbers (2,000, 4,000, or 8,000 cells/well), in triplicate. Cells were counted at several time points over a 96 h period of incubation at 37ºC. At 24, 48, 72, 96, and 120 h, 10 µL of Cell Counting Kit 8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and incubated for 2 h. Absorbance values at a wavelength of 450 nm were recorded using a microplate reader (Bio-Rad, Hercules, CA). Growth curves were plotted using the seeding day as the horizontal axis, and the absorbance value at 450 nm as the vertical axis.

Colony formation assay

The bottoms of 35-mm dishes were coated with 0.5% low melting agarose (Noble agar, Sigma Aldrich, St. Louis, Missouri, America), with DMEM supplemented with 10% fetal bovine serum and penicillin streptomycin (Thermo Fisher Scientific, San Jose, CA), and covered with 0.33% agarose containing 5 × 10⁶ cells. The plates were incubated at 37ºC for 4 weeks. After incubation, the presence of colonies containing more than 50 cells was confirmed by microscopic observation (ZEISS Primo Vert Inverted Microscope, Carl Zeiss, Jena, Germany). All assays were performed in duplicate.

Immunohistochemical verification

The cells were suspended and solidified using iPGeell (Genostaff, Tokyo, Japan), according to the manufacturer’s instructions. Cell blocks were fixed with 10% formalin neutral buffer solution, and embedded in paraffin. Four-micrometer-thick paraffin sections were prepared and HE stained. Immunostaining was performed according to a previously published method⁴. The primary antibodies used were mouse monoclonal antibodies against α-smooth muscle actin (SMA; 1:100; 1A4; Dako, Glostrup, Denmark), cytokeratin (1:100; AE1/ AE3; Dako), desmin (1:100; D33; Dako), and myogenin (1:100; F5D; Dako); rabbit polyclonal antibody was used against S-100 (1:2000; Dako). The reactions were detected using the EnVision system (Dako).

Sensitivity to anti-cancer drugs

Resuspended NCC-UPS1-C1 cells were seeded at a density of 1250 cells per well on 384-well cell culture plate in DMEM with 10% FBS, and grown overnight. On the following day, anti-cancer drugs or vehicle were added by automated liquid handling platform (Bravo, Agilent Technologies, Santa Clara, CA, USA). After 72 h of treatment, the suppression rates were assessed using a CCK-8. The experiments were conducted two times on different days. At each time point, the experiments were performed in duplicate. Half-concentration of inhibitory effects were measured for the anti-cancer drugs, which reduced the cell viability by more than 99%.
Mass spectrometric protein expression profiling

Proteins were extracted from NCC-UPS1-C1 cells using urea lysis buffer (6M urea, 2M thiourea, 3% CHAPS, 1% Triton X-100), following a method we reported previously\(^5\). The protein samples (25 \(\mu\)g) were processed into tryptic digests using the filter-aided sample preparation (FASP) method\(^6\). The tryptic digests were subjected to liquid chromatography coupled with nanoelectrospray tandem mass spectrometry (Finnigan LTQ Orbitrap XL mass spectrometer; Thermo Fisher, MA, USA). The Mascot software package (version 2.5.1; Matrix Science, London, U.K.) was used to search for the mass of each peptide ion peak against a SWISS-PROT database (Homo sapiens, Swiss prot_2015_09.fasta file containing 20,205 sequences), using the following parameters: 1 missed cleavage; variable modifications: oxidation (Met), phosphorylation (ST), phosphorylation (Y); peptide tolerance: 10 ppm; MS/MS tolerance: 0.8 Da; peptide charge: 2+ and 3+.

Data analysis

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations for each protein group in our proteome data were inferred using the Database for Annotation, Visualization and Integration Discovery (DAVID) software (http://david.abcc.ncifcrf.gov/). The KEGG database was used to classify gene sets into their respective pathways\(^7\). Significance is indicated by the p-value for each category, and the process groups with p<0.05 were considered significant. KEGG analysis results were plotted using the R package “treemap”\(^8\).

Results

Histological appearance of original tumor tissue

Figure 1D shows a typical histological image of the resected primary tumor tissue. The tumor showed high grade histology comprising pleomorphic spindle cells with storiform growth, many mitoses, and necrosis. No histologic evidence of specific line of differentiation was observed. Although myxoid degeneration was present in less than 5% of the tumor tissue, this was too limited to support myxofibrosarcoma diagnosis. Immunohistochemical studies showed that the tumor was diffusely positive for SMA, but negative for AE1/AE3, S100, desmin, caldesmon, myogenin, MDM2, and CDK4 (data not shown). Diffuse pos-
itivity for SMA suggested myofibroblastic differentiation. However, this phenotype is not specifically used for high-grade sarcomas classification proposed by the WHO (ie, no definition of high-grade myofibroblastic sarcoma was provided), and such actin-positive sarcomas are typically considered as belonging to the category of undifferentiated pleomorphic sarcoma.

Chromosome aberrations
Chromosome analysis was performed for the established cell lines. Chromosome analysis revealed complex karyotypes. The average total number of chromosomes was 126 in NCC-UPS1-C1 cells. Many chromosomes had abnormal appearances, and were difficult to classify (data not shown).

Mycoplasma test
Mycoplasma-specific DNAs were not detected in conditioned media from the NCC-UPS1-C1 cell culture. We concluded that the cell line was free of Mycoplasma contamination.

Authentication of the cell lines
We examined the status of STRs of nine microsatellites in the donor’s normal and tumor tissue, and in NCC-UPS1-C1 cells. Most of the STRs were identical in all samples examined (Supplementary Table 1). We did not find any previously reported cell lines with the same STR patterns as the ones that we observed. Thus, we confirmed that NCC-UPS1-C1 cell line was a novel UPS cell line.

Phenotypic characterization of tissue cultured cells
A morphological characterization was performed by light microscopy. NCC-UPS1-C1 cells were spindle, round, or polygonal in shape (Figure 2A). We did not clone the cells from mixed tissue cultures. The morphology of established cell line was concordant with that of the original tumor tissue, which comprised a mixture of atypical spindle cells, round cells, and giant cells in storiform pattern (Figure 1D). The growth rates of the cell line was assessed by seeding cells at different numbers per well. The doubling time of the cell

![Figure 2](image-url)

Figure 2. Phenotypic characterization of NCC-UPS1-C1 cells; phase contrast micrographs for NCC-UPS1-C1 (A). Bar: 200 μm. The growth rates of NCC-UPS1-C1 cells was plotted (B). Different numbers of cells (shown on the plots) were plated in the 96-well plates. The colony formation capability was examined for NCC-UPS1-C1 cells (C). Bar: 100 μm.
lines were approximately 40.9 hours. Colony formation capability of NCC-UPS1-C1 cells was assessed on soft agar plates (Figure 2C). Immunocytochemistry indicated that the NCC-UPS1-C1 cells were positive for smooth muscle actin, and cytokeratin. The cells were negative for the S100, desmin, and myogenin antigens (Figure 3).

Proteomic study

The protein expression profiles of primary tumor tissue and NCC-UPS1-C1 cells were examined by mass spectrometry (Supplementary Tables 2 and 3). We did not perform the quantitative comparison. Instead, we functionally characterized all proteins, which were observed by proteomic experiments in the tumor tissue and the NCC-UPS1-C1 cells. Figure 4 depicts a functional classification of identified proteins in treemap format. Treemaps of KEGG pathways were created for the primary tumor tissue, NCC-UPS1-C1 cells (Figure 4). Each treemap includes KEGG pathways with adjusted p-values < 0.05, colored according to their enrichment score and sized according to the number of proteins in that pathway. Supplementary Table 4 summarizes the annotations of the proteins used in the treemaps. Sixteen KEGG pathways were identified in the primary tumor tis-

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**Figure 3.** Immunohistochemical assessment. Tissue biomarker expression was examined in NCC-UPS1-C1 cells (A). The cells were stained with specific antibodies (antigens are denoted beneath panels). The arrows indicate the SMA-positive cells, and the arrow heads indicate the cytokeratin-positive cells. Bar: 50 μm.

