

Frequent overexpression of cyclin D1 in sporadic pancreatic endocrine tumours

S S Guo, X Wu, A T Shimoide, J Wong, F Moatamed¹ and M P Sawicki

Department of Surgery, West Los Angeles VA Medical Center and the UCLA School of Medicine, Los Angeles, California 90095, USA

¹Department of Pathology, West Los Angeles VA Medical Center and the UCLA School of Medicine, Los Angeles, California 90095, USA

(Requests for offprints should be addressed to M P Sawicki, Division of General Surgery, UCLA School of Medicine, Room 72-215, CHS10833 Le Conte Avenue, Los Angeles, CA 90095-6904, USA; Email: msawicki@ucla.edu)

Abstract

Pancreatic endocrine tumours (PETs) occur sporadically or are inherited as part of the multiple endocrine neoplasia type-1 syndrome. Little is known about the molecular events leading to these tumours. Cyclin D1, a key regulator of the G1/S transition of the cell cycle, is overexpressed in a variety of human cancers as well as certain endocrine tumours. We hypothesized that similar to other endocrine tumours, cyclin D1 is overexpressed in human sporadic PETs. Cyclin D1 protein overexpression was found in 20 of 31 PETs (65%) when compared with normal pancreatic tissue. Furthermore, Northern blot analysis suggests that cyclin D1 up-regulation occurs at the post-transcriptional level in some PETs. Because the key cell growth signalling pathways p42/p44/ERK (extracellular signal-regulated kinase), p38/MAPK (mitogen-activated protein kinase), and Akt/PKB (protein kinase B)

can regulate cyclin D1 protein expression in other cell types, pancreatic endocrine tumours were analysed with phospho-specific antibodies against the active forms of these proteins to elucidate a tissue-specific regulatory mechanism of cyclin D1 in PETs. We found frequent activation of the p38/MAPK and Akt pathways, but down-regulation of the ERK pathway, in cyclin D1 overexpressing PETs. This study demonstrates that cyclin D1 overexpression is associated with human sporadic PET tumorigenesis, and suggests that this up-regulation may occur at the post-transcriptional level. These findings will direct future studies of PETs towards cell cycle dysregulation and the identification of key growth factor pathways involved in the formation of these tumours.

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Introduction

Pancreatic endocrine tumours (PETs) develop from neuroendocrine cells residing in and around the pancreas. These rare tumours occur either sporadically or are inherited as part of the multiple endocrine neoplasia type-1 syndrome (MEN-1). While PETs are well known for characteristic hormonal production related syndromes, such as Zollinger Ellison syndrome, they may also cause symptoms from local invasion as well as metastatic spread. There are few effective therapies for patients with PETs besides surgical management, and prognosis varies between excellent for those without metastasis to poor for those with metastasis. Knowledge of the molecular mechanisms leading to PET tumorigenesis would greatly facilitate the development of rational anti-tumour therapy, as well as provide diagnostic and prognostic markers.

Little is known regarding the molecular pathogenesis of PETs. The gene responsible for the MEN-1 syndrome, *MEN1*, was cloned in 1997 (Chandrasekharappa *et al.*

1997), and is mutated in 23% of PETs (Guo & Sawicki 2001). The tumour suppressor *Smad4/DPC4* (Bartsch *et al.* 1999) is mutated in 50% of nonfunctional tumours. Other tumour suppressors and oncogenes implicated in PET tumorigenesis include *HER-2/neu* (Evers *et al.* 1994, Terris *et al.* 1998), *p16^{INK4}* (Muscarella *et al.* 1998), *p27* (Guo *et al.* 2001), and *p53* (Evers *et al.* 1994, Lin *et al.* 1997). Loss-of-heterozygosity studies have also suggested the involvement of tumour suppressor genes from chromosomes 1 (Ebrahimi *et al.* 1999, Guo *et al.* 2002b), 3p (Chung *et al.* 1997), 3q (Guo *et al.* 2002a), 11p, 16p (Chung *et al.* 1998), 17p (Beghelli *et al.* 1998) and 22q (Chung *et al.* 1998).

Cyclins are the regulatory subunits of the cyclin/Cdk (cyclin-dependent kinase) complexes. Cyclin D1 binds to *Cdk4/6*, and the resultant complex phosphorylates the retinoblastoma susceptibility gene product, pRb. Sequential phosphorylation of pRb by cyclin D/Cdk4/6 and cyclin E/Cdk2 inactivates pRb and allows cell-cycle progression through G1 (Fagan *et al.* 1994). Once this

restriction-point is traversed, cells lose their mitogen-dependence and progress through the cell cycle until the restriction point of the next cell cycle is reached. Although there is variability amongst different cell systems, the synthesis and accumulation of the regulatory cyclins, in particular cyclin D1, is an important control of pRb phosphorylation (Grana & Reddy 1995) (reviewed in Hatakeyama & Weinberg 1995). Not surprisingly, many human cancers (e.g. breast, parathyroid, head and neck, gastric, oesophageal) have increased cyclin D1 expression (reviewed in Hunter & Pines 1994), and cyclin D1 is a prognostic marker for many cancers (Bova *et al.* 1999, Keum *et al.* 1999, Samejima *et al.* 1999, Cuny *et al.* 2000, Itoi *et al.* 2000). Moreover, forced overexpression of cyclin D1 results in a shortened G1 phase, reduced cellular dependence on exogenous mitogens, and cell transformation (Bodrug *et al.* 1994, Wang *et al.* 1994). Finally, activation of Cdk4 causes pancreatic β -islet proliferation while inactivation of Cdk4 results in β -islet hypoplasia and insulin-dependent diabetes (Rane *et al.* 1999).

Cyclin D1 expression is regulated by transcriptional and post-transcriptional mechanisms. At the transcriptional level, kinase pathways such as p38/MAPK (mitogen-activated protein kinase), c-Jun N-terminal kinase/SAPK (stress-activated protein kinase), and p42/p44/ERK (extracellular signal-regulated kinase) transmit exogenous growth signals to up-regulate cyclin D1 (Lavoie *et al.* 1996, Lee *et al.* 1999). Similarly, activation of the Akt/PKB (protein kinase B) pathway can increase cyclin D1 expression through either transcriptional (Gille & Downward 1999) or post-transcriptional mechanisms (Diehl *et al.* 1998, Muisé-Helmericks *et al.* 1998). There is, however, tremendous tissue specificity in cyclin D1 regulation and conflicting data regarding the effects of these pathways on cyclin D1 expression between cell types. Such studies have not been performed on human pancreatic endocrine tissues.

We hypothesized that sporadic PETs have dysregulation of cyclin D1 similar to parathyroid adenomas and other endocrine tumours. Western blot analysis demonstrated frequent overexpression of cyclin D1 in sporadic PETs compared with normal pancreas tissues. Cyclin D1 overexpression was confirmed by immunohistochemistry (IHC) analysis on a subset of 5 sporadic PETs. Northern analysis suggests that cyclin D1 up-regulation in these tumours occurs at the post-transcriptional level. Using phospho-specific antibodies, we also show frequent activation of the p38/MAPK and Akt pathways, but down-regulation of the ERK pathway, in cyclin D1 overexpressing sporadic PETs. Our findings are strengthened by a recent report of frequent cyclin D1 overexpression in human sporadic PETs (Chung *et al.* 2000). Together, these two studies strongly suggest the importance of cyclin D1 up-regulation in the tumourigenesis of sporadic PETs.

Materials and Methods

Tissue isolation

Tumour samples were obtained from patients who have received extensive pre-operative evaluations at exploratory laparotomy. Tissues were snap-frozen in liquid nitrogen and stored at -80°C .

Institutional review and informed consent

This study was performed under the auspices of the West LA, VA, Institutional review board, with appropriate institutional oversight. Informed consent was obtained from all patients from whom tissue samples were harvested.

Protein isolation

Tissues were homogenized using a Kontes pellet pestle (Fisher Scientific, Pittsburg, PA, USA) in CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl_2 , 1 mM EGTA, 0.1 mM benzamidine, 5 mM β -mercaptoethanol, 0.5% CHAPS, 10% glycerol). After 30 min incubation, centrifugation was performed and cellular debris discarded. The supernatant was stored at -80°C until use. Protein concentration was determined using the Bichinchonic method (Pierce Biotechnology, Rockford, IL, USA).

Western blot analysis

Protein samples were denatured in loading dye (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% Bromophenol Blue, 50 mM dithiothreitol) and boiled at 100°C . Equal amounts (25 μg) of protein were loaded into each well and resolved in 10% SDS-PAGE gel. The protein samples were transferred to Hybond-C Extra protein membrane (Amersham, Piscataway, NJ, USA), agitated in blocking solution (20 mM Tris, 140 mM NaCl, 5% dry milk, 0.1% Tween 20), and probed with primary antibodies in primary antibody dilution buffer (20 mM Tris, 140 mM NaCl, 5% bovine serum albumin, 0.1% Tween 20). After hybridization with the appropriate secondary antibody (1:2000 dilution), blots were developed with ECL reagents according to the manufacturer's instructions (Amersham). Equal protein loading and transfer were reconfirmed with Ponceau S staining (Sigma, St Louis, MO, USA) of the blots. The expression level of cyclin D1 was normalized to that of β -tubulin, and the expression levels of phospho-Akt, phospho-p38, and phospho-ERK were normalized to those of total-Akt, total-p38, and total-ERK respectively.

Antibodies

Monoclonal mouse anti-cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at 1:500 dilutions. Monoclonal mouse anti- β -tubulin (Sigma Scientific) was used at 1:2000 dilutions. Rabbit polyclonal anti-total p38, anti-phospho-specific p38, anti-total Akt,

anti-phospho-specific Akt, and anti-total ERK (New England Biolabs, Beverly, MA, USA) were used at 1:1000 dilutions. Mouse monoclonal anti-phospho-specific ERK (New England Biolabs) was used at 1:1000 dilutions. For IHC, monoclonal cyclin D1 (NeoMarkers, Fremont, CA, USA) was used at 1:50 dilutions.

Immunohistochemistry

Tissues (3 PETs with cyclin D1 overexpression – 14T, 78T, 111T; 2 PETs without cyclin D1 overexpression – 226T, 250T) were taken from the frozen tissue bank, and fixed in formalin, ethanol, and xylene in an automated tissue processor (LX300 Tissue Processor, Fisher Scientific, Tustin, CA, USA). After tissues were embedded in paraffin, they were cut into 4 µm thick slices and mounted onto slides. Target retrieval was performed with DAKO Target Retrieval Solution (DAKO Corporation, Carpinteria, CA, USA) at 95 °C for 20 min in a steamer, then cooled to room temperature. Slides were then placed into the DAKO Autostainer and stained using the DAKO LSAB2 System, Peroxidase (DAKO Corporation). Primary antibody was diluted in DAKO Antibody Diluent, and blocking was performed in DAKO Protein Block Serum-Free (DAKO Corporation). Signals were obtained using DAKO Liquid DAB Large Volume Substrate-Chromogen System (DAKO Corporation). Specificity of the antibody was confirmed by peptide blocking using the cyclin D1 peptide (NeoMarkers). Appropriate positive and negative controls were used.

Northern blot analysis

Tissues (2 PETs with cyclin D1 overexpression – 67T, 113T; 2 PETs without cyclin D1 overexpression – 7T, 43T) were homogenized (see above) and RNA was isolated according to the RNeasy Mini protocol (Qiagen, Santa Clarita, CA, USA). RNA was resolved in 1.2% FA Gel (1.2% agarose, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, adjusted to pH 7.0), and transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) by capillary transfer (Sambrook *et al.* 1989). Cyclin D1 cDNA was labelled by a random primed DNA labelling kit (Boehringer Mannheim, Mannheim, Germany) using [α -³²P]dCTP (ICN Biomedical, Costa Mesa, CA, USA). Hybridization was performed with ExpressHyb Solution (Clontech, Palo Alto, CA, USA) according to the manufacturer's protocol.

Results

Overexpression of cyclin D1 in sporadic PETs

Cyclin D1 is a key regulatory protein for the G1-S transition of the cell cycle, and was first shown to be up-regulated in human endocrine tumours (Motokura *et al.* 1991). We hypothesized that similar to other

Table 1 Activation of growth signalling pathways in sporadic PETs with increased cyclin D1 expression. Western blots with tumour protein were probed with antibodies to cyclin D1 and phospho-specific as well as total forms of Akt/PKB, p38/MAPK, and p42/p44/ERK. The Western data were normalized to the pancreas control band to eliminate the effect of different exposure times between the different Western blots, and quantified to a scale between 3– to 3+ by two independent reviewers. 3+ represents the strongest signal while 0 denotes a signal comparable to that of the control. Clinical phenotype and the presence of hepatic involvement in each tumour are also shown. There is up-regulation of the Akt and p38 pathways, but down-regulation of the ERK pathway in sporadic PETs. There is no particular correlation between growth pathway activation and clinical behaviour or tumour type

	Phenotype	Hepatic involvement	Akt	p38	ERK
Case					
1T	Gastrinoma	No	1+	3+	2–
4T	Gastrinoma	Yes	0	0	3–
8T	Gastrinoma	No	1+	0	3–
14T	Gastrinoma	Yes	1+	2+	0
39T	VIPoma	No	0	0	3–
67T	Gastrinoma	No	3+	0	3–
78T	Gastrinoma	No	1+	0	2–
83T	Non-functional	Yes	0	2+	3–
111T	Non-functional	No	0	0	3–
113T	Gastrinoma	No	0	0	3–
119T2	Gastrinoma	No	2+	1+	1+
124T	Insulinoma	No	1+	0	0
200TL3	Gastrinoma	Yes	0	1+	3–
201T2C	Gastrinoma	No	2+	1+	1–
202T	Gastrinoma	No	1+	2+	2–
206T1	Gastrinoma	No	2+	2+	1–
208T	Gastrinoma	No	1+	1+	3–
213T	Gastrinoma	No	1+	0	0
214T2	Gastrinoma	No	2+	1+	0
225T	Gastrinoma	No	1+	0	2–

VIPoma, vasoactive intestinal peptide tumour.

endocrine tumours there is dysregulation of cyclin D1 in sporadic PETs. To assess the expression of cyclin D1 at the protein level, Western blot analysis was performed on 31 sporadic PETs. As shown in Table 1, 20 of 31 sporadic PETs (65%) demonstrated overexpression of cyclin D1 by Western blot analysis compared with two normal pancreas controls. A representative Western blot is shown in Fig. 1. Protein concentration of tumour samples was performed by the Bichinchonic assay, and equal loading was demonstrated with Ponceau S staining (data not shown) as well as by stripping the blots and re-probing with β -tubulin antibody (Fig. 1).

To validate the Western blot data, IHC was performed in 5 sporadic PETs for which we had sufficient tumour material for analysis (Fig. 2 and data not shown). Of these 5 PETs analyzed by IHC, all three PETs found to have cyclin D1 overexpression by Western blot analysis (tumours 14T, 78T, and 111T) also demonstrated both nuclear and cytoplasmic staining of cyclin D1 by IHC.

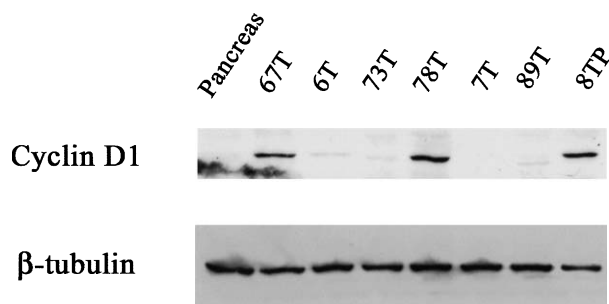


Figure 1 Cyclin D1 expression by Western blot in sporadic PETs. Tumours were snap-frozen at exploratory laparotomy, and stored at -80°C . After protein isolation (see Materials and Methods), PETs were probed with cyclin D1 and representative Western blots are shown. Protein from two normal pancreases was used for control. Results from both pancreases were similar, and data from one is shown. The same blot was stripped and re-probed with β -tubulin to demonstrate similar protein loading among samples.

In contrast, two PETs without cyclin D1 overexpression by Western blot analysis (226T and 250T) showed undetectable cyclin D1 staining by IHC. Peptide blocking confirmed the specificity of our cyclin D1 antibody (Fig. 2a and b). IHC demonstrated minimal contamination in the surrounding normal tissue in our samples.

Cyclin D1 overexpression occurs at the post-transcriptional level in sporadic PETs

One mechanism for cyclin D1 overexpression in many human tumours is through DNA amplification or rearrangement (e.g. breast cancer and parathyroid tumours), resulting in transcriptional up-regulation. However, previous studies did not reveal gross cytogenetic rearrangement or DNA amplification in the region of cyclin D1 in sporadic PETs (Sawicki *et al.* 1992, Terris *et al.* 1998, Speel *et al.* 1999). Cyclin D1 expression in sporadic PETs is therefore likely to occur through a transcriptional or a post-transcriptional mechanism. We investigated these possibilities by Northern blot analysis of 4 sporadic PETs (2 PETs with cyclin D1 overexpression - 67T, 214T2; 2 PETs without cyclin D1 overexpression - 7T, 43T). Figure 3 shows that although the level of cyclin D1 is dramatically different between these 4 sporadic PETs, their cyclin D1 mRNA expression is not significantly altered. This suggests that cyclin D1 overexpression may occur at the post-transcriptional level in sporadic PETs.

Activation of MAP kinase pathways in pancreatic endocrine tumours

Ras and its multiple effector pathways such as Akt, p38/MAPK, and p42/p44/ERK are known to have regulatory roles, through either transcriptional or post-transcriptional mechanisms, in the expression of cyclin D1

(Diehl *et al.* 1998, Muise-Helmericks *et al.* 1998, Lee *et al.* 1999). The active regulatory pathway is cell-type specific and context sensitive. No studies have been performed on human pancreatic endocrine tumour tissues.

We therefore investigated which Ras effector pathways might play an important role in cyclin D1 overexpression in sporadic PETs. Using phospho-specific antibodies and total-form antibodies against activated- and total-Akt, -p38/MAPK, and -p42/p44/ERK respectively, the activation status of these growth pathways in sporadic PETs was examined. Protein gel loading was equalized according to the total forms of these antibodies, and Western bands were quantified on a scale between 3- and 3+ by two independent reviewers. As shown in Table 1, within the group of sporadic PETs that show cyclin D1 overexpression, there is activation of the Akt and/or the p38/MAPK growth pathways in 80% (16 of 20). In contrast, there is down-regulation of the ERK pathway in 75% (15 of 20) of the same group of samples. A representative Western blot is shown in Fig. 4.

Cyclin D1 expression does not correlate with tumour aggressiveness or differentiation in sporadic PETs

Because other studies have shown cyclin D1 to be a prognostic factor in human cancers (Bova *et al.* 1999, Keum *et al.* 1999, Samejima *et al.* 1999, Cuny *et al.* 2000, Itoi *et al.* 2000), cyclin D1 expression levels were analyzed for correlation with clinical tumour behaviour of these sporadic PETs. Tumours were categorized as benign or malignant based on the presence or absence of hepatic metastases. Of the six sporadic PETs that metastasized to the liver, four expressed increased levels of cyclin D1 (67%) versus two that did not (Table 1 and data not shown). Conversely, of the 25 sporadic PETs without hepatic involvement, 16 expressed increased levels of cyclin D1 (64%). Analysis according to tumour hormonal status (gastrinoma vs insulinoma vs nonfunctional tumours, etc.) also did not reveal any association with cyclin D1 overexpression. Thus there is no correlation between cyclin D1 overexpression and sporadic PET clinical aggressiveness or differentiation.

Discussion

In this study, 31 sporadic pancreatic endocrine tumours were analysed for the expression of cyclin D1 and 20 PETs (65%) showed overexpression by Western blot analysis. These findings were validated by IHC in a subset of tumours. Although our sample size is limited, this is one of only two such studies in these rare and generally small tumours.

Previous studies showed no gross chromosomal rearrangement or DNA amplification in the region of cyclin D1 in sporadic PETs. This was confirmed by our

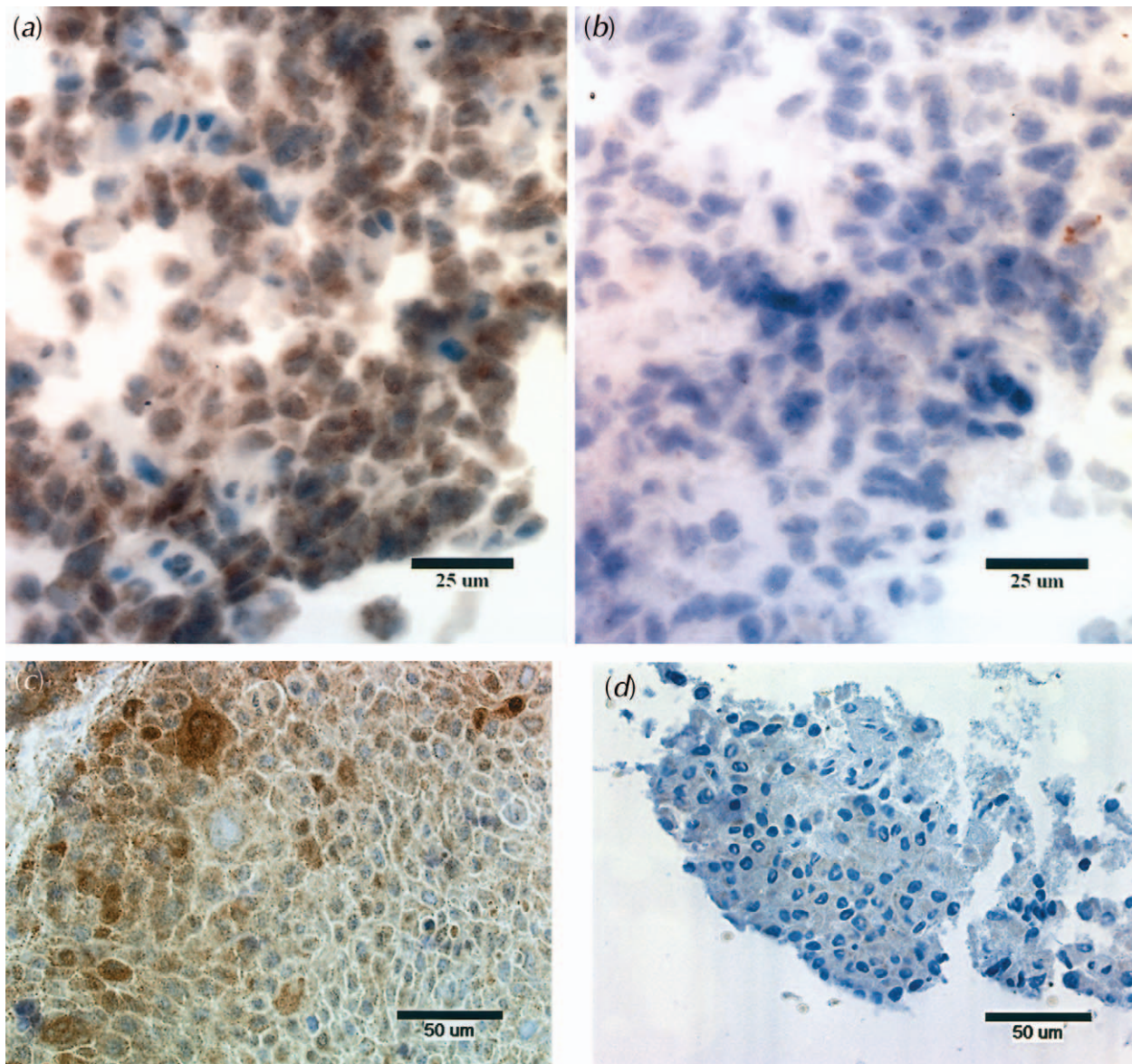


Figure 2 Cyclin D1 expression by IHC in sporadic PETs. Cyclin D1 overexpression was confirmed by IHC in 5 tumours (3 tumours with cyclin D1 overexpression – 14T, 78T, 111T; 2 PETs without cyclin D1 overexpression – 226T, 250T). Representative photomicrographs are shown. (a) Tumour (14T) found to have cyclin D1 overexpression by Western analysis has nuclear as well as cytoplasmic staining of cyclin D1 by IHC. (b) Specificity of the antibody used in immunohistochemistry is demonstrated with peptide blocking. Cyclin D1 staining in the same tumour is completely abolished with peptide blocking. (c) Large dysmorphic tumour cells can also be seen in another tumour with cyclin D1 overexpression. (d) A PET found to have no cyclin D1 overexpression (226T) by Western analysis also has no significant staining of cyclin D1.

Northern blot analysis that suggests in some sporadic PETs, post-transcriptional mechanisms are responsible for the up-regulation of cyclin D1 expression. Current studies in other cellular contexts demonstrated that the control of cyclin D1 expression by key growth regulatory pathways may be cell-type specific. We found activation of either the p38/MAPK or the Akt pathway in 80% (16 of 20) of sporadic PETs with cyclin D1

overexpression. In contrast, the ERK pathway is down-regulated in 75% of these tumours (15 of 20). These observational studies on these rare human tumours may provide important clues for growth dysregulation in sporadic PETs.

In addition, no correlation between cyclin D1 overexpression and clinical behaviour or tumour phenotype was found. Studies of various human cancers suggest that

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