Original paper

Pan-p63 but not ΔNp63 (p40) expression in undifferentiated carcinoma of the pancreas

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Undifferentiated carcinoma of the pancreas (UC) is a carcinoma without a definitive direction of differentiation. Tumour protein p63 is a regulator of squamous phenotype, which may also be engaged in tumour development. N-terminal isoforms of p63 are TAp63 and ΔNp63. Pan-p63 antibodies are able to detect both isoforms, whereas p40 antibodies recognise the ΔNp63 isoform only. The aim of the study was to describe pan-p63/p40 immunohistochemical expression patterns in pancreatic neoplasms: UC, ductal adenocarcinomas, neuroendocrine tumours, neuroendocrine carcinomas, serous cystic neoplasms, and solid pseudopapillary neoplasms. DAK-p63 and BC28 antibodies were used for pan-p63 and p40 detection, respectively. Moderate-to-strong pan-p63 was found in anaplastic (pleomorphic giant cell) UC (n = 4), sarcomatoid UC (n = 2), UC with osteoclast-like giant cells (n = 3), and ductal carcinomas with partial squamous differentiation. Weak and focal pan-p63 expression was found in monomorphic UC (n = 3) and in the majority of neuroendocrine carcinomas (6/7 cases). Pan-p63 expression was infrequent in ductal carcinomas without squamous differentiation and in neuroendocrine tumours. Serous cystic and solid pseudopapillary neoplasms were pan-p63-negative. Ductal carcinomas with partial squamous differentiation were the only tumours with evident p40 expression. Pan-p63(+)/p40(–) immunohistochemical status may be supportive for UC diagnosis. The pan-p63 expression was not equivalent to squamous differentiation in pancreatic neoplasia.

Key words: TP63 gene, tumour protein p63, undifferentiated carcinoma, pancreas, pancreatic neoplasms.

Introduction

Ductal adenocarcinoma (DA) is the most common malignant neoplasm of the pancreas. Most cases of DA represent the ordinary (ductal) type. Several histopathological variants of DA were distinguished: adenosquamous carcinoma, colloid carcinoma, medullary carcinoma, hepatoid carcinoma, signet-ring cell carcinoma, undifferentiated carcinoma (UC), and undifferentiated carcinoma with osteoclast-like giant cells (UCOGC) [1].

Undifferentiated carcinoma of the pancreas is defined as malignant epithelial neoplasm without the definitive direction of differentiation [1, 2, 3]. Undifferentiated carcinoma exist in three histopathological patterns: (1) anaplastic (pleomorphic giant cell/large cell) UC, (2) sarcomatoid UC, and (3) carcinosarcoma. Anaplastic UC consist of definitely malignant, polymorphous, poorly cohesive atypical cells (both mononuclear and giant multinuclear), usually located in scant stroma. Spindle-shaped neoplastic cells are predominant in sarcomatoid carcinomas. Carcinosarcomas are composed of coexisting adenocarcinomatous and spindle cell (sarcoma-like) components [1]. Some rare UC may show small cell/round cell anaplastic

type [4, 5] or monomorphic [6] histopathological appearance. Importantly, different patterns of growth may coexist within a single UC [1, 2, 3, 5, 7, 8, 9]. The histopathological picture of UCOGC shows mononuclear atypical cells and multinucleated, histiocytic, osteoclast-like, benign cells [1, 2, 3, 5, 7, 10, 11, 12]. Carcinoma cells in UCOGC may be small ("histocyte-like cells") or large (i.e. pleomorphic giant cells) [12]. A component of (differentiated) DA may be found in more than 50% of UC cases and in 40-60% of UCOGC cases [3, 10, 13]. Squamous and rhabdoid cells, as well as heterologous elements, may be found in some UC/UCOGC [1, 3, 6, 7, 8, 9, 11]. Focal squamous differentiation may be found in 8-33% of UC cases [3, 8, 9], in particular in spindle-cell sarcomatoid carcinomas [7]. Adenosquamous carcinomas may contain a UC component in up to 13% of cases [14]. The UC component may be sometimes found in metastatic deposits of adenosquamous carcinoma [15]. UC/UCOCG may arise in mucinous cystic neoplasms (MCN) and in intraductal papillary mucinous neoplasms (IPMN) [2, 3, 10, 11, 12, 13].

Prevalence of UC is 6.8% among epithelial neoplasms of the exocrine pancreas [4]. The frequency of UC is distinctly higher among autopsied patients with previously recognised DA (16%), which suggests dedifferentiation of DA to UC during tumour progression [16]. Undifferentiated carcinoma with osteoclast-like giant cells constitutes 1.4% of invasive ductal carcinomas of the pancreas [12]. The prognosis in UC is very poor [1, 8]. Today the prognosis in UCOGC seems to be significantly better than in conventional DA and UC [12], contrary to earlier reports that included smaller numbers of cases [11].

Tumour protein p63 [17, 18], encoded by the *TP63* gene, is a crucial regulator of epidermal development and epidermal-mesenchyme interaction [19, 20]. It is a transcription factor involved in the regulation of cell cycle, cell proliferation and migration, apoptosis, senescence, development and morphogenesis, maintenance of stem cells, metabolism, and stress response (reviewed in [19, 21, 22, 23, 24]). The p63 is responsible for determination of squamous differentiation [19, 25]. Owing to its multidirectional activity, p63 may be engaged in both tumour progression and tumour suppression [26, 27, 28, 29, 30, 31, 32, 33, 34]. The role of *TP63*/p63 protein in the biology of DA was recently comprehensively investigated [30].

The p63 protein has several *N*-terminal and *C*-terminal isoforms. Major *N*-terminal isoforms (TAp63 and ΔNp63) are generated using two promoters. TAp63 isoforms contain a transactivation domain, in contrast to N-terminally truncated ΔNp63 isoforms. Both TAp63 and ΔNp63 have *C*-terminal isoforms, generated with alternative splicing: alpha, beta, gamma, delta, and epsilon [18, 35, 36]. TAp63 and ΔNp63 may have opposite functions because TAp63 has features of the tumour suppressor, and ΔNp63 may serve as an oncogene [18, 21, 22, 33, 34, 35, 37]. There are significant differences regarding TAp63 and ΔNp63 expression patterns across the body [38]. Gene targets of TAp63 and ΔNp63 are also partially different [18, 24]. The distinction of ΔNp63 and TAp63 in clinical samples may be important because p63 expression patterns are associated with clinicopathological features of tumours and long-term prognosis [39, 40]. ΔNp63 has a dominant-negative effect on p53 and p63 function [18] because it is able to functionally inactivate p53 [27]. ΔNp63 is able to overcome cellular senescence and to increase the population of stem-like cells [33]. ΔNp63 increases the growth, motility, and invasion potential of DA cells, and determines and regulates the molecular profile of DA of squamous subtype [30]. TAp63 is able to induce senescence and inhibit proliferation irrespective of p53 status [34]. Loss of TAp63 in p53-null DA cells results in an increase of their metastatic abilities [37]. TAp63 suppresses tumorigenesis and tumour progression through modulation of *Dicer* expression [31], and it controls stem cell population by its influence on senescence and cellular aging [41]. In some conditions, e.g. in thyroid cancer cells, TAp63 may also have a dominant-negative role in p53-mediated suppression and act as a tumour promoter [32]. TAp63 takes part in the regulation of gemcitabine resistance of DA cells [42, 43]. TAp63 and ΔNp63 may also directly transactivate genes involved in proliferation without interaction with *TP53* [28, 34]. Interaction with the miR-130b/TAp63 pathway may be an effective therapeutic approach [44].

Immunohistochemistry (IHC) is a competent way to show protein expression patterns in a microanatomical context. The p63 immunostain has wide utility in diagnostic pathology, in particular in differential diagnostics of a variety of breast and prostate lesions [45, 46, 47, 48]. The p63 immunoassay is also a valuable tool for confirmation of squamous differentiation in poorly differentiated carcinomas [49]. Several p63 antibodies are commercially available, but mouse monoclonal 4A4 and DAK-p63 antibodies are probably the most widely used for diagnostic purposes. They recognise both TAp63 and ΔNp63 isoforms, so they serve as pan-p63 markers [35, 50]. In contrast, p40 antibodies are able to detect ΔNp63 isoforms but not TAp63 isoforms [35, 46]. Because ΔNp63 is the main isoform present in squamous cell carcinomas [29, 35, 51], the specificity of the p40 antibody for detection of squamous differentiation is higher than pan-p63 antibody [35, 46]. Moreover, pan-p63 immunostain is not fully specific for squamous cell carcinoma because its expression may be seen in 31% and 54% of pulmonary adenocarcinomas and "large cell" lymphomas, respectively [35].

In contrast, only 3% of pulmonary adenocarcinomas and virtually no "large cell" lymphoma are p40-positive [35]. However, in some other diagnostic scenarios, pan-p63 and p40 antibodies have similar effectiveness. They are comparably competent in the detection of myoepithelial and basal cells in breast and prostate lesions, respectively [45, 46, 47, 48].

During the diagnostic workup, this author noticed pan-p63 expression in a case of pancreatic UC. This anecdotal observation was the rationale for the present investigation. It is possible that p63 may serve as a diagnostic immunomarker for UC. Therefore, pan-p63 expression should not be *per se* indicative of squamous/squamoid differentiation in pancreatic neoplasia. The aim of the study was to examine comprehensively the IHC expression patterns of tumour protein p63 in UC/UCOGC of the pancreas as well as in some other types of pancreatic neoplasia. This was performed using both pan-p63 and p40 antibodies.

Material and methods

Study samples

Cases of UC and UCOGC of the pancreas diagnosed between 2008 and 2018 were retrospectively retrieved from a prospective institutional database of pancreatic specimens. The inclusion criterion for the study was a histopathological diagnosis of UC or UCOGC [1], made in a resection specimen or in an incisional (surgical) biopsy of the primary tumour or metastatic lesion. Metastatic samples used in the present study were obtained from patients who presented with primary non-resectable pancreatic mass. For comparative purposes, samples of other pancreatic neoplasms arranged in tissue microarray (TMA) blocks (diameter of cores 1.5 mm) were utilised:

- paired samples of primary and metastatic DA assembled in a single TMA block (n = 19). These samples were obtained from patients who presented with DA and synchronous liver metastasis and underwent surgical open biopsy between 2006 and 2012. A single core was taken from both primary tumour and hepatic metastasis [52];
- samples of serous cystic neoplasms in two TMA blocks (n = 27); each tumour represented by five cores [53];
- samples of neuroendocrine tumours (NET) in two TMA blocks (n = 29); each tumour represented by three cores taken from primary lesions (some cases also had additional cores from regional or distant metastases) [54];
- samples of neuroendocrine carcinomas (NEC) in a single TMA block (n = 7); each tumour represented by five cores [54];
- samples of solid pseudopapillary neoplasms (SPN) in a single TMA block (n = 12); each tumour represented by four cores [54, 55].

Samples of normal pancreatic tissue were included in TMA block for control and orientation purposes.

Tissue processing and histopathological diagnoses

Specimens were fixed in 10% buffered formalin for 48-72 hours at room temperature. Tissue processing, paraffin embedding, and haematoxylin-eosin staining were performed in a routine manner. Each case was diagnosed based on the World Health Organisation (WHO) histopathological criteria [1, 56].

p63 and p40 immunostains

For UC/UCOGC cases, a single representative whole paraffin-embedded tissue block from each tumour was selected for IHC study. Blocks containing abundant undifferentiated neoplastic tissue as well as differentiated tumour component (if present) were preferentially chosen for IHC. Freshly cut 4 μm-thick sections were placed on adhesive glass slides (Menzel Gläser, Thermo Fisher, Braunschweig, Germany). Details of the IHC procedure are presented in Supplementary Data 1.

In brief, for detection of p63 protein, DAK-p63 mouse monoclonal antibody purchased from Dako/Agilent was selected. This antibody was shown to react with both TAp63 and ΔNp63 isoforms, so it is a pan-p63 antibody [50]. For detection of ΔNp63 isoforms, two p40 antibodies (both clone BC28, purchased from Abcam and Ventana) were used. Abcam antibody was available in a limited amount for the present study, so a portion of samples was stained with Ventana antibody. Several samples were examined using both p40 antibodies, aiming to confirm their diagnostic equivalence (see below). Diaminobenzidine was used for visualisation. Slides were counterstained with haematoxylin. In negative controls, primary antibodies were omitted. IHC slides and haematoxylin-eosin slides were digitised using a slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) using 40× mode (0.23 μm/pixel) and evaluated using dedicated software (NDP.view2, Hamamatsu).

IHC scoring

IHC scoring was performed by a single investigator. Nuclear expression was considered positive, but cytoplasmatic expression was also reported. The extent of expression was estimated visually and reported in percentages. Stain intensity was reported as weak (1+), moderate (2+), or strong (3+). Histoscore values were obtained by multiplication of stain extent and intensity (scores ranged from 0 to 300) [35].

In this study, cells expressing pan-p63 protein but not p40 protein were recognised as TAp63 positive [35, 57, 58, 59, 60].

In silico analysis

Sequences of p63, p73, and p53 proteins were compared with sequences of immunogens of DAK-p63 and BC28 antibodies. Human Protein Atlas data [61], The Cancer Genome Atlas (TCGA) dataset [62, 63], and the Australian Pancreatic Cancer Genome Initiative – International Cancer Genome Consortium (APGI-ICGC) dataset [64] were examined for p63/*TP63* status in the pancreas and in pancreatic DA. Details of *in silico* analysis are provided in Supplementary methods.

Statistical analysis

For quantitative variables, Mann-Whitney U tests, Kruskal-Walis ANOVA, and Wilcoxon-signed rank tests were used appropriately. Additionally, Spearman rank correlation coefficients were calculated. For qualitative variables, χ^2 tests were used. Survival data were examined using log-rank tests. Statistical calculations were performed using Statistica 13 software (Tibco Software, Palo Alto, CA, USA). Data obtained from APGI-ICGC cohort were statistically examined within R2 genomic analysis platform [65].

Ethics and guidelines

The Institutional Review Board gave permission to perform this observational study without full review needed for experimental studies. Histopathology-adjusted REMARK guidelines were followed [66].

Results

p63 protein and p63/p40 antibodies

There are at least 12 isoforms of p63 protein (Supplementary Data 2). Sequence alignments of isoforms of p63 protein are presented in Supplementary data 3. The immunogen of pan-p63 (DAK-p63) antibody is a synthetic peptide derived from the core DNA-binding domain of the human p63 protein [50]. The sequence of the DNA-binding domain of p63 protein is identical among 10 p63 isoforms (100% identity) and highly similar to another two isoforms (Q9H3D4-9 and Q9H3D4-10). There is a high similarity between the DNA-binding domain of human p63 protein versus a sequence of p73 protein (85% of identities by BlastP alignment), but not versus a sequence of p53 protein (57% identities). The immunogen of p40 (BC28) antibody is a synthetic peptide corresponding to human p40 – ΔNp63 amino acids 5-17 (ENNAQTQFSEPQY) [67]. This sequence is present in the exact form in 5 ΔNp63 isoforms (13/13 amino acids), and partially in another ΔNp63 isoform (Q9H3D4-10). In contrast, the BC28 immunogen sequence does not show significant similarity with TAp63 isoforms, p73, and p53 proteins.

Performance of pan-p63 and p40 antibodies/protocols in the present study was examined in control tissue of tonsil, as recommended [68, 69]. Both pan-p63 (Supplementary Fig. 1 [SF 1]) and p40 (SF 2) antibodies showed strong nuclear reactivity in basal and parabasal cells of squamous epithelium. Abluminal cells in adjacent minor salivary glands were also pan-p63 positive (SF 3) and p40 positive (SF 4). In contrast, weak to moderate reactivity in some lymphoid cells of the tonsil was seen using pan-p63 antibody (SF 5), but not using p40 antibody (SF 6). This was consistent with previous data, showing expression of both isoforms in squamous epithelium, but only TAp63 isoform in lymphoid cells [36]. Both p40 antibodies from Abcam and Ventana showed the same expression patterns in tissue controls.

In concordance with sequence alignment analysis, pan-p63 antibodies may cross-react with p73 protein [70]. Results of examination of pan-p63/p40 expression in a sample of normal fallopian tube suggested that DAK-p63 may indeed cross-react with p73 protein, in contrast to p40 antibody (SF 7, SF 8). Cross-reactivity of pan-p63/p40 antibodies with p53 was unlikely (details in Supplementary Data 4) [51, 70].

Normal pancreatic tissue was pan-p63 negative and p40 negative (SF 9 and SF 10, respectively), as reported previously [71]. This was concordant with gene expression data [72].

Histopathological features of UC/UCOGC samples

Nine UC and three UCOGC cases were available for the study. Detailed clinicopathological data of the study cases are presented in Supplementary Data 5. No case was treated with neoadjuvant chemotherapy. Follow-up data were not available for the present study.

Based on the predominant growth pattern [5], four cases of anaplastic (pleomorphic giant cell) type (Fig. 1A, SF 11, SF 12), two cases of sarcomatoid (spindle cell) type (Fig. 1D), three cases of monomorphic type (Fig. 1G), and three cases of UCOGC (Fig. 1J, SF 13) were distinguished. Nine cases were associated with the invasive differentiated component (six conventional DA, two adenosquamous carcinomas, and a single colloid carcinoma). Five cases were derived from macroscopic precursor lesions: MCN (n = 3) or IPMN (n = 2). In particular: (1) three anaplastic UC cases developed from tubular adenocarcinoma (one of them arose in IPMN and another one in MCN; a single case of adenocarcinoma had a minor [less than 30%] squamous component); (2) one anaplastic

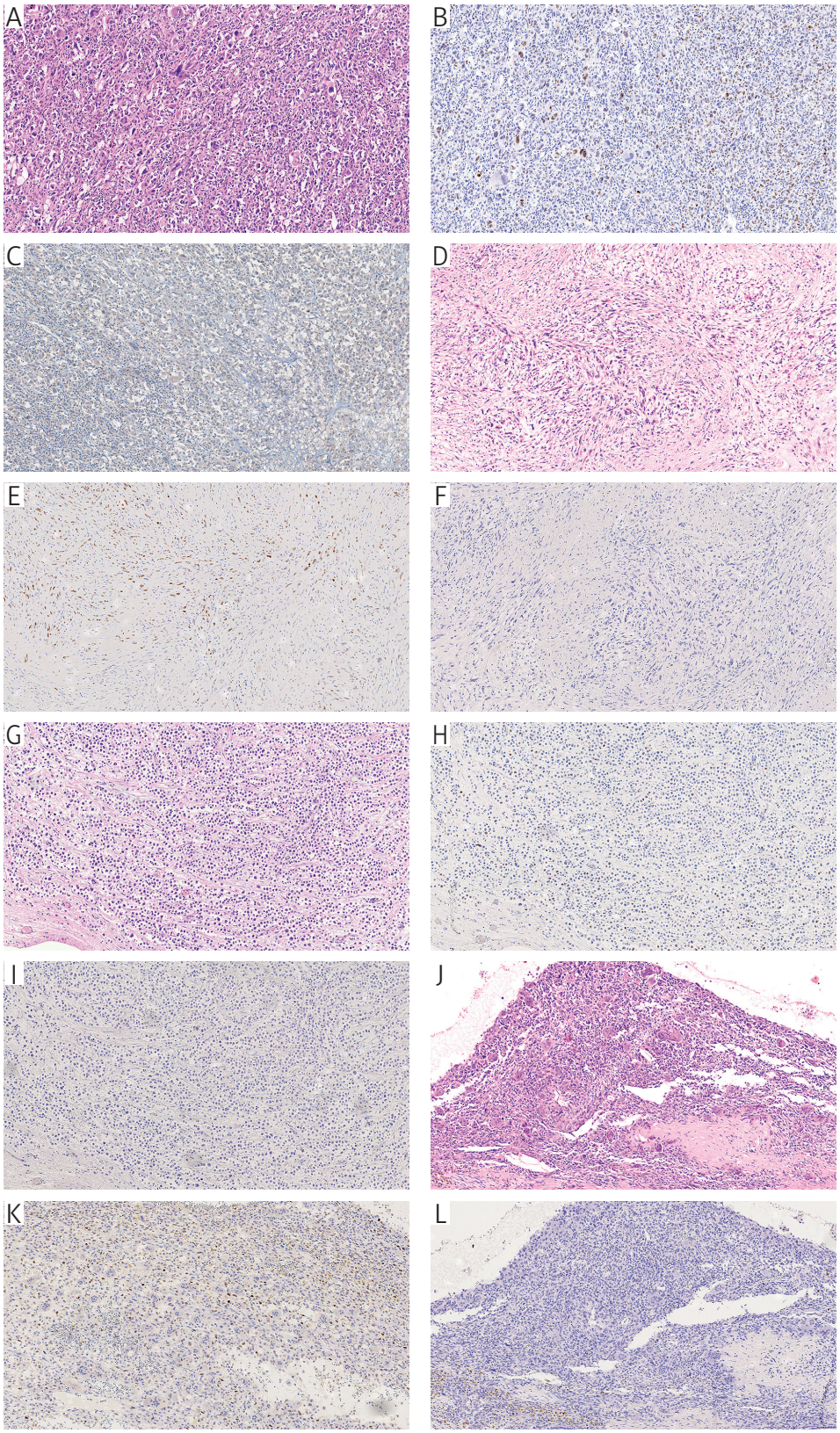

Fig. 1. Undifferentiated carcinoma of the pancreas. Original magnification 20×. A) Anaplastic type. Haematoxylin-eosin stain. B) Expression of pan-p63 in anaplastic type. C) Expression of p40 in anaplastic type. D) Sarcomatoid type. Haematoxylin-eosin stain. E) Expression of pan-p63 in sarcomatoid type. F) Expression of p40 in sarcomatoid type. G) Monomorphic type. Haematoxylin-eosin stain. H) Expression of pan-p63 in monomorphic type. I) Expression of p40 in monomorphic type. J) Undifferentiated carcinoma with osteoclast-like giant cells. Haematoxylin-eosin stain. K) Expression of pan-p63 in undifferentiated carcinoma with osteoclast-like giant cells. L) Expression of p40 in undifferentiated carcinoma with osteoclast-like giant cells

UC developed from colloid carcinoma associated with IPMN; (3) two cases of sarcomatoid carcinoma developed from adenosquamous carcinoma; (4) two UCOGC developed from tubular adenocarcinoma associated with MCN; and (5) one case of UCOGC was associated with foci of adenocarcinoma with partial (less than 30%) squamous differentiation. Three monomorphic samples of UC did not have distinct differentiated component, but importantly all three were diagnosed in incisional biopsies. In a single monomorphic UC rare scattered signet-ring cells were noted, and they did not form any topographically distinct tumour component.

The pan-p63 expression in UC

Results of IHC examinations are summarised in Table I. Surprisingly, pan-p63 expression was found in all nine examined UC cases. However, the extent of stain ranged widely, from 3% up to 60% of cells (median 20%). Stain intensity was weak in three cases and moderate to strong in six cases (histoscore ranged from 3 to 150; median 60). The pattern of pan-p63 expression in UC was clearly related to its histopathological type: (1) cases with predominant anaplastic (pleomorphic giant cell) type showed moderate to strong expression in 15-50% of cells (median 25%) (Fig. 1B, SF 14, SF 15). Pan-p63 expression in UC was heterogeneous in terms of stain intensity – in general, pleomorphic cells showed slightly stronger expression than histiocyte-like cells, but not all carcinomatous cells were positive. The reason for this observation remained unknown because the heterogeneity of expression could be explained by differences in the microscopical picture of positive and negative cells or by fixation/staining artefacts. (2) Two cases of sarcomatoid carcinoma showed moderate to strong expression in 30% and 60% of cells (Fig. 1E). (3) Monomorphic UC showed only weak and focal (median 5%) pan-p63 immunoexpression (Fig. 1H). No clear association between the extent of p63 stain or p63 histoscore versus clinicopathological data other than tumour histotype were noticed.

