Overexpression of Oncogenic STK15/BTAK/Aurora A Kinase in Human Pancreatic Cancer[1]

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ABSTRACT

Purpose: **Multiple chromosome abnormalities, including gain of chromosome 20q, have been detected frequently in human pancreatic cancers. Overexpression of the *STK15/BTAK/Aurora A* gene located on chromosome 20q13, which encodes a centrosome-associated serine/threonine kinase, has been shown to induce chromosomal instability, leading to aneuploidy and cell transformation in multiple *in vitro* experimental systems. The purpose of this study was to investigate the expression and copy number alteration of STK15 in pancreatic cancer.**

Experimental Design: **STK15 expression at both the mRNA and protein levels together with the copy number of *STK15* gene was measured in nine pancreatic carcinoma cell lines: (*a*) HPAF-II; (*b*) Aspc-1; (*c*) Panc-1; (*d*) Panc-3; (*e*) Panc-28; (*f*) Panc-48; (*g*) HS766T; (*h*) MIAPaCa-2; and (*i*) BxPc3. STK15 protein expression was also examined in normal pancreatic tissues and tumors by Western blotting and immunohistochemistry.**

Results: **STK15 was overexpressed in all of the nine cell lines examined, but gene amplification was infrequent. Western Blot analysis of primary tumor tissues revealed 2–10 times overexpression of STK15 protein compared with normal adjacent tissues from pancreatic cancer patients. Concurrent overexpression of cdc20, an STK15-associated protein, and reduced expression of cdc25, a mitosis-activating protein phosphatase, were detected in the same tumor samples. Elevated STK15 protein expression was detected in 22 of 38 tumor sections (58%) from pancreatic cancer patients. The extent of STK15 expression was not significantly correlated with the size, degree of differentiation, and metastasis status of the tumors.**

Conclusions: **These results show that STK15 is overexpressed in pancreatic tumors and carcinoma cell lines and suggest that overexpression of STK15 may play a role in pancreatic carcinogenesis.**

INTRODUCTION

Chromosome copy number aberrations or aneuploidy is the most prevalent somatic cell genomic alteration identified in human solid tumors (1, 2). It has been proposed that aneuploidy drives tumor progression by enhancing genomic instability, resulting in massive alterations of the cellular phenotypes (3). This hypothesis appears compelling in view of recent reports that human and rodent cell lines undergoing transformation display elevated rates of chromosome instability and that aneuploidy precedes immortalization (4–6). A strong correlation between the degree of CIN[3] and tumor behavior has also been reported. Tumors showing minimal deviation of their chromosome copy number, *i.e.*, near diploid, are clinically less aggressive than those that have major increases in their total nuclear DNA content often manifested with extra copies of multiple chromosomes (7–12).

During normal cell proliferation, centrosomes ensure equal segregation of chromosomes by organizing the bipolar mitotic spindle. In cancer cells, on the other hand, multipolar mitotic spