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A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an in vitro research platform

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Abstract There are a large number of stable pancreatic ductal carcinoma cell lines that are used by researchers worldwide. Detailed data about their differentiation status and growth features are, however, often lacking. We therefore attempted to classify commonly used pancreatic carcinoma cell lines according to defined cell biological criteria. Twelve pancreatic ductal adenocarcinoma cell lines were cultured as monolayers and spheroids and graded according to their ultrastructural features. The grading system was based on the integrity of membrane structures and on the presence of mucin granules, cell organelles, nuclear and cellular polymorphism, cell polarity, and lumen formation. On the basis of the resulting scores the cell lines were classified as well, moderately, or poorly differentiated. In addition, immunocytochemistry was performed for the markers cytokeratin 7, 8, 18, 19, carcinoembryonic antigen, MUC1 MUC2, MUC5, and MUC6. The population doubling time of monolayer cultures, determined by a tetrazolium salt based proliferation assay was correlated with the ultrastructural grade. The grading of the ultrastructural features of the monolayers, and particularly of the spheroids, revealed that Capan-1 and Capan-2 cells were well differentiated; Colo357, HPAF-2, Aspc-1, A818-4,

BxPc3, and Panc89 cells were moderately differentiated and PancTu-1, Panc1, Pt45P1, and MiaPaCa-2 cells poorly differentiated. Membrane-bound MUC1 staining was a characteristic of well differentiated cell lines. The population doubling time of the monolayer cultures was related to the differentiation grade. No relationship was found between the p53, K-ras, DPC4/Smad4, or p16^{INK4a} mutation status and the grade of differentiation. We conclude that the proposed ultrastructural grading system combined with the proliferative activity provides a basis for further comparative studies of pancreatic ductal adenocarcinoma cell lines.

Keywords Pancreatic ductal carcinoma · Cell lines · Differentiation · Ultrastructure · Proliferation

Introduction

As is the case with other carcinomas, studies on the cell biology and genetics of ductal adenocarcinoma (DAC) of the pancreas are largely based on in vitro investigations employing various cell lines that are commonly available through cell culture collections. The data produced in these studies are often used to make general statements regarding the biological features of DACs and their response to certain stimuli, even if only a few cell lines were examined. However, DACs are quite heterogeneous. This is reflected in their grade of differentiation, which varies from tumor to tumor. It must therefore be anticipated that the various cell lines also reveal a great deal of diversity in structure and function, so that generalized interpretations of results need to be viewed cautiously. Interpretations of data would, however, be more meaningful if there were a cell line catalogue listing cell lines according to their morphological differentiation, functional properties, growth kinetics, and main genetic changes. In 1993 we made a first attempt to grade pancreatic carcinoma cell lines on the basis of their ultrastructural features [22]. Similar studies were also performed by Vila et al. [34], who detected ductal differentiation markers

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such as MUC1, cytokeratin (CK) 7, and carbonic anhydrase II in four newly established pancreatic carcinoma cell lines and classified these cell lines on the basis of their phenotypical features into more and less differentiated groups. As helpful as these early studies were in providing a first phenotypic profile of pancreatic carcinoma cell lines, they were not yet systematic and failed to relate the structural, functional, and genetic features of a greater number of well established cell lines to their proliferation status. In an earlier study we analyzed the genetic alterations in 22 pancreatic carcinoma cell lines of known or potential ductal origin and found that more than 90% of the cell lines harbored alterations of the p16^{INK4a}, K-ras, and p53 genes, 36% of them DPC4/Smad4 genetic alterations [25].

In this study we systematically screened 12 DAC cell lines for their in vitro growth, fine structural differentiation, expression of functional markers, and cell population doubling time. To analyze in vitro growth we used not only the monolayer culture system but also the spheroid tumor model. Being a three-dimensional in vitro culture it facilitates direct cell-cell interactions and may therefore simulate in vivo growth more closely than the monolayer conditions. Our analysis revealed a conspicuous degree of heterogeneity among these 12 cell lines, which is best reflected by the ultrastructural grade of differentiation of spheroids and the population doubling time.

Material and methods

Table 1 lists the pancreatic ductal carcinoma cell lines investigated in this study. The origin of each cell line was traced back to the respective sources and publications, to ascertain that they were truly generated from DACs. The histological typing of the tumors and their grade of differentiation according to the WHO classification [8] were deduced, if not explicitly mentioned in the original articles, from the descriptions and illustrations of the tumor's morphology. The histology and grade of the xenotransplants were

studied in experiments by the authors [15] or have been described by other authors (see Table 1).

Culture conditions

Cells were cultured as monolayers in RPMI 1640 with Glutamax (Gibco, Karlsruhe, Germany) containing 10% fetal calf serum (PAA Laboratories, Cölbe, Germany) under standard culture conditions (5% CO₂, 95% air in humidified chamber at 37°C).

Generation of spheroids

Cells were trypsinized, counted in a hemocytometer and seeded onto nonadherent 96-well plates with U-form wells at a concentration of 10⁴/well in RPMI medium containing 10% fetal calf serum supplemented with 20% methyl cellulose (M-0512, Sigma-Aldrich, Sternheim, Germany). Spheroids were harvested after 96 h, pelleted, and washed in phosphate-buffered solution.

Transmission electron microscopy

Monolayer cultures were grown to confluency on glass slides, washed twice in phosphate-buffered solution and fixed in 1–2.5% glutaraldehyde in 0.1 M cacodylate buffer. Some cell lines and the spheroids were fixed in a solution containing 0.05% picric acid, 2.5% glutaraldehyde, and 2% freshly prepared formaldehyde in 0.1 M cacodylate buffer. All samples were postfixed in 1% osmium tetroxide in cacodylate buffer. Following dehydration monolayer cultures were embedded in Epon in situ on the glass slides. Ultrathin sections, cut on a Leica Ultracut UCT ultramicrotome, were stained with uranyl acetate and lead citrate and examined under a Zeiss E902 electron microscope. At least 20 cells from each of two independent cultures of each cell line were photographed on Kodak electron microscope film.

Ultrastructural grading

The ultrastructure of the monolayers and spheroids was evaluated on microphotographs according to the slightly modified criteria of Kern et al. [14]. The analysis included cellular polymorphism (variability of cell shape, amount of cytoplasm), nuclear polymorphism (nuclear membrane, chromatin, nucleoli), mucin granules (rare, abundant), cell organelles (Golgi apparatus, endoplasmic

Table 1 Origin, histology and grade of differentiation of pancreatic ductal adenocarcinoma (PDAC) cell lines

Cell line	Source of tumor cells	Histology and grade ^a of primary tumor	Histology and grade of transplanted tumor (in nude or SCID mice)	Principal investigator
A818-4	Ascites	PDAC, G2	G2 ^b	H. Kalthoff (Germany) [25]
ASPC-1	Ascites	PDAC, G2	G2/G3 (focally G1) [5]	W.H. Chen and (USA) [5]
BxPc3	Primary tumor	PDAC, G2	G2/G3 [33]	M.H. Tan (USA) [33]
Capan-1	Liver metastasis	PDAC, G1	G1 [16]	J. Fogh (USA) [6]
Capan-2	Primary tumor	PDAC, G1	G1 [19]	J. Fogh (USA) ^c
Colo357	Lymph node metastasis	PDAC, G1-G2	G2/G3 (focally G1 and adenosquamous components) [23]	R. Morgan (USA) [26]
HPAF-2	Ascites	PDAC, G2?	not known	R.S. Metzgar (Germany) [24]
MiaPaCa-2	Primary tumor	PDAC, G3	G3 ^b	A. Yunis (USA) [35]
Panc1	Primary tumor	PDAC, G3	G3 [18]	M. Lieber (USA) [18]
Panc89	Lymph node metastasis	PDAC, G2	G2/G3 [28]	T. Okabe (Japan) [28]
PancTu1	Primary tumor	PDAC, G2-G3	G3 ^b	M. von Bülow (Germany) [25]
Pt45P1	Primary tumor	PDAC, G3	G3 ^b	H. Kalthoff (Germany) [25]

^a WHO grading system

^b Unpublished data

Table 2 Grading of ultrastructural features of monolayer cultures of PDAC cell lines (*grade 1* score 5–8, *grade 2* score 9–12, *grade 3* score 13–15)

Cell line	Cellular polymorphism	Nuclear polymorphism	Cell membrane structures	Mucin granules	Cell organelles	Final score	Grade
Capan-1	1	2	1.5	1–2 (1.5)	1	7	1
Capan-2	1	2–3 (2.5)	2	1–2 (1.5)	1	8	1
BxPc3	3	2	1–2 (1.5)	1	1–2 (1.5)	9	2
HPAF-2	3	3	1	1	1	9	2
Panc89	2	2	2	1	2	9	2
A818-4	3	2	3	1	1	10	2
Aspc-1	3	3	2	1	1	10	2
Colo357	3	2	2	2	1	10	2
PancTuI	3	3	2–3 (2.5)	1–2 (1.5)	3	13	3
MiaPaCa-2	3	2–3 (2.5)	3	2	3	13.5	3
Panc1	3	3	3	3	2–3 (2.5)	14.5	3
Pt45P1	3	3	3	2–3 (2.5)	3	14.5	3

Table 3 Grading of ultrastructural features of spheroids generated from cultured PDAC cells (*grade 1* score 7–11, *grade 2* score 12–16, *grade 3* score 17–21)

Cell line	Cellular polymorphism	Nuclear polymorphism	Cell membrane structures	Mucin granules	Cell organelles	Lumen formation	Cell polarity	Final score	Grade
Capan-1	1	1	1–2 (1.5)	1	1	1	1	7.5	1
Capan-2	1–2 (1.5)	2	1	1–2 (1.5)	1	1	1–2 (1.5)	9.5	1
Colo357	2	1	2	1	1	2	3	12	2
HPAF-2	3	3	1	1	1	1	3	13	2
Aspc-1	2–3 (2.5)	3	2	1	1	1	3	13.5	2
A818-4	3	2	2	1	1	2	3	14	2
BxPc3	3	2	1	1–2 (1.5)	2	1–2 (1.5)	3	14	2
Panc89	3	3	1–2 (1.5)	1	1–2 (1.5)	1–2 (1.5)	3	14.5	2
PancTu-I	3	3	2	2	2–3 (2.5)	1	3	16.5	3
Panc1	3	3	2	2	2	2	3	17	3
Pt45P1	3	3	2	2	3	2	3	18	3
MiaPaCa-2 ^a	–	–	–	–	–	–	–	20 ^b	–

^a No spheroid formation

^b Estimated score

Table 4 Immunocytochemical features of monolayer cultures and spheroids generated from cultured PDAC cells (*m* membrane-bound staining pattern, *CK* cytokeratin, *CEA* carcinoembryonic antigen; – negative, + <10%, ++ 10–50%, +++ >50% of the cells stained)

Cell line	Grade	CK7	CK8, 18, 19 ^a	Pan-CK ^a	Vimentin	MUC1	MUC2	MUC5	MUC6	CEA ^a
Capan-1	1	+++	+	+++	++	+++ m	–	++	+++	+
Capan-2	1	+++	+++	+++	–	+++ m	–	–	–	–
Colo357	2	+++	+	+++	+	+++	–	++	–	+
HPAF-2	2	+++	+++	+++	+	++	–	+	–	++
Aspc-1	2	++	+++	+++	+++	++	+	+	–	++
A818-4	2	+++	–	+++	+++	+	+++	+	–	–
BxPc3	2	+++	+	+++	+	++	+	+	–	++
Panc89	2	+++	+	++	–	+	+	+	–	++
PancTuI	3	+++	++	+++	+++	+	–	–	+++	+
Panc1	3	–	+++	+++	+++	+	–	–	–	–
Pt45P1	3	+++	++	+++	+++	++	+	+++	–	–
MiaPaCa-2 ^a	3	–	++	+++	+++	++	–	–	n.d.	–

reticulum, mitochondria, microfilaments), cell membrane structures (cell junctions, interdigitations, microvilli), and in the case of spheroids additionally lumen formation and cell polarity (well to poorly developed). Each feature was evaluated in at least 20 intact cells from each series and given an individual score ranging from 1 to 3. The final score was calculated by adding the individual scores obtained for each cell line. To define the grade, the final scores were arbitrarily divided into three groups designated as grade 1 (final score, monolayer, 5–8; spheroid 7–11), grade 2 (final score,

monolayer, 9–12; spheroid, 12–16), and grade 3 (final score, monolayer, 13–15; spheroid, 17–21; Tables 2, 3).

Immunocytochemistry

Immunocytochemistry was performed on monolayer cultures and spheroids (Table 4). For monolayer cultures cells were grown on glass slides and fixed in 4% buffered paraformaldehyde for 1 h.

Then the cells were permeabilized by heating in a pressure cooker in Tris-EDTA-trisodium citrate buffer for 3 min. The spheroids were embedded in OCT medium, snap-frozen in isopentane-dry ice, and cut into 10- μ m-thin cryostat sections. Primary antibodies were used to MUC1 (1:100, Novocastra, Newcastle, UK), MUC2 (1:100, Novocastra), MUC5 (1:500, Mab2011, Chemicon, Temecula, Calif., USA), MUC6 (1:100, Novocastra), cytokeratin 7 (1:25, M 7018, Dako, Glostrup, Denmark), cytokeratin 8, 18, and 19 (1:10, CAM 5.2, 349205, Becton Dickinson, San Jose, Calif., USA), pan-cytokeratin (1:200, KI-1, 1918, Immunotech, Marseille, France), vimentin (1:100, M 7020, Dako), carcinoembryonic antigen (1:100, M7072, Dako), synaptophysin (1:50, Dako), and chromogranin A (1:2, Linaris, Wertheim-Bettingen, Germany). Serum blocking and detection were performed with a peroxidase-based mouse and rabbit Vectastain Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. The staining intensity was graded as mild, moderate or strong.

Proliferation assay

Confluent cultures were trypsinized, counted in a hemocytometer, and seeded into 96-well plates in concentrations of 5×10^3 , 10^4 , and 2×10^4 cells/well. The number of viable cells was detected with a colorimetric tetrazolium salt based assay (Cell Proliferation Assay, Roche Diagnostics, Mannheim, Germany) after 24, 48, 72, and 96 h. Each measurement was performed 12-fold in three independent series. Optical densities were plotted against incubation time. The highest proliferation rate was calculated from the curve.

Genetic data

The data on the mutational status of the most frequent genetic changes (p53, K-ras, DPC4/smud4, p16) in the cell lines were obtained from earlier publications [3, 4, 13, 25, 27].

Results

Features of monolayer and spheroid cultures

The pancreatic ductal adenocarcinoma (PDAC) cell lines grew as adherent monolayer cultures. Capan-1 and Colo357 and to some extent Panc1 cells tended to form bi- or multilayers in postconfluent cultures. A subset of Capan-1 and MiaPaCa-2 cells detached after the cultures became confluent and grew as floating cell aggregates (Capan-1) or single cells (MiaPaCa-2). The cells varied highly in shape from cell line to cell line. Capan-1 and Capan-2 cultures were rather monomorphic and consisted of polygonal, cuboid, or round-oval cells. Panc89 cells were moderately polymorphic, polygonal, or oval. All other cultures exhibited marked polymorphism. Cell shapes ranged from polygonal, oval-round to spindle-shaped fibroblastoid. HPAF-2, Colo357, A818-4 cultures additionally contained bizarre giant cells.

All PDAC cell lines formed spheroids within 24 h except for MiaPaCa-2, the cells of which disaggregated during harvesting. Capan-1 and Capan-2 cells formed cohesive spheroids consisting of smaller cell aggregates with lumen formation. A818-4, BxPc3, Colo357, HPAF-2, Panc89, PancTu-I, and Pt45P1 cells developed well circumscribed spheroids. Aspc-1 and Panc-1 spheroids had an irregular outer surface.

Ultrastructural features

Tables 2 and 3 show the results of the grading of the cell lines. The grade 1 cell lines, Capan-1 and Capan-2, differed little in their features. The nuclei of the monomorphous cells showed homogeneous chromatin and one to four round nucleoli. The abundant cytoplasm contained many electron dense or lamellar mucin granules, Golgi complexes, and elements of rough endoplasmic reticulum. The cells were connected by numerous cell junctions such as desmosomes and junctional complexes. Networks of intermediate filaments were anchored to the desmosomes. Capan-1 cells formed many interdigitations with neighboring cells. Capan-2 cells had well developed microvilli and glycocalyx on their surface. In spheroids both cell lines revealed marked lumen formation. Capan-1 cells and to some extent Capan-2 cells showed a polarization towards the outer surface of the spheroids, with mucin granules in apical position and nuclei at the opposite side. The lateral plasma membranes at the apical pole of the cells were connected by regular junctional complexes (Fig. 1a).

The grade 2 cell lines exhibited marked heterogeneity from cell line to cell line. The most striking difference between these lines and the grade 1 cell lines was the pronounced cellular and nuclear polymorphism. The nuclei of the polymorphic cells were irregular and sometimes highly segmented, occasionally with crude chromatin structure and with up to four nucleoli. In some cell lines a reduced number of interdigitations (A818-4, Aspc-1, Colo357) and desmosomes (Aspc-1, Colo357) was noted. In general the cells were well equipped with cell organelles, i.e., Golgi complexes, rough endoplasmic reticulum, and mucin granules. Lumen formation was seen in the spheroids, but the lumina were smaller and more irregular than in grade 1 spheroids. None of the grade 2 spheroids showed signs of polarization (Fig. 1b, c).

The grade 3 cell lines showed significantly fewer cell organelles, specialized membrane structures and mucin granules than the grade 2 cell lines. The cytoplasm contained elements of rough endoplasmic reticulum which were rarely organized in parallel structures. Golgi complexes were only seen in PancTu-I cells. Interdigitations were completely absent. Interestingly, PancTu-I spheroids formed well defined lumina. MiaPaCa-2 cells were not able to generate spheroids. We interpreted this phenomenon as loss of the capability to form cell-cell contacts and hence considered the MiaPaCa-2 cell line to be the most poorly differentiated line (Fig. 1d).

Immunocytochemistry

Since there is no specific marker for PDACs or ductal differentiation, we used a panel of antibodies directed against various antigens (Table 4). CK7 was strongly expressed in 10 of the 12 cell lines. All but one cell line (A818-4) reacted with an antibody (CAM5.2) against

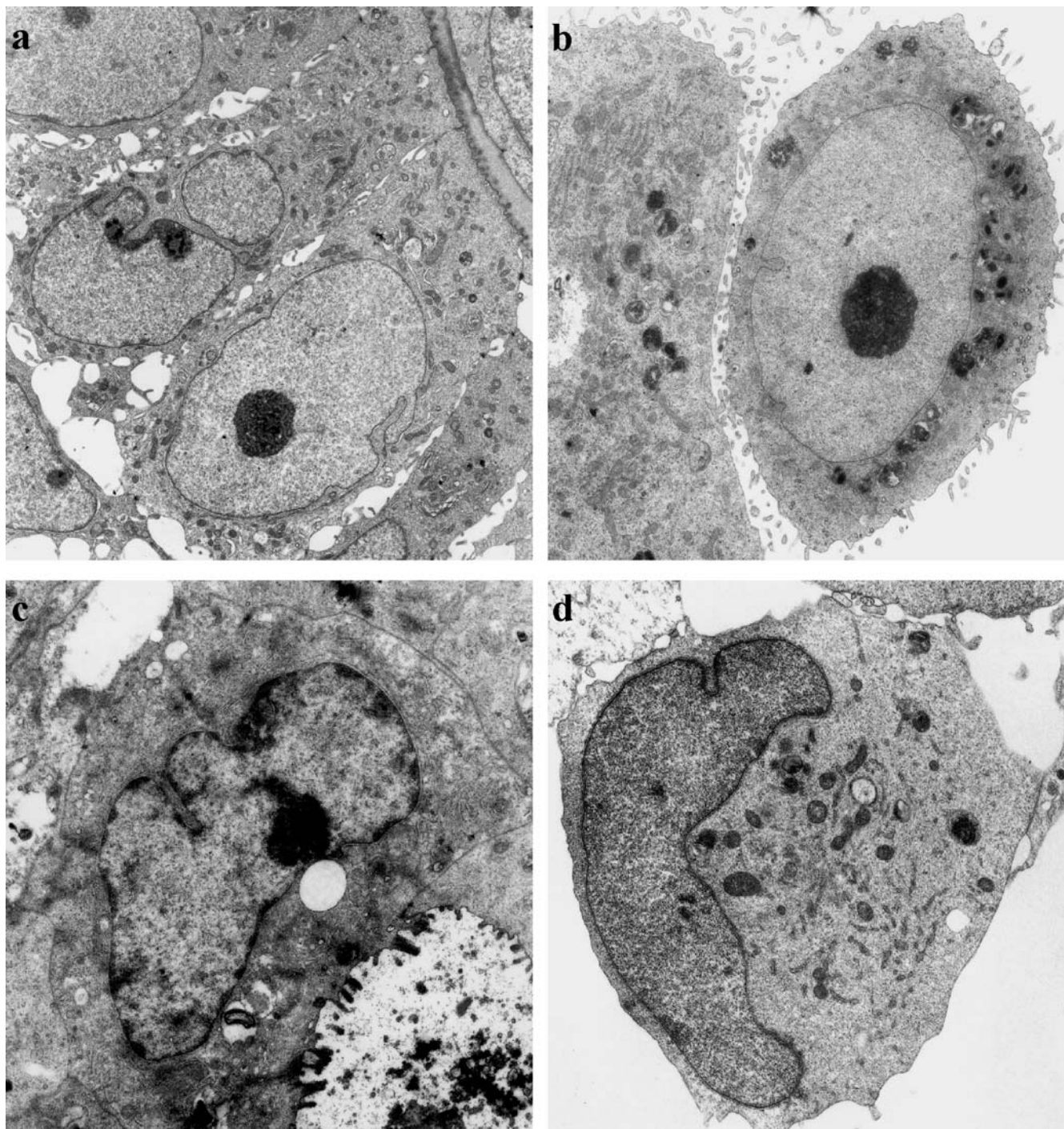


Fig. 1 Ultrastructural features of PDAC cell lines. **a** Spheroid of Capan-2 cells showed polarity toward the surface, a number of microvilli and cell junctions, glycocalyx on the surface. **b** Colo-357 cells in spheroid showed many microvilli, mucin granules, well developed Golgi apparatus and endoplasmic reticulum. **c** HPAF-2 cells in spheroid formed lumina with microvilli, numerous cell

junctions. Note the markedly irregular nucleus with crude chromatin structure and membrane invagination. **d** MiaPaCa-2 cells in monolayer culture exhibited poorly developed membrane structures, cytoplasmic organelles and few mucin granules. Original magnification, $\times 3000$

