

Establishment and Characterization of a New Pancreatic Ductal Adenocarcinoma Cell Line Capan-26

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Abstract. *Background/Aim: Pancreatic ductal adenocarcinoma is one of the deadliest forms of human cancer. Since only a vast panel of cell lines can fully recapitulate disease heterogeneity, our aim was to establish a new pancreatic cancer cell line. Materials and Methods: Newly established pancreatic ductal adenocarcinoma cell line Capan-26 was characterized by assessing growth rate, tumor and stem cell marker expression, colony forming efficiency, mutations of KRAS and TP53 genes, karyotype and sensitivity to drug treatment. Results: Cell doubling time was 74 h. We detected CA19-9, CEACAM6, CD44, OCT4 and ZEB1 expression in Capan-26 cell line. Cells formed colonies in soft agar, have a deletion of KRAS exon 3 and a point mutation V172F in TP53 exon 5. They are a mixed aneuploid/polyploid population with high sensitivity to gemcitabine. Conclusion: Capan-26 is a unique cell line that may be used to study the mechanism of pancreatic cancer.*

Pancreatic cancer accounts for only 3% of cases among the leading cancer types in the United States; however, it places fourth according to the number of deaths. When metastatic pancreatic cancer is diagnosed, the 5-year patient survival decreases about ten-fold in comparison to an early detected disease (1). Most of the time pancreatic cancer is diagnosed in an advanced stage. Since there is a great genetic and phenotypic diversity in pancreatic tumors, there is a lack of sensitive and specific biomarkers (2, 3). Disease variability

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also makes pancreatic cancer difficult to combat using mainstream treatment modalities such as chemotherapy and radiotherapy, which are usually the only solutions at an advanced cancer stage, when surgical intervention is not applicable. For many years, cell lines have proved to be a useful *in vitro* model for cancer research (4) and American Type Culture Collection (ATCC) provides a panel of them for different cancer types, including pancreatic. However, the strength of cell lines lies in their diversity: each cell line represents a unique cancer case. Thus, we are constantly in need of new cell lines that can recapitulate primary tumors. This study presents establishment and characterization of the new pancreatic ductal adenocarcinoma cell line Capan-26. To our knowledge, it is the first documented pancreatic cancer cell line derived from a Lithuanian patient.

Materials and Methods

Cell line establishment. Primary tumor tissue was obtained from a 65-years old female Lithuanian patient with pancreatic ductal adenocarcinoma, stage T2N0M0, differentiation grade 3. The patient did not have any remarkable past medical history. After pancreatic resection, the patient received adjuvant chemotherapy with FOLFIRINOX. Fifty-two months later, the patient's condition is stable and no cancer relapse is detected. The patient has read and signed the form of informed consent for taking part in this research, approved by Vilnius Regional Biomedical Research Ethics Committee (Protocol No. PancCa001-3).

For the establishment of the cell line, tumor tissue was washed three times with PBS and homogenized. Tissue fragments were transferred to a plastic culture dish containing Iscove's Modified Dulbecco's Media (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 15% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). After two days, islands of epithelial cells were observed. Cells were grown at 37°C in a humidified atmosphere with 5% CO₂ and routinely passaged with 0.25% trypsin/EDTA (Gibco). Frozen stocks of early passages were stored

in liquid nitrogen in culture medium with 8% dimethyl sulfoxide and 25% FBS. After thawing, the stored cells successfully attached and proliferated in culture. Currently, cells have undergone 50 passages. The established cell line was named Capan-26 (abbreviated *Cancer Pancreatic*, “26” indicating the number of the specimen).

Cell doubling time. Cells were seeded into a 48-well plate. Viable cells were counted with Guava easyCyte 8HT Flow Cytometer (Merck-Millipore, Darmstadt, Germany) at 24-h intervals for 5 days. Cell doubling time was calculated using the formula $v = \lg N_t / \lg N_0 - 1$, where doubling time = $1/v$, N_t – cell number at day 5, N_0 – cell number at day 1.

Immunofluorescence. Cells grown in 24-well plates on glass coverslips (2D) or in suspension on a non-adhesive plastic surface (3D) were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.2% of Triton X-100 in PBS. The nonspecific binding sites were blocked by incubating with 1% bovine serum albumin in PBS. Slides were then stained with primary anti-CEACAM6 (9A6, Santa Cruz Biotechnology, Dallas, TX, USA), anti-CA19-9 (116-NS-19-9, Invitrogen, Rockford, IL, USA), anti-E-cadherin (ECCD-2, Thermo Fisher Scientific, Waltham, MA, USA), anti-cytokeratin 5/6/18 (0.N.345, Santa Cruz Biotechnology), anti-CD44 (8E2, Cell Signaling Technology, Danvers, MA, USA) or anti-Ki-67 (MIB-1, Agilent, Santa Clara, CA, USA) antibodies, followed by Alexa Fluor 488 or Alexa Fluor 594 conjugated goat anti-mouse or Alexa Fluor 594 conjugated goat anti-rat secondary antibodies (Thermo Fisher Scientific). Cell nuclei were stained with 300 nM 4',6-diamidino-2-phenylindole dihydrochloride dye (Thermo Fisher Scientific). After washing, slides were mounted in ProlongGold antifade (Molecular Probes, Invitrogen) and observed using a confocal laser scanning microscope (Nikon Eclipse TE2000-S, Tokyo, Japan).

Colony forming assay. Five thousand cells per well were seeded into a 6-well plate in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 0.3% agarose (Thermo Fisher Scientific) and 1% insulin-transferrin-selenium (ITS, Life Technologies, NY, USA) on top of the 0.5% agarose layer. After two weeks, formed colonies were stained with MTT dye (Roth, Karlsruhe, Germany) and counted.

RT-qPCR. Total RNA was extracted from cells grown in a monolayer (2D) or in aggregates (3D) using GeneJET RNA Purification Kit (Thermo Fisher Scientific). cDNA was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). RT-qPCR was performed on the MasterCycler RealPlex4 RT-PCR system (Eppendorf, Hamburg, Germany) using Luminaris HiGreen qPCR Master Mix (Thermo Fisher Scientific). The reaction conditions were as follows: pre-denaturation at 95°C for 3 min followed by amplification of 40 cycles. Results were normalized to *TBP* gene expression. Primer sequences were as follows: *OCT2* 5'-AATCTCTACCCGCTCCCTT-3' and 5'-CACAGAGCTGCTCGTGAACCA GT-3', *OCT4* 5'-CCTCCTGAGTAGCTGGGATT-3' and 5'-GCTGAATACCTTCCCAAATAGAA-3', *ZEB1* 5'-CGCAATAACGC TGTTAAAGG-3' and 5'-GTGCAGGAGGGACCTCTTTA-3', *NANOG* 5'-CAGCTACAAACAGGTGAAGACC-3' and 5'-CATCC CTGGTGGTAGGAAGA-3', *TBP* 5'-CCACTCACAGACTCTCA CAAC-3' and 5'-CTGCGGTACAATCCCAGAACT-3'.

Mutational analysis. Full length *KRAS* and *TP53* exon 5-9 cDNA was ligated into pJET1.2 vector (CloneJET PCR Cloning kit, Thermo Fisher Scientific) and sequenced using MacroGen sequencing service (Seoul, Republic of Korea).

Chromosomal analysis. Capan-26 cells were subjected to karyotyping analysis as described previously (5).

Cell viability assays. Cell viability after gemcitabine hydrochloride (Sigma, St. Louis, MO, US), 5-fluorouracil (TEVA, Vilnius, Lithuania), oxaliplatin (TEVA) and cisplatin (TEVA) treatment was determined with MTT dye. Proportion of apoptotic and necrotic cells was evaluated by flow cytometry using CellEvent Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific) and propidium iodide (Sigma) staining, respectively. Cells in S-phase were detected with Click-iT Edu Cell Proliferation Kit (C10337, Thermo Fisher Scientific). Quantification was done with ImageJ software.

Results

Morphology and growth characteristics. In adhesive plates, Capan-26 cells grew in monolayer and formed epithelial-like islands. However, when plated on a non-adhesive surface they clustered in aggregates of irregular shape (Figure 1A and B). Cell doubling time of Capan-26 cells was 74 ± 1 h (Figure 1C).

Expression of cancer and epithelial markers. The cells that grew in monolayer or in 3D expressed CEACAM6 and CA19-9 (Figure 1D and E). Also, Capan-26 cells stain positively for E-cadherin and cytokeratin 5/6/18 (Figure 1F).

Colony forming efficiency and stemness. At an early passage (20th) Capan-26 cells formed three types of colonies in soft agar (Figure 2A): aggregate, organ-like cystic structure and compact sphere, the latter consisting the majority. At later passages (40th) sphere morphology tended to become uniform (compact), while colony forming efficiency increased approximately 3 times (Figure 2B). Microscopy confirmed positive expression of the stem cell marker CD44 in Capan-26 cells (Figure 2C). Also, in comparison to monolayer, cells growing in 3D showed increased expression of *OCT4* and *ZEB1*, but the levels of *OCT2* and *NANOG* transcripts remained basal (Figure 2D).

Oncogene mutations. About one sixth (3 out of 18 clones investigated) of the Capan-26 cell population was found to bear a deletion of *KRAS* exon 3 (Figure 3A). No point mutations in the *KRAS* gene were detected by sequencing. Also, the cells were found to have the V172F (G→T) point mutation in exon 5 of *TP53* (Figure 3B). Pancreatic cancer related oncogene *SMAD4* was not detected in Capan-26 cells (data not shown).

Karyotyping. Capan-26 cells comprise a mixed population of aneuploids (approx. 44 chromosomes, 50% of cells) and pseudotetraploids (approx. 77 chromosomes, 50% of cells).

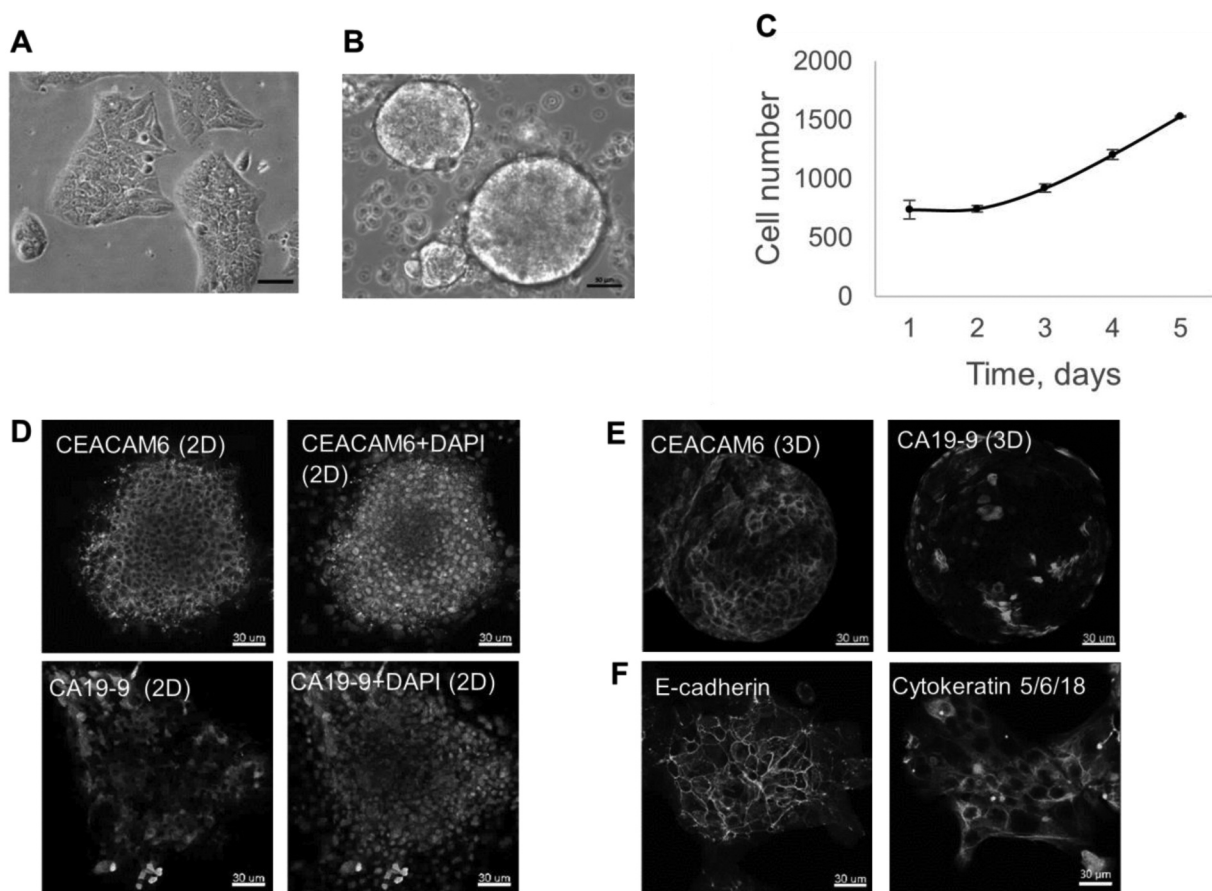


Figure 1. Growth characteristics and marker expression. A and B) Morphology of Capan-26 cells growing in a monolayer (2D) and in aggregates/spheres on an adhesive and a nonadhesive surface (3D), respectively. Scale bar – 50 μ m. C) Growth curve of Capan-26 cells. $n=3$. D and E) Expression of CEACAM6 and CA19-9 in cells that grow in 2D and 3D systems, respectively. F) E-cadherin and cytokeratin 5/6/18 expression in Capan-26 cells.

One translocation and 1-2 marker chromosomes were detected in each of the 100 metaphase plates inspected (Figure 3C).

Sensitivity to chemotherapeutic drugs. MTT cell viability test showed that Capan-26 cells were rather resistant to traditional pancreatic cancer chemotherapeutics, such as 5-fluorouracil (5-FU), oxaliplatin (OxaPt) and cisplatin (CisPt), but were sensitive to gemcitabine (Gm) (Figure 4A). On the other hand, significant changes in the viable cell population were detected after OxaPt and CisPt treatment, both drugs increased apoptosis (Figure 4B). As seen from EdU staining, Gm significantly decreased the number of cells in the S-phase, while OxaPt and CisPt had effect only on Edu incorporation rate (Figure 4C, D and E).

Discussion

Here, we present the novel pancreatic ductal adenocarcinoma cell line Capan-26. Success rate of pancreatic cancer cell line

establishment rarely exceeds 10%; in our case it was 4%. The main difficulties we faced was fibroblast outgrowth and low cell viability after tissue dissociation. However, in our case primary tissue easily disaggregated, thus increasing our chances of successful cell line establishment.

An interesting observation might be drawn from the functional characterization of Capan-26 cells: only cells that grew in aggregates expressed pancreatic cancer markers. Although both CA19-9 and CEACAM6 were secreted and might have just become “trapped” in cell clusters, CEACAM6 immunofluorescence also showed a membrane staining pattern. It is known that CEACAM6 over-expression mediates anoikis resistance in pancreatic cancer cells (6), and induces epithelial-to-mesenchymal transition (EMT) (7), which explains its expression in cells growing in 3D. Also, such cells have increased expression of ZEB1 and OCT4, established indicators of EMT and stemness (8, 9). CD44 and OCT4 are also known as pancreatic cancer stem cell markers (10). The anoikis resistant cell population especially