The pan-p63 expression in UCOGC

Three UCOGC cases had moderate to strong pan-p63 expression in 10-50% of cells. Importantly, osteoclast-like giant cells were consistently pan-p63 negative (Fig. 1K).

The p40 expression in UC/UCOGC

Six UC/UCOGC cases were examined with Abcam p40 antibody, three with Ventana p40 antibody, and another three cases with both antibodies. In sharp contrast to pan-p63, p40 expression was not seen in any UC case, irrespective of the used antibody (Fig. 1C, SF 16, SF 17, Fig. 1F, Fig. 1I). Similarly, no UCOGC case was p40-positive (Fig. 1L), irrespective of the used antibody.

The pan-p63 and p40 expression in differentiated invasive component coexisting with UC/UCOGC

Expression of pan-p63 in the differentiated invasive component of the tumours was heterogeneous. As expected, two cases of adenosquamous carcinomas showed moderate to strong pan-p63 expression stain in 50% of cells (one case) and strong stain in 90% of cells (one case). This was paralleled with p40 positivity in 10% and 80% of cells, respectively. Adenocarcinomatous component in another two cases (one UC and one UCOGC) showed partial squamous differentiation, with focal pan-p63/p40 expression. The remaining the conventional adenocarcinomatous components coexistent with UC showed weak and focal pan-p63 expression in 1-10% of cells and no p40 expression. A single case of colloid carcinoma was pan-p63/p40 negative. Expression patterns of Abcam and Ventana p40 antibodies in differentiated component coexisting with UC/UCOGC (SF 18, SF 19) were very similar.

The pan-p63 and p40 expression in non-invasive component coexisting with UC/UCOGC

Two UC cases were associated with IPMN: a single IPMN case had a weak pan-p63 expression in less than 1% cells, and another case showed weak expression in approximately 5% of cells (SF 20). Both cases were p40-negative. Three UC/UCOGC cases coexisted with MCN: a single MCN showed weak pan-p63 expression in 5% of cells and no p40 expression. Another two MCN had similar expression patterns: pan-p63 expression in moderate-to-high-grade areas was focal and weak-to-moderate in intensity (SF 21). In contrast, in some low-to-moderate grade areas strong and diffuse pan-p63 expression was evident (SF 22). The p40 expression was very focal in low-grade areas (SF 23), whereas moderate-to-high-grade areas were p40-negative (SF 24). In a single UC case, foci of pancreatic intraepithelial neoplasia-3 were visible in the tissue section submitted to IHC – moderate-to-strong pan-p63 expression was found in 10% of cells (SF 25); p40 was negative.

The pan-p63 and p40 in pancreatic islets

Interestingly, pancreatic islets showed cytoplasmic but not nuclear pan-p63 stain in six out of seven UC/UCOGC cases (in other five cases islets were not present in the tissue section submitted to IHC). Staining was preferentially detected in residual islets in atrophic peri/intratumoural parenchyma (SF 26) –

Table I. Expression of pan-p63 and p40 in the study samples

TUMOR TYPE	PAN-P63			P40			P-VALUES [1]	
	STAIN EXTENT (IN PERCENTAGE); MEDIAN (RANGE)	STAIN INTENSITY	HISTOCORE; MEDIAN (RANGE)	STAIN EXTENT (IN PERCENTAGE); MEDIAN (RANGE)	STAIN INTENSITY	HISTOCORE; MEDIAN (RANGE)	STAIN EXTENT (IN PERCENTAGE)	HISTOSCORE
UC								
Anaplastic (pleomorphic giant cell) (n = 4)	25% (15-50)	Moderate to strong: 4	60 (30-150)	0%	None: 4	0	< 0.001/0.068	< 0.001/0.068
Sarcomatoid (n = 2)	45% (30-60)	Moderate to strong: 2	90 and 120	0%	None: 2	0	NP	NP
Monomorphic (n = 3)	5% (3-10)	Weak: 3	5 (3-10)	0%	None: 3	0	NP	NP
UC total (n = 9)	20% (3-60)	Weak: 3 Moderate to strong: 6	60 (3-150)	0%	None: 9	0	< 0.001/0.008	< 0.001/0.008
UCOGC								
UCOGC (n = 3)	20% (10-50)	Moderate to strong: 3	60 (30-150)	0%	None: 3	0	NP	NP
Differentiated invasive component coexisting with UC/UCOGC								
DA (n = 6)	5% (1-25)	Weak: 4 Moderate to strong: 2[2]	7.5 (1-75)	0% (0-25)	None: 4 Weak: 1 Strong: 1[2]	0 (0-75)	0.054/0.043	0.056/0.043
Adenosquamous carcinoma (n = 2)	70% (50-90)	Moderate to strong: 2	210 (150-270)	45% (10-80)	Moderate: 2	90 (20-160)	NP	NP
Colloid carcinoma (n = 1)	0%	None	0	0%	None	0	NP	NP
Non-invasive component coexisting with UC/UCOGC								
Intraductal papillary mucinous neoplasm (n = 2)	less than 1% and 5%	Weak	1 and 5	0%	None	0	NP	NP
Mucinous cystic neoplasm (n = 3)	5% (5%)	Weak: 1 Strong: 2	15 (5-15)	0 and less than 1%	None: 1 Weak: 2	1 (0-1)	NP	NP

Table I. Cont.

Tumor type	pan-p63			p40			p-values [1]	
	Stain extent (in percentage); median (range)	Stain intensity	Histocore; median (range)	Stain extent (in percentage); median (range)	Stain intensity	Histocore; median (range)	Stain extent (in percentage)	Histoscore
Other neoplasms of the pancreas								
DA – primary tumors (n = 19)	0% (0-80) [4]	None: 10 Weak: 6 Moderate to strong: 3[3]	0 (0-240) [4]	0% (0-80)[4]	None: 15 Weak: 1 Moderate to strong: 3[3]	0 (0-240) [4]	0.129/0.050	0.142/0.161
DA - liver metastases (n = 19)	0% (0-60) [4]	None: 12 Weak: 4 Moderate to strong: 3[3]	0 (0-180) [4]	0% (0-70)[4]	None: 14 Weak: 2 Moderate to strong: 3[3]	0 (0-210) [4]	0.436/0.402	0.501/0.463
Serous cystic neoplasms (n = 27)	0%	None: 27	0%	Not performed			NA	NA
Solid pseudopapillary neoplasms (n = 12)	0%	None: 12	0%	Not performed			NA	NA
Neuroendocrine tumors (n = 29)	0% (0-30)	None: 25 Weak: 3 Moderate: 1	0 (0-60)	0%	None: 29	0	0.042/0.068	0.042/0.068
Neuroendocrine carcinomas (n = 7)	20% (0-25)	None: 1 Weak: 5 Moderate: 1	20 (0-50)	0%	None: 7	0	0.004/0.028	0.004/0.028

[1] *comparison of results obtained using pan-p63 versus p40 antibody; p values calculated with Mann-Whitney U tests (first given value) / and Wilcoxon signed-rank tests (second given value)*

[2] *focal (less than 30%) adenosquamous differentiation*

[3] *adenosquamous carcinoma or focal (less than 30%) adenosquamous differentiation*

[4] *primary tumors versus liver metastases, non-significant (Wilcoxon signed-rank test)*

UC – undifferentiated carcinoma, UCOGC – undifferentiated carcinoma with osteoclast-like giant cells, DA- ductal adenocarcinoma; NP – not performed due to the limited number of samples; NA – not applicable

islets in preserved parenchyma at a distance from the tumour were consistently pan-p63-negative. In all seven available cases, pancreatic islets were p40 negative (SF 27).

The pan-p63 and p40 in other tumours

Neoplasms in TMA format were examined using Ventana p40 antibody.

Nineteen cases of paired samples of primary and metastatic DA in TMA (out of 32 assembled pairs) were available for analysis (Table I). Pan-p63 expression was detected in nine primary tumours and seven secondary lesions. p40 expression was seen in four primary tumours and in five secondary lesions. The extent of expression did not differ significantly between primary and secondary lesions. In general, patterns of pan-p63 and p40 expression in DA were similar: (1) a single case of adenosquamous carcinoma showed strong and diffuse pan-p63 and p40 expression (SF 28 and SF 29, respectively); (2) a similar observation concerned two DA samples with focal squamous differentiation; and (3) another sample of DA with partial micropapillary differentiation showed weak pan-p63 and p40 expression in approximately 5% and 2% of cells, respectively (SF 30 and SF 31).

Discordant expression of pan-p63 and p40 was detected in a single case of UC and in four cases of conventional DA: (1) a sample of sarcomatoid carcinoma showed very weak pan-p63 expression (and no p40 expression) in approximately 10% of cells (SF 32 and SF 33, respectively); and (2) four DA cases without overt squamous differentiation had very weak pan-p63 expression (and no p40 expression) in rare cells (not more than 5% of cells) (SF 34 and SF 35, respectively).

All examined cases of serous cystic neoplasms and solid pseudopapillary neoplasms were pan-p63-negative (p40 immunostains in these neoplasms were not performed).

Among 29 NET cases assembled in TMA, four (13.8%) were pan-p63 positive. Three cases showed weak staining in less than 1% of cells (two cases) and 3% of cells (a single case). Another case showed weak to moderate stain in 30% of cells (SF 36) – this was a sample of NET G3 diagnosed in distal pancreatectomy specimen in a 45-year-old male. This tumour invaded the colon (ENETS pT4N1 stage). Ki67 labelling index in that case ranged from 1/2000 cells (0.05%) in a cold spot up to 487/2000 cells (24.4%) in a hot spot. Pan-p63 expression was retained in metastatic lymph node with extent and intensity similar to the primary tumour. All examined NET cases (n = 29) were p40-negative, including pan-p63-positive samples (SF 37).

Unexpectedly, six out of seven examined NEC cases showed pan-p63 expression (SF 38, SF 39), including a single NEC with partial squamous differentiation (SF 40). Stain intensity was weak in five cases, and moderate in a single case only. The extent of expression in positive cases ranged from 3% to 25% of cells (median 20%). All NEC cases were p40-negative (SF 41).

p63 in Human Protein Atlas resources

The Human Protein Atlas [61] contains a digitised collection of TMA of normal and neoplastic tissues. For p63 IHC assessment three antibodies were utilised: CAB00083 (monoclonal 4A4 antibody provided by Agilent/Dako), as well as HPA006288 and HPA007010 (two polyclonal antibodies developed by Sigma-Aldrich). All available TMA were evaluated for the purpose of this study (results in Supplementary Data 6). Unfortunately, samples stained with 4A4 antibody and polyclonal antibodies were not matched, with the exception of a single case (sample no. 834). This precluded comparative evaluation of results obtained using all three antibodies.

As expected, normal pancreatic tissue (n = 3) was p63-negative with 4A4 antibody (not shown). Twelve DA cases were stained with 4A4 antibody (samples obtained from six females and six males, aging from 52 years to 79 years; four G1 cases, three G2 cases, and five G3 cases, as assessed by this author). A single case was, in fact, adenosquamous carcinoma; it showed strong expression of p63 in approximately 90% of cells (case no. 582, SF 42). Seven cases were p63-negative or had only trace stain (up to 2% of cells with weak stain intensity). Two other cases showed weak or weak-to-moderate stain in 10% of cells; another one case showed a moderate-to-strong stain in approximately 3% of cells. Surprisingly, a single case of G1 DA without any overt features of squamous differentiation (case no. 646) showed a moderate-to-strong stain in approximately 60% of cells (SF 43).

TP63 in TCGA cohort

The role of *TP63* in pancreatic cancer was also investigated using the TCGA dataset [62]. The analysis was based on a curated cohort of 139 samples [73] (details in Supplementary Methods). *TP63* gene mutations, amplifications, and overexpression (defined based on z-score above 2.0) were found in three (2.2%); two (1.4%), and 13 (9.4%) cases, respectively. *TP63* gene expression was not correlated with patients' age ($p = 0.117$), tumour cellularity ($p = 0.415$), tumour purity ($p = 0.909$), or tumour ploidy ($p = 0.388$). *TP63* gene expression was not related with patients' gender ($p = 0.928$), tumour localisation within pancreas ($p = 0.337$), tumour grade ($p = 0.357$), pT tumour stage ($p = 0.336$),

pN tumour stage (p = 0.190), overall tumour stage (p = 0.375), presence of residual tumour at surgical margins (p = 0.504), presence of *KRAS* mutation (p = 0.232), or *CDKN2A* gene status (p = 0.073).

Importantly, *TP63* gene expression was related to transcriptomic tumour classes, as defined by Moffitt *et al*. [74], because *TP63* expression was significantly higher in "basal-like" cancers in comparison to "classical" cancers (p < 0.0001). "Quasi-mesenchymal" samples (as defined by Collisson *et al.* [75]) also showed higher *TP63* expression than "classical" and "exocrine" samples (p = 0.002). As expected, tumours of "squamous" transcriptomic type (as defined by Bailey *et al.* [64]) had higher *TP63* expression than "immunogenic", "progenitor", or "aberrantly differentiated endocrine exocrine' tumours (p = 0.003).

As examined by this investigator, at least focal squamous (adenosquamous) differentiation was found in one out three cases with *TP63* mutation, in one out of two cases with *TP63* amplification, and in five out of 13 cases with *TP63* overexpression. Three cases in the curated TCGA cohort were originally diagnosed as adenosquamous carcinomas – TP63 expression in these cases was at the 72nd, 86th, and 97th percentile of the curated TCGA cohort. Clinicopathological data regarding patients with *TP63* gene alterations are described in Supplementary Data 7.

Upregulation of *TP63* gene (defined as expression equal to the median or higher in the curated cohort) was not related significantly to overall survival (log-rank test, p = 0.163). A best expression cut-off value equal to 143.2 showed that higher expression of *TP63* (n = 31) resulted in significantly lower overall survival in comparison to lower expression (n = 108), p = 0.003 (SF 44). Upregulation of *TP63* gene (defined as expression equal to the median or higher in the curated cohort) was associated with worsening of progression-free survival (log-rank test, p = 0.015). A best expression cut-off value equal to 261.5 showed that higher expression of *TP63* (n = 21) resulted in significantly lower progression-free survival in comparison to cases with lower expression (n = 118), p = 0.005 (SF 45).

Importantly, *TP63* expression in curated TCGA samples did not show statistically significant correlation with *TP73* expression (R = 0.143; p = 0.092) (SF 46), nor with *TP53* expression (R = 0.151; p = 0.077) (SF 47).

A single case of UC was included in the TCGA cohort (TCGA-2J-AABP, not included in the curated subgroup examined above). This case represented anaplastic (pleomorphic giant cell) type UC with rhabdoid cells. *TP63* gene expression in that case was low (fourth percentile of the curated TCGA cohort), and *TP63* gene mutations or copy number alterations were not detected. *TP73* expression in UC case was at the 80th percentile of the curated TCGA cohort.

TP63 isoforms in the TCGA cohort

The most prevalent *TP63* isoform in DA samples in the curated TCGA cohort was ΔNp63-epsilon (uc010hzd.1) (Supplementary Data 8). Lower levels of uc003fsc.2 (ΔNp63-alpha) and uc003fsd.2 (ΔNp63-beta) were also detected. Other isoforms were expressed at very low levels. ΔNp63-epsilon and ΔNp63-alpha were the most prevalent isoforms in DA cases with *TP63* gene aberrations. Surprisingly, in a single case with *TP63* overexpression (TCGA-IB-7646) the most abundant isoform was TA*-alpha; that case had focal spindle cell differentiation. The only isoform detected in the UC case (TCGA-2J-AABP) was ΔNp63-epsilon.

TP63 in APGI-ICGC cohort

The APGI-ICGC cohort consisted of 96 resected samples of DA, DA variants, and DA associated with IPMN acinar cell carcinomas [64]. The results of *TP63* gene expression patterns in APGI-ICGC cohort were similar to those in the TCGA cohort. *TP63* gene expression was related to the histological type of tumour (ANOVA, p < 0.001), being significantly higher in adenosquamous carcinomas (median expression 6.40) than in conventional DA (median 1.24) (SF 48). *TP63* gene expression was higher in tumours of higher histological grade (ANOVA, p = 0.017) (SF 49). *TP63* gene upregulation did not show any association with patients' age (R = 0.02; p = 0.851), gender (t-test, p = 0.356), tumour localisation within the pancreas (ANOVA, p = 0.274), and overall tumour stage (ANOVA, p = 0.456). A best expression cut-off value equal to 6.1 showed that higher expression of *TP63* (n = 26) resulted in significantly lower survival in comparison to cases with lower expression (n = 70), p = 0.0002 (SF 50). However, the setting of the *TP63* gene expression cut-off value at median expression (2.36) resulted in loss of its prognostic significance (p = 0.205). *TP63* expression was significantly but weakly positively correlated with *TP73* expression (R = 0.28; p = 0.005) (SF 51), but not with *TP53* expression (R = –0.18, p = 0.08) (SF 52).

Two cases of UC with gene expression data were included in the APGI-ICGC cohort [64]: a single UCOGC (ICGC_0009) and a single UC "with some multinucleated cells but not osteoclast-like cells" (ICGC_0061). ICGC_0009 showed high *TP63* expression (6.2), similar to values seen in cases of adenosquamous carcinoma. IGCC_0061 had low *TP63* expression (0.02). Importantly, *TP73* gene expression in ICGC_0009 was low.

Discussion

There are several findings of the present study: (1) evident pan-p63 expression is a prevalent finding among anaplastic UC, sarcomatoid UC, and UCOGC, but not in cases of monomorphic UC; (2) UC and UCOGC do not express ΔNp63 (as detected using p40 antibody), so it may be presumed that pan-p63 expression in UC/UCOGC is related to the presence of TAp63 isoforms; (3) some cases of conventional (ductal) adenocarcinomas without visible squamous differentiation may show focal and weak pan-p63 expression but no p40 expression; (4) some rare cases of conventional (ductal) adenocarcinomas without visible squamous differentiation may show more diffuse, evident pan-p63 expression but no p40 expression; (5) serous cystic neoplasms and solid pseudopapillary neoplasms do not express pan-p63; (6) rare cases of biologically aggressive NET may express pan-p63 but not p40; (7) weak pan-p63 (but not p40) expression is a frequent finding in NEC; (8) some MCN may show strong pan-p63 expression in areas of low-to-moderate grade dysplasia (p40 expression in MCN is weak and very focal); and (9) pan-p63 expression in pancreatic neoplasia is not unambiguously diagnostic of squamous/squamoid differentiation. Results of the study and their potential clinicopathological significance are summarised in Table II.

The p63 expression in human tissues

The p63 is expressed in stratified squamous and transitional epithelia (basal cells layers but not differentiated cells layers), basal cells of bronchial epithelium, basal cells of the prostate, myoepithelial cells of the breast and salivary glands, cytotrophoblast, subset of cells in Bowman's capsules of renal glomeruli, epithelial cells of the thymus, germinal centres of lymph nodes, seminiferous tubules, and oocytes [18, 45, 47, 48, 49, 57, 76]. The p40 expression was observed in stratified squamous and transitional epithelia, basal cells of the prostate, myoepithelial cells of the breast, and epithelial cells of the thymus [45, 46, 47, 48]. TAp63 isoforms were detected not only in stratified epithelia, but also in most epithelial lining cells in normal breast and in normal prostate, as well as in epithelial cells of the normal colon [36]. As mentioned above, TAp63 isoforms, but not ΔNp63, may be detected in some lymphoid cells of benign germinal centres and mantle zone [36].

Squamous carcinomas (both pulmonary and extrapulmonary), areas of squamous differentiation in other neoplasms (adenocarcinomas, teratomas), transitional cell carcinomas, myoepithelial, trophoblastic, and thymic epithelial neoplasms, and selected B-cell lymphomas are also pan-p63-positive [35, 49, 57, 58, 59, 60, 76, 77]. Pan-p63 may be detected in up to 50% of lymphomas, which are entirely p40-negative [35, 58, 60]. Some "non-squamous" carcinomas may show p63-reactivity in a small proportion of cases [49].

Both pan-p63 and p40 stains seem to have good sensitivity for the detection of squamous differentiation; squamous cell carcinomas are frequently positive with both markers irrespective of tumour grade [35, 49, 78, 79, 80, 81, 82]. Expression of pan-p63 and p40 in squamous cell carcinomas is typically strong and diffuse [35, 46, 49, 51, 77, 79, 80, 81, 83] although the most differentiated, keratinised areas are p63-negative [82]. Importantly, up to 19% and up to 23% of squamous cell carcinomas may be pan-p63-negative and p40-negative, respectively [46, 49, 83]. The pan-p63 reactivity in pulmonary adenocarcinomas may be present in a wide range of tumoural cells (1-90%) [35, 77] in up to 65% of cases [35, 49, 51, 77, 78, 79, 80, 82, 83]. In contrast, adenocarcinomas may show p40 expression only in rare cases (up to 7%) [35, 46, 78, 80, 83] and in a small proportion of tumour cells (range 1-5%) [35, 83]. p40/p63 reactivity in adenocarcinomas may concern scattered cells or peripheral layer of cellular nests [35, 82].

p63 expression in normal pancreas and in pancreatic DA

Normal pancreatic ducts, pancreatic islets, pancreatic intraepithelial neoplasia, and conventional DA are usually p63-negative/ΔNp63(p40)-negative [71, 84, 85]. In the pancreas, p63 (clone not given) [84] and ΔNp63 (clone not given) [71] may be seen in squamous cell metaplasia, centroacinar/squamoid microcysts, adenosquamous carcinomas, and regions of squamous differentiation of DA. Rare reserve cells in pancreatic ducts may be p63 positive [49].

Identification of molecular subtypes of DA [64, 74, 75] may contribute to better treatment outcome in the future. As shown above, *TP63* gene expression was higher in squamous/quasi-mesenchymal/basal-like DA [64, 74, 75], which all are associated with unfavourable prognosis. In their transcriptome analysis, Khatri *et al.* showed that *TP63* was upregulated at the stage of metastatic DA [86]. Interestingly, squamous molecular type of DA defined by Bailey *et al*. [64] was enriched in adenosquamous carcinomas (as expected), but the majority of cases in this molecular subtype were conventional DA. The frequency of this subtype in the study cohort (26%) exceeded the prevalence of adenosquamous carcinomas in large series (less than 5% of DA) [87]. This indicates that histopathological examination may be not sensitive enough to detect DA with squamous molecular features. Pan-p63 (but not p40) was found in some conventional DA examined in the present study.

Table II. The pan-p63/p40 expression patterns in examined samples of pancreatic neoplasia

Histopathological type of tumor	pan-p63	p40	Potential clinicopathological significance
UC (anaplastic/ pleomorphic giant cell type) and UCOGC	Positive in up to 50% of cells	Negative	pan-p63(+)/p40(–) expression pattern may be useful for UC diagnosis in the appropriate clinical and histopathological context
UC (sarcomatoid type)	Positive in approximately 50% of cells	Negative	Sarcomatoid carcinoma may develop from adenosquamous carcinoma [7], and this progression may be associated with p40 loss (limited data in this study)
UC (monomorphic type)	Usually focal and weak expression	Negative	Lack of pan-p63 expression does not exclude diagnosis of UC
Conventional DA without overt squamous differentiation	Rare, usually focal and weak expression	Negative	Squamous molecular type of DA may be associated with worse prognosis [64], but it is not known if weak and focal pan-p63 IHC expression is a surrogate of squamous molecular type
Adenosquamous carcinoma	Positive	Positive	p40 expression is diagnostic of squamous differentiation in DA [71]
IPMN	Focal and weak	Negative	Limited data in this study, unknown significance
MCN	Strong in some areas with low-to-moderate grade dysplasia, weak to moderate and focal in areas of moderate-to-high-grade dysplasia	Weak and very focal in some areas with low-to-moderate grade dysplasia, negative in areas of moderate-to-high-grade dysplasia	Limited data in this study, unknown significance
Serous cystic neoplasm	Negative	Not examined in this study	p63 is not probably related to the pathogenesis of serous cystic neoplasms
Solid pseudopapillary neoplasm	Negative	Not examined in this study	p63 is not probably related to the pathogenesis of solid pseudopapillary neoplasm
Neuroendocrine tumor	Rare	Negative	Biologically aggressive neuroendocrine tumors may show p63 expression (limited data in this study)
Neuroendocrine carcinoma	Frequent, but the expression is usually focal and weak	Negative	pan-p63 expression does not exclude diagnosis of neuroendocrine neoplasm; pan-p63 expression does not distinguish neuroendocrine tumors and neuroendocrine carcinomas

UC – undifferentiated carcinoma; UCOGC – undifferentiated carcinoma with osteoclast-like giant cells; DA –ductal adenocarcinoma; IHC – immunohistochemical; IPMN – intraductal papillary mucinous neoplasm; MCN – mucinous cystic neoplasm

However, the stain was usually weak and seen only in a minor cell population (up to 10%). Wartenberg *et al*. detected focal p63 (clone 7JUL) expression in 20/110 (18%) cases of pancreatic DA (adenosquamous carcinomas were not included), usually in the tumour buds [88]. A subgroup of patients with p63 expression in tumour cells ("immune-escape" subtype) were characterised by the unfavourable outcome in that study [88]. It is possible that DA with focal pan-p63 expression represent samples of squamous molecular subtype.

ΔNp63-epsilon was the most prevalent isoform in the curated cohort of DA and in a single UC case in TCGA resources. ΔNp63-epsilon serves as an opera-

tive transcriptor factor, which is able to transactivate p63 response elements, to induce *TP63* target genes, and to increase the rate of proliferation [89].

TP63/p63 expression in UC

Data on p63/p40 expression in UC of the pancreas is limited. Kane *et al*. described pan-p63-negative sarcomatoid UC associated with DA [90]. Sekulic *et al*. reported p40 (Biocare) expression in cell-block preparation of one out five UCOGC; expression was found in rare mononuclear cells with squamous morphology [91]. Maksymov *et al*. described a case of UCOGC with pan-p63 (4A4) expression in epithelial and "non-epithelial" component of tumour [92]. This finding suggested squamous, transitional, or myoepithelial tumour differentiation [92]. It was also postulated that tumour could have arisen from reserve cells of the pancreatic ducts [92]. p40 expression was not examined in that case.

In the present study p63 immunopositivity was found in all UC/UCOGC cases, but it was found more in anaplastic, sarcomatoid, and UCOGC types. All UC/UCOGC cases were p40-negative. The reason for p63 overexpression in UC/UCOGC of the pancreas is unknown. The p63 upregulation may be caused by *TP63* fusions or amplifications [51, 58, 93, 94]. *TP63* amplifications are particularly common in pulmonary squamous cell carcinomas (88%), but the relationship between *TP63* amplification and p63 protein expression is not direct [51]. Two cases of DA with *TP63* amplifications in curated TCGA cohort had *TP63* mRNA expression levels at the 28th and 69th percentile of the entire cohort. No case in that cohort had *TP63* fusions. It is unlikely that *TP63* rearrangements are the main reason for p63 overexpression in UC/UCOGC. It is also unlikely that the presence of pan-p63 expression in UC/UCOGC is caused by its "occult" squamous differentiation. ΔNp63 isoforms are essential for the development and maintenance of squamous differentiation in DA [30]. Transcriptomic expression pattern of UC examined in a small number of cases (n = 4) showed that UC did not have squamous molecular phenotype as defined by ΔNp63 expression, but rather revealed upregulation of MAPK signalling pathway, epithelial-to-mesenchymal transition, and embryonic transcription program, as well as increased tissue infiltration by lymphoid cells [95]. It is possible that the pan-p63(+)/p40(-) IHC profile is caused by upregulation of TAp63 mRNA, as in other tumours [35, 57, 58, 59, 60]. This is supported by the observation of a single UCOGC in the APGI-ICGC cohort, which had high *TP63* mRNA expression (ICGC_0009).

The biological significance of TAp63 upregulation in UC/UCOGC is undetermined. Because TAp63 has features of tumour suppressor [18, 31, 34], its overexpression in highly malignant anaplastic UC is counterintuitive. However, TAp63 may sometimes be upregulated in neoplasms [32, 60]. The mechanisms of TAp63 action are complicated and only partially understood [60].

p63 IHC stain in differential diagnostics of UC/UCOGC

The differential diagnostics of UC/UCOGC is broad. Importantly, pan-p63 expression in high-grade neoplasm is not proof of epithelial differentiation. Some mesenchymal and lymphoid tumours may express pan-p63, including samples of pleomorphic undifferentiated sarcoma (38%) [96], giant cell tumour of soft tissue (30%) [97], epithelioid sarcoma (10%) [97], spindle cell melanoma (5%) [97], and anaplastic large cell lymphoma (35.3%) [58]. Reactive stromal proliferations may also be pan-p63 positive (5-30%) [96, 98]. The pan-p63(+)/p40(–) IHC profile may be supportive for UC/UCOGC diagnosis in the appropriate clinical and histopathological context.

Biological features of UC/UCOGC of the pancreas

The mutational profile of UCOGC resembles conventional DA [10]. *KRAS* mutant allele-specific imbalance and *KRAS* amplification are frequent in pancreatic UC/UCOGC (64% and 38-42%) in comparison to conventional DA (6% and 0%, respectively) [6, 13]. *KRAS* amplification or chromosome 12 hyperploidy may be involved in the progression of DA to UC [13]. Downregulation of epithelial markers (in particular E-cadherin) and upregulation of epithelial-to-mesenchymal transition markers (vimentin, zeb1, slug, twist) are essential in UC pathogenesis [6, 8, 16, 99, 100, 101]. UC with rhabdoid cells [100], in particular monomorphic UC [6], may show loss of SMARCB1/INI1 expression. Overexpression of autophagy-related proteins is evident in UC with rhabdoid features [100]. Prevalence of programmed cell death ligand 1 expression in UC/UCOGC (63%) is greater than in DA (15%) [102]. Based on the present study, it may be presumed that TAp63 upregulation is another event during dedifferentiation of DA to UC.

UCOGC and giant cell tumour of the bone

UCOGC share some morphological features with giant cell tumour of the bone [7, 11, 103]. It is intriguing that giant cell tumours of bone, such as UCOGC in the present study, express p63 [104, 105]. Expression of mRNA TAp63 isoforms and p63 protein, but not mRNA ΔNp63 isoforms, in giant cell tumour of the bone was reported by Dickson *et al*. [104] and Lee *et al.* [105], among others. Moderate-to-strong pan-p63 expression may be found in 15-85% of mononuclear cells in 69-100% samples of giant cell tumour of bone but not in osteoclast-like cells [104, 105].

TP63 regulates cell cycle progression in giant cell tumour of bone [26]. Moreover, p63 expression may have prognostic value in giant cell tumour of bone because recurrent tumours showed a higher extent and intensity of p63 expression in comparison with non-recurrent samples [106]. One may speculate that p63 is involved in the biology of lesions with osteoclast-like giant cells irrespective of their cellular origin.

Extrapancreatic UC and heterogeneity of UC

The p63/p40 status was examined in some extrapancreatic UC. Head and neck sarcomatoid carcinoma associated with squamous cell carcinoma may retain p63 (62-63%) [96, 98] and p40 (54%) expression [96]. Sarcomatoid carcinomas of the lung, urinary bladder, and skin express pan-p63 in 50% [98], 36% [98], and 81% [107] of cases, respectively. p40 may be expressed in 8.1% [108] and in 56% [107] of sarcomatoid carcinomas of the lung and skin, respectively. It is clear that the p63/p40 expression profile in UC of the pancreas is not necessarily reflected in extrapancreatic UC.

It is difficult to develop a clinically useful and biology-driven classification of UC. Weissferdt *et al*. proposed a new classification of sarcomatoid carcinomas of the lung based on (1) the presence of differentiated component (adenocarcinomatous or squamous cell), and (2) expression patterns of pneumocytic (TTF-1) and squamous (p40) markers in sarcomatoid component [108]. Cases with a retained expression of lineage markers in sarcomatoid component were classified as sarcomatoid (adenocarcinomas or squamous cell carcinomas), whereas cases with a lost expression of lineage markers were classified as dedifferentiated (adenocarcinomas or squamous cell carcinomas) [108]. Translation of proposed classification to the pancreatic UC would be difficult for a number of reasons: (1) one third of UC cases developed from DA with partial squamous features; (2) expression of squamous marker p40 in UC cases was nil; and (3) expression of adenocarcinomatous markers (e.g. CEA, Ca19.9, MUC1) was not examined in the present study. However, the lack of p40 expression in pancreatic UC is compatible with "dedifferentiated" expression profile. Although the number of spindle cell carcinomas in the present study was small (n = 2), it was documented that adenosquamous carcinomas of the pancreas may lose their p40 expression during progression to UC, similarly to some cases of sarcomatoid carcinomas of the lung [108].

p63 expression in neuroendocrine neoplasms

Pan-63 expression was found in four (13.8%) NET cases in the present study, but it was diffuse in one case only (NET G3). Simtniece *et al*. detected p63 with DAK-p63 antibody in the nuclei of 4 out of 14 NET of the pancreas (28.6%), but the proportion of immunopositive cells was low (median 2%, range 2-7%) [85]. The presence of p63-positive cells in NET in that report correlated with the presence of vascular invasion and p53 immunoreactivity [85]. Other investigators reported focal pan-p63 expression but no p40 expression in 6% of pulmonary carcinoids [80, 109]. The pancreatic islets examined by Simtniece *et al*. (n = 14 cases) were DAK-p63 negative [85]. In this investigation, pancreatic islets in atrophic peri/intratumoural parenchyma showed evident cytoplasmic pan-p63 expression. Pathogenesis of cytoplasmic pan-p63 stain in human disease is not clear; it may be related to increased proliferation and apoptosis, the presence of altered protein isoforms or protein aggregates, protein interactions, or inadequate cellular protein transport [110, 111].

The p63/p40 expression can be found also in some neuroendocrine carcinomas. Pan-p63 expression in pulmonary large cell neuroendocrine carcinoma seems to be more frequent (18%) than in small cell carcinoma (4%) [112]. In another report focal pan-p63 expression was found in 13.5% and 22.1% of pulmonary large cell and small cell neuroendocrine carcinomas, respectively [109]. Pan-p63 expression was also detected in 43% of neuroendocrine carcinomas of the cervix [113]. Diffuse but weak pan-p63 expression was reported in 89% of neuroendocrine carcinomas of the head and neck [114]. Additionally, pan-p63 expression (found in 53% of cases) was an independent unfavourable prognostic factor in Merkel cell carcinoma [115]. In contrast, the p40 expression is not typical for pulmonary large cell and small cell neuroendocrine carcinomas [46]. For example, p40 expression was reported in 7% and 3% of oesophageal and pulmonary small cell carcinomas, respectively [81]. However, Nakajima *et al*. described p40 expression in rare cells (usually up to 5%) in as many as 34.1% of small cell lung cancers [116]. In the present study pan-p63 but not p40 expression was documented in the majority of pancreatic NEC cases. The reason for this finding is unknown. It must be emphasised that pan-p63 expression in a limited sample of malignant neoplasm of the pancreas is not diagnostic of ductal-(adeno) squamous differentiation and it does not exclude the diagnosis of NEC. The role of p63 cytoplasmic and nuclear expression in neuroendocrine cells and lesions of the pancreas requires further study.

The role of TP63/p63 in other pancreatic diseases

Based on the results of this study, one may suspect that *TP63*/p63 probably does not fulfil a critical function in IPMN, serous cystic neoplasms, and solid pseudopapillary neoplasms. Observation of pan-p63 expression in some areas of MCN requires further study.

Limitations of the study

There were several limitations of the present study: (1) the number of study cases was not large. However, UC of the pancreas is a very rare disease; (2) a single tissue block from each UC/UCOGC case was submitted to IHC procedure, and other tumour types were examined in TMA format – for this reason, some heterogeneity p63/p40 expression might stay undetected; (3) DAK-p63 rather than another pan-p63 antibody (clone 4A4) was utilised in the present study – as mentioned, DAK-p63 antibody was shown to react with both ΔNp63 and TAp63 isoforms [50]; (4) scoring of IHC assays was performed by a single observer – this precluded assessment of interobserver variability; (5) scoring of IHC was performed manually – heterogeneous cellular composition of tumoural tissues (presence of different tumour components and non-neoplastic stromal cells) made digital image analysis difficult to apply; (6) follow-up data were not available; therefore, the potential prognostic significance of the protein expression patterns could not be analysed; (7) cross-reactivity of DAK-p63 antibody with p73 protein in UC could not be reliably excluded; however, lack of association of expression levels of *TP63* and *TP73* genes in TCGA DA cohort has made this phenomenon unlikely; (8) TAp63 expression levels were not examined directly. Observation of pan-p63-positivity in p40-negative tissues is suggestive of the presence of TAp63 isoform [35, 57-60]. Gene fusions involving *TP63* and *TP63* amplifications may influence p63 expression patterns [39, 58], but *TP63* fusions were not reported in DA – in high-grade follicular lymphoma the prevalence of pan-p63-positive cells correlated with TAp63 mRNA expression [59] – TAp63 is an unstable isoform [20], so its mRNA and protein expression levels may not be fully concordant [70]; (9) other markers of squamous differentiation (e.g. CK5/6, CK34βE12, desmocollin-3, desmoglein-3, S100A2, S100A7, SOX2, and glypican-3 [35, 77, 78, 80]) were not examined; however, their diagnostic performance seems to be worse than p40 immunostain, due to compromised specificity or sensitivity [35, 77, 78, 80]. – the p40-negativity argues strongly against squamous differentiation in UC/UCOGC.

Conclusions

The pan-p63 expression was a constant feature of anaplastic UC, sarcomatoid UC, and UCOGC. It was not paralleled with p40 expression; therefore, it suggested that TAp63 isoforms rather than ΔNp63 isoforms are present in UC/UCOGC. Pan-p63(+)/p40(–) IHC status may be supportive of UC diagnosis in the appropriate clinical and histopathological context. In DA without overt (adeno)squamous differentiation, pan-p63 expression was usually, but not always, weak and limited to a small fraction of tumour cells. The pan-p63 expression was rare in NET, MCN, and IPMN. Weak pan-p63 expression but not p40 expression was frequent in pancreatic NEC. Serous cystic neoplasms and solid pseudopapillary neoplasms were pan-p63-negative. Ductal carcinomas with partial squamous differentiation were the only examined pancreatic tumours with evident p40 expression. The pan-p63 expression pattern was not equivalent to squamous differentiation in pancreatic neoplasia.

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