**Figure 4.** Treemaps of KEGG pathway categories for enrichment analyses of the original tumor tissue (A), and NCC-UPS1-C1 cells (B). The box sizes represent the numbers of genes in that category, and the colors represent the enrichment scores. All process groups were considered significant at p < 0.05. The enlarged figure is attached as Supplementary Figure 1.
PATIENT-DERIVED CELL LINE OF UNDIFFERENTIATED PLEOMORPHIC TUMOR TISSUE (Figure 4A). In the NCC-UPS1-C1 cell line, we identified 14 KEGG pathways (Figure 4B). Between the primary tumor tissue and the NCC-UPS1-C1 cells, most of the enriched KEGG pathways were similar; they consisted of the ribosome; proteasome; fatty acid metabolism; glycolysis/glucogenesis; valine, leucine and isoleucine degradation; citrate cycle (TCA cycle); and aminoacyl-tRNA biosynthesis pathways (Figure 4).

![Graph showing effects of anti-cancer drugs on the growth of NCC-UPS1-C1 cells](image)

**Figure 5.** Effects of anti-cancer drugs on the growth of NCC-UPS1-C1 cells. The effects of anti-cancer drugs with fixed concentration on the cell growth were monitored in NCC-UPS1-C1 cells (A). The anti-cancer drugs examined were listed in Supplementary Table 5. Eleven anti-cancer drugs reduced the cell growth by more than 99% (Supplementary Table 6). The half-maximal inhibitory concentration values were monitored for these 11 anti-cancer drugs (B) (Supplementary Table 7).
Sensitivity to anti-cancer drugs

Sensitivity to 164 anti-cancer drug treatments was assessed in the NCC-UPS1-C1 cells (Figure 5A, Supplementary Table 5). The cells were treated with anti-cancer drugs with the fixed concentration of 10 μM. Among 164 anti-cancer drugs examined, 11 compounds reduced the cell viability by more than 99% (Supplementary Table 6). Half-maximal inhibitory concentrations of these drugs were calculated by treating the cells with different concentrations (Figure 5B), and the results were summarized in Supplementary Table 7.

Discussions

We established a cell line, NCC-UPS1-C1, from a tumor tissue of a UPS patient. Accumulation of clinical data and in vitro and in vivo omics data, through more extensive omics studies of the cells as well as the original tumor tissues, will be required to evaluate the utility of established cell line. Characters of NCC-UPS1-C1 cells and their proteome data suggested the similar but not identical features between the original tumor tissue and NCC-UPS1-C1 cells. The proteomic characters should be further investigated using multiple proteomics modalities, and other omics studies are also worth considering. We showed that the NCC-UPS1-C1 cells could be used as a pre-clinical in vitro model to assess the effects of drugs on the cell viability. The clinical utility of anti-cancer drugs should be evaluated considering the possible adverse effects at certain drug concentrations, and the in vitro IC50 values may be useful for the re-purposing of existing anti-cancer drugs to UPS. The effects of combination of several anti-cancer drugs are also worth investigating as a pre-clinical examination. The recent studies indicated the possible utilities of patient-derived xenografts (PDXs) and the use of PDXs may be the next challenge to apply the in vitro examinations in this study to the treatments of patients. Continuous efforts to develop and characterize patient-derived cell lines will be necessary to realize novel clinical applications for UPS patients, and to explain the tumor heterogeneity and clinical diversity of UPS.

Conflict of interest

The authors declare that they have no conflicting interests.

Sources of support

This research was supported by the Practical Research for Innovative Cancer Control Program (15ck0106089h0002) from the Japan Agency for Medical Research and Development (AMED), and the National Cancer Center Research and Development Fund (26-A-3 and 26-A-9).

References


(Accepted 13 November 2017)