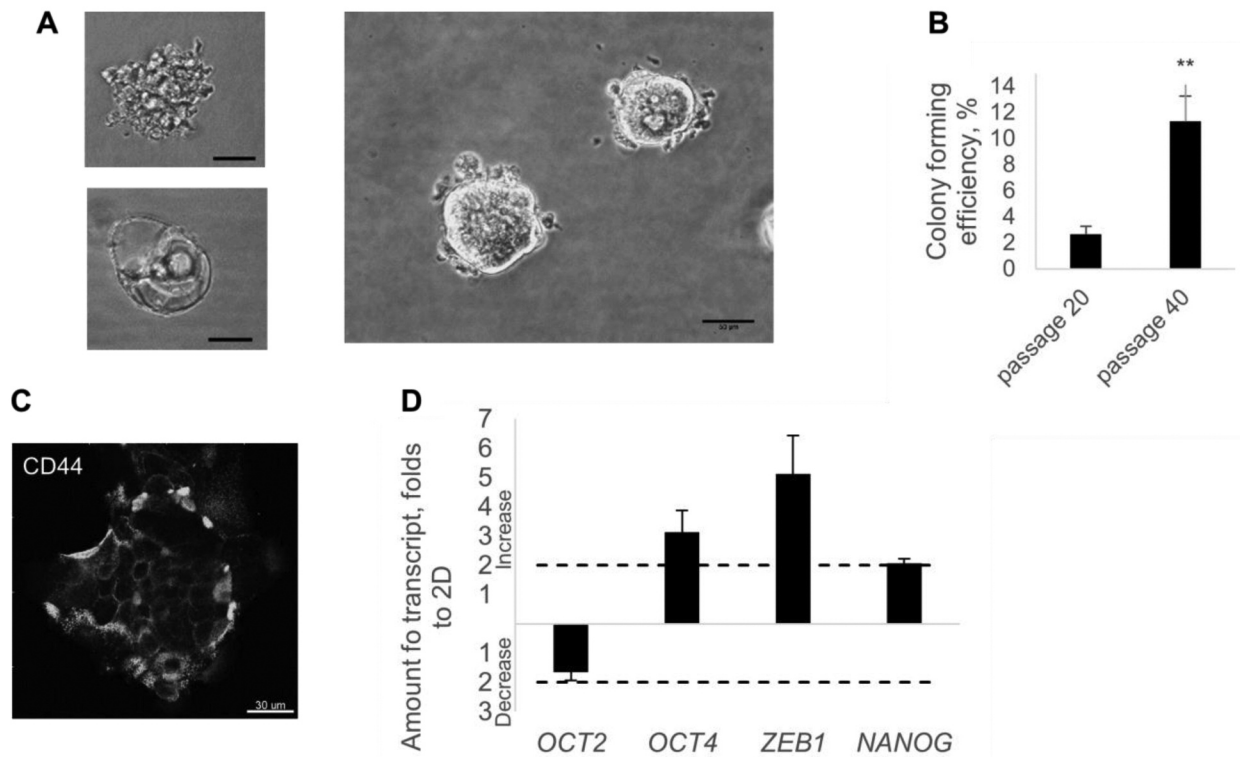


Figure 2. Colony forming efficiency and stemness. A) Different types of colonies formed by Capan-26 cells in soft agar. Scale bar – 50 μ m. B) Colony forming efficiency of early (20th) and late (40th) passage. C and D) Stem cell marker expression was assessed by immunofluorescence (C) or qPCR (D). n=3. In D, higher than twofold expression change was considered statistically significant.