CK8, 18 and 19, and all cells were strongly positive for the broad spectrum CK marker KI-1. Vimentin was variably expressed in grade 1 and grade 2 cell lines, and strongly expressed in all grade 3 cell lines. MUC1 was

expressed in all cell lines. Grade 1 cell lines displayed a membrane-bound staining pattern and grade 2 and 3 cell lines faint to moderately strong cytoplasmic staining. The MUC2, MUC5, and MUC6 staining patterns appeared to

Table 5 Genetic aberrations in PDAC cell lines [25]

Cell line	Grade	K-ras	p53	p16	DPC4/smad4
Capan-1	1	Mut (p.m.) [4, 13]	Mut (p.m.) [4]	Mut ^a [27]	Mut (p.m.) [32]
Capan-2	1	Mut (p.m.) [4, 13]	W.t. [4]	Mut (ins) [27]	N.d. (low protein expression) ^b
Colo357	2	Mut (p.m.) [13]	W.t. [3, 13]	W.t. [27]	Mut (h.d.) [32] ^c
HPAF-2	2	Mut (p.m.)	Mut (p.m.)	Mut (del)	W.t.
Aspc-1	2	Mut (p.m.)	Mut (frameshift)	Mut (frameshift)	W.t./Mut (p.m.) [32]
A818-4	2	Mut (p.m.)	Mut	Mut (h.d.)	W.t.
BxPc3	2	W.t. [4, 13]	Mut (p.m.) [4, 13]	Mut ^a [27]	Mut (h.d.) [7]
Panc89	2	W.t. [13]/Mut (p.m.) [36]	Mut (p.m.)	Mut (meth)	W.t.
PancTu-I	3	Mut (p.m.)	Mut (p.m.)	Mut (meth)	W.t.
Panc1	3	Mut (p.m.)	Mut (p.m.)	Mut (h.d.)	W.t.
Pt45P1	3	Mut (p.m.)	Mut (p.m.)	Mut (h.d.)	W.t.
MiaPaCa-2	3	Mut (p.m.)	Mut (p.m.)	Mut (h.d.)	W.t.

^a No gene product detected

^b Personal communication I. Schwarte-Waldhoff (Immunologisch-Molekularbiologisches Labor, Medizinische Universitätsklinik Bochum, Knappschafts Krankenhaus, Ruhr-Universität, Bochum, Germany)

^c DPC4/Smad4 expressing Colo357 cell line exists also (personal communication, I. Schwarte-Waldhoff)

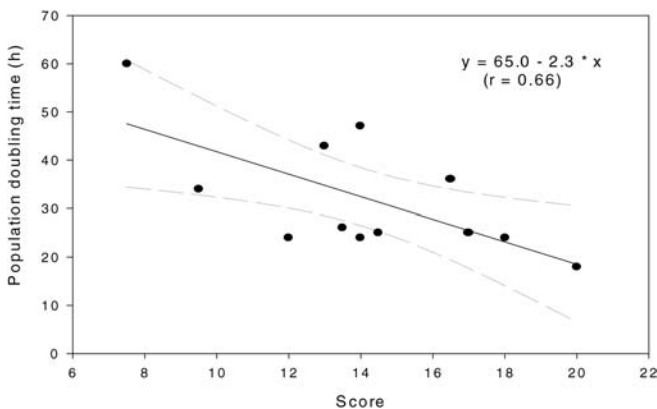


Fig. 2 Correlation between ultrastructural grade (final score) of spheroids and population doubling time of monolayer cultures

be unrelated to the grade of differentiation. Carcinoembryonic antigen expression was quite evenly distributed among the cell lines. The neuroendocrine markers chromogranin and synaptophysin were negative in all cell lines (data not shown).

Growth kinetics and correlation with ultrastructural grade

The population doubling time of the monolayer cultures ranged between 18 and 60 h. Grade I cell lines tended to have a higher population doubling time (47 ± 13 h) than grade II (31.5 ± 9.6 h) and grade III (25.7 ± 6.5 h) cell lines. The limited number of cell lines did not allow a statistical analysis. The shortest doubling time was seen in MiaPaCa-2 cells and the longest in Capan-1 cells. These two cell lines represented the opposite ends of the ultrastructural grading spectrum (Fig. 2).

Relationship between genetic alterations and ultrastructural grade

Table 5 shows the mutational status of the cell lines for K-ras (10/12), p53 (10/12), p16 (11/12), and DPC4/smad4 (1/8). There was no correlation between the K-ras, p53, p16, or DPC4/smad4 mutation rate and the ultrastructural grade of the cell lines.

Discussion

Pancreatic carcinoma cell lines are so widely used for the study of tumorigenesis, tumor progression and tumor biology that it is definitely worth establishing a catalog of their principal features based on a comparative characterization of such cell lines. In a previous study we analyzed the K-ras, p53, p16, and DPC4/Smad4 status of 22 cell lines derived from pancreatic ductal carcinomas to determine their genetic profile [25]. In this study we analyzed the light microscopic, ultrastructural, and immunocytochemical features of 12 established pancreatic ductal carcinoma cell lines (including six of the previously tested series of 22 cell lines) when grown in monolayers and in three-dimensional cell cultures, i.e., spheroids. By establishing an ultrastructural grading system we were able to relate the fine structure of the individual cell lines to their population doubling time. This revealed a relationship between differentiation and growth.

We traced the source of the various cell lines tested to ensure that all derived from human pancreatic carcinomas showing ductal differentiation in the primary and, where available, in the xenograft. All cell lines were found to originate from PDACs, although of different degrees of differentiation.

The morphology of the monolayer cultures, as analyzed by phase contrast microscopy, differed from cell line to cell line. However, the differences allowed only a rough distinction between monomorphic and polymorphic

cell lines. Multicellular spheroids are thought to represent a model that is intermediate in complexity between monolayer cultures and solid *in vivo* tumors, in that they facilitate direct cell-cell interactions, which may influence metabolism, proliferation, and differentiation. Indeed, in the spheroids formed by several of our PDAC cell lines we saw cell polarization and lumen formation. On the basis of these criteria we found Capan-1 cells to be the most highly differentiated because their spheroids even resembled hollow spheres, a recently developed culture model of ductal differentiation characterized by cellular polarization towards the surface of the three dimensional cell aggregates [17]. On the other end of this spectrum were MiaPaCa-2 cells, which, in contrast to Capan-1 cells and all other PDAC cell lines analyzed, completely failed to grow as spheroids. These spheroids may therefore be regarded as a test system in which lumen formation and polarization, two of the most important *in vivo* criteria for PDAC differentiation, can be studied *in vitro*.

According to the WHO grading system [8], PDACs may be classified into three grades that can be used as prognostic markers [15, 20]. Originally this grading system also included an analysis of the ultrastructural features of the tumor cells [14, 15]. We applied similar criteria to the electron microscopic grading of our PDAC cell lines grown either as monolayers or spheroids. The main features that were scored, and that defined the grade were cellular and nuclear polymorphism and the presence of specialized membrane structures, cell organelles, polarity, and lumen formation, the latter two criteria applying only to spheroids. The features discriminating between grade 1 and grade 2 cell lines were cellular and nuclear polymorphism and loss of polarity of the outer cell layer of the spheroids. Grade 2 and grade 3 cell lines were distinguished mainly by the decreased number of mucin granules and cell organelles. Interestingly, all cell lines that were able to form spheroids also revealed lumen formation, even if the histology of the xenotransplants of the respective cell lines failed to show any clear glandular formations, as in the Panc1 cell line. This seems to indicate that the feature of glandular/ductal formation of a carcinoma may be retained longer *in vitro* under adequate growth conditions than *in vivo*.

The ultrastructural grading revealed that most (>80%) of the analyzed cell lines fall into either the grade 2 (50%) or grade 3 (33.3%) category, while the grade 1 PDAC cell lines formed the smallest group (16.7%). This does not reflect the distribution of grades among PDAC, which is 33% G1, 51% G2 and 16% G3 tumors [20], but is related to the general difficulty in culturing cell lines from well differentiated tumors. The grade assigned to the monolayer of a PDAC cell line did not differ from that of the accompanying spheroid. However, the two usually differed in their final scores. When we compared the final scores of the monolayer and spheroid of a cell line with the histological differentiation of its respective xenotransplant, it appeared that in the grade 2 group the final score for the spheroid reflected the capacity of the respective cell line for glandular differentiation in the

xenotransplant better than the final score for the monolayer. For instance, the monolayer of BxPc3, a grade 2 cell line, had a lower final score than Aspc-1 or Colo357, although after xenotransplantation the latter two cell lines formed, at least focally, better differentiated tumors than BxPc-3 cells, which formed only moderately to poorly differentiated tumors. The spheroid scores for Aspc-1 and Colo 357 were, however, lower than that for BxPc3 and compared better with the cell lines' *in vivo* capacity for tumor differentiation. This suggests that their spheroids' ultrastructural score is the best determinant for the *in vivo* differentiation status of the tested PDAC cell lines. This is a notion, however, that needs to be confirmed in a larger study.

The expression patterns of cytokeratins are well defined characteristics of epithelial malignancies. CK7, 8, 18, and 19 are expressed in all human PDACs [9, 10, 29, 30], but decreasing expression of these cytokeratins is reported in the undifferentiated variant [12]. Vimentin, conversely, appears in undifferentiated carcinomas and is virtually absent from differentiated PDACs [12]. The disappearance of these cytokeratins and the appearance of vimentin may therefore be considered as a sign of dedifferentiation of PDAC *in vivo*. *In vitro* this observation is apparently of no relevance, since, irrespective of their ultrastructural differentiation, 10 of 12 PDAC cell lines expressed vimentin and to some extent also CK7, 8, 18, and 19, thereby confirming a number of other reports [12, 31]. None of the other immunocytochemical markers examined, i.e., MUC1, MUC2, MUC5, MUC6, CEA, synaptophysin, and chromogranin A, revealed any clear relationship with the ultrastructural differentiation of the cell lines. Hence they seem to be of no use in the characterization of PDAC cell lines. This applies particularly to MUC1 expression, which is a functional feature of PDACs [34]. MUC1 was expressed in all of our PDAC cell lines, confirming the data of other authors [2, 11]. Whether the membrane-bound MUC1 staining pattern seen in the spheroids of the Capan-1 and Capan-2 cell lines is a feature of highly differentiated PDAC cell lines needs to be confirmed by other studies. Interestingly, MUC2, which is virtually absent from PDACs *in vivo* but characterizes certain intraductal papillary mucinous neoplasms [1, 21], was expressed in 5 of 12 cell lines in this series, indicating that, as with vimentin, MUC2 is one of the markers whose reexpression is favored by *in vitro* conditions.

Because of the highly standardized culture conditions the population doubling time of monolayer cultures is a well reproducible feature of cell lines. We asked whether there is a relationship between the proliferation rate of the PDAC cell lines and their ultrastructural differentiation. As Fig. 2 shows, the population doubling time is related to the ultrastructural differentiation status of the cell lines. This is best exemplified by the cell lines with the highest degree of differentiation, Capan-1, and the lowest degree of differentiation, MiaPaCa-2, the two cell lines that also exhibited markedly differing proliferation rates. The fact that the correlation for the other cell lines is not better

may be explained in several ways. First, monolayer cultures are optimally supplied with nutrients and oxygen but have reduced cell-cell contacts and grow on an artificial surface. These conditions are highly artificial and may affect both the differentiation program and the proliferation rate. Second, the number of cell lines tested is too limited to reveal statistically significant differences. Third, in normal cells differentiation and proliferation are usually controlled by different signal transduction pathways, which may be affected differently by the genetic and epigenetic changes that lead to malignant transformation. Therefore differentiation and proliferation are not necessarily affected to the same extent in the PDAC cell lines.

In a previous publication on 22 PDAC cell lines we analyzed the most common genetic alterations in human PDAC, K-ras, p53, p16^{Ink4}, and DPC4/smad 4. The K-ras, p53, and p16^{Ink4} genes were found to be mutated in over 90% of the cell lines, followed by mutations of DPC4/Smad4 in approximately 50% [25]. Eleven of the 12 cell lines in this series (which included six cell lines from the previous series [25]) also harbored p16^{Ink4} mutations and 10/12 p53 and K-ras mutations, while DPC4/smad4 was mutated in only one of nine examined cell lines. From these results it was not possible to determine a correlation between differentiation and genetic alterations. It is possible that the genetic alterations that accompany the differentiation of tumor cells will not be detected until more is known about the genes that control the cellular differentiation process.

In conclusion, our study shows that the differentiation status of PDAC cell lines can be reliably determined by means of a detailed grading system based on the ultrastructural features. This system is best applied to spheroids, because this three dimensional culture model seems to offer a good correlate for the in vivo situation and is also related to the proliferation status of the monolayer cultures. The data from this study may be used to create a biological catalogue of PDAC cell lines listing their differentiation status and may be helpful in interpreting the relevance of many cell biological observations.

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