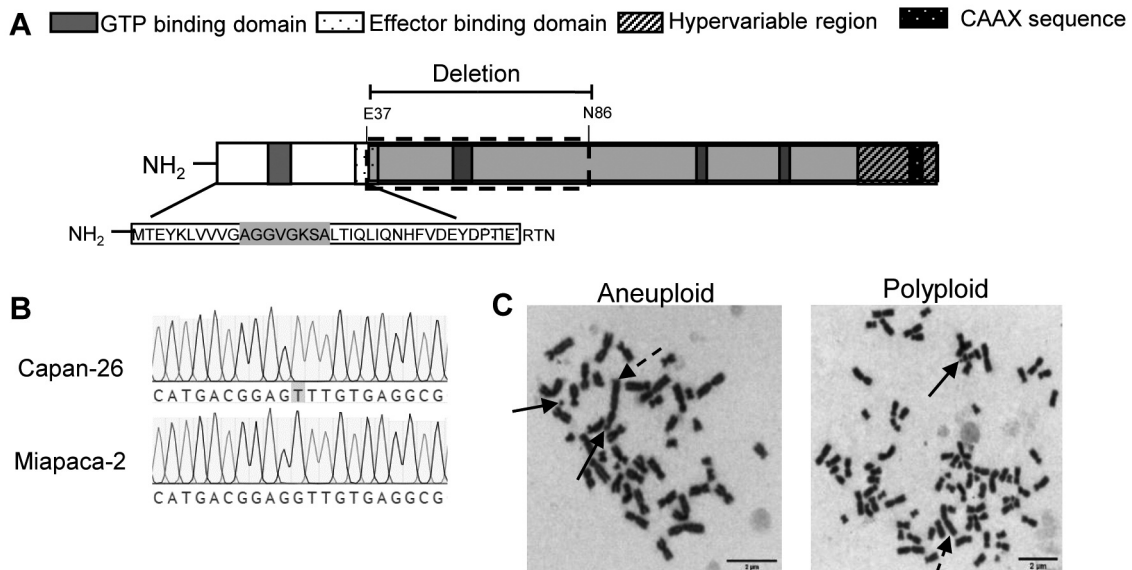


Figure 3. Genetic alterations in Capan-26 cells. A) A schematic representation of KRAS deletion in Capan-26 cells. A translated truncated peptide is also depicted. B) TP53 point mutation in Capan-26 cells; Miapaca-2 cells are used as a positive control. C) Karyotyping of Capan-26 cells. Metaphase plates of aneuploid and polyploid cells are shown. Arrows depict translocation (dashed) and marker chromosomes (solid). Scale bar – 2 μ m.

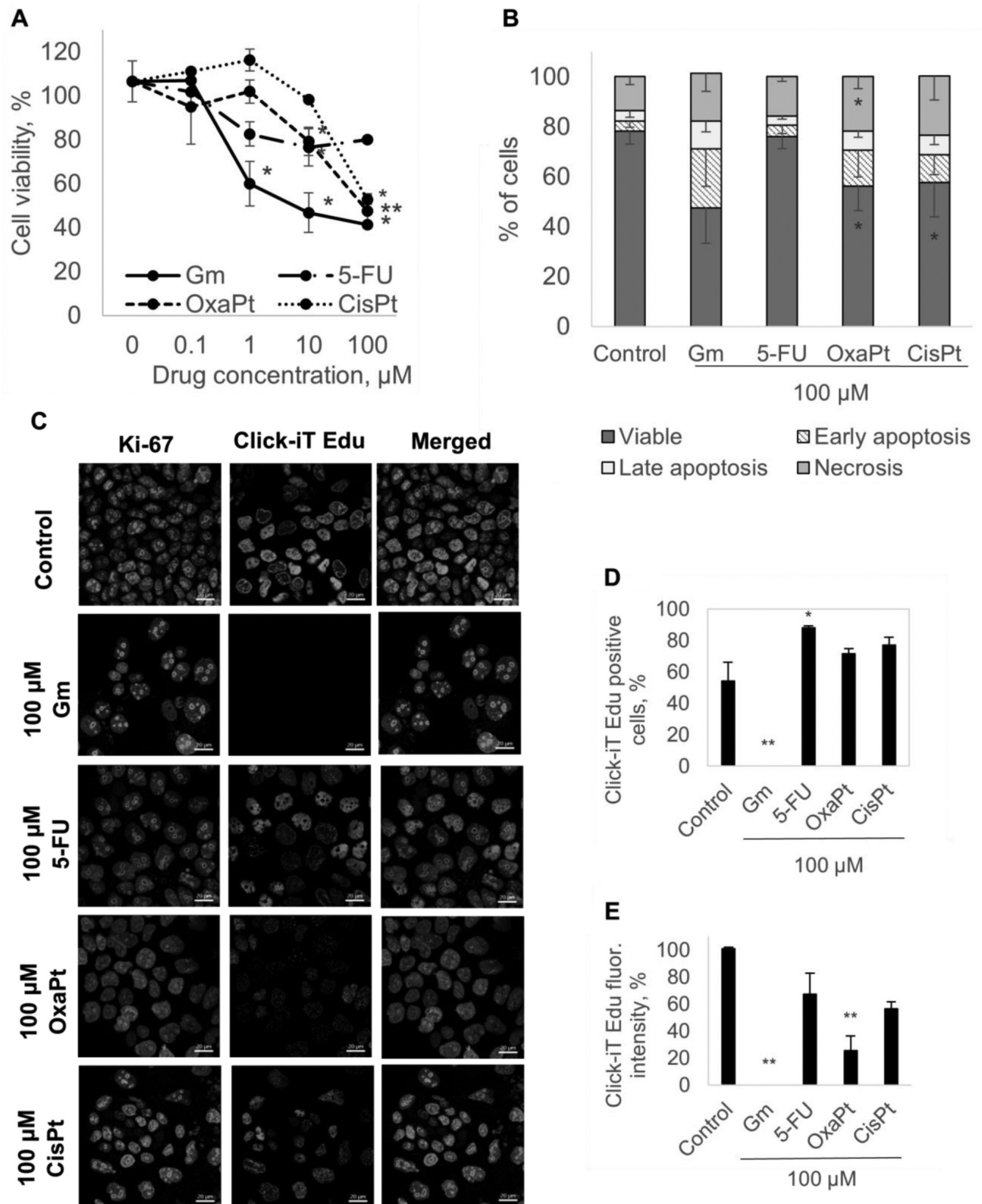


Figure 4. Sensitivity of Capan-26 cells to drug treatment. A) Capan-26 cell viability after drug treatment, determined by MTT assay. B) Cell death assessment after drug treatment. C) Proportion of cells in S phase (Click-iT Edu staining) after drug treatment. Ki-67 staining shows cells in cell cycle. Scale bar – 20 µm. D and E) Quantification of number of cells in the S phase and average Click-iT Edu fluorescence intensity, respectively. n=3.

expands at late passages. Interestingly, at early passages, a minority of the spheres exhibited vacuolar morphology, typical for mucin secreting cells.

Capan-26 line has a dual polyploid/aneuploid nature. Polyploidy is not uncommon in pancreatic cancer: in established cell lines (Miapaca-2, Capan-2, Panc-1), modal chromosome number varies between 60-70. However, Capan-26 cells are extremely unique considering their other genetic abnormalities: they have an oncogene *KRAS* exon 3 deletion and V172F point mutation in exon 5 of the *TP53* gene, none of which have been documented for pancreatic cancer to date. In ovarian cancer cells, the V172F mutation promotes cisplatin resistance (11). Indeed, Capan-26 cells were less sensitive to cisplatin (and oxaliplatin) compared to gemcitabine. Interestingly, FOLFIRINOX was administered to the patient after surgery, although Capan-26 seem to be resistant to one of its drug components, 5-fluorouracil. In conclusion, the novel cell line Capan-26 will be a valuable tool for preclinical human pancreatic cancer studies in the future.

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

EZ established cell line, performed the experiments, analyzed data and wrote the manuscript. VD performed karyotyping. BK and AS managed and provided information about the patient. AS performed the operation. BK and PS edited the manuscript. MV supervised the project. PS acquired funding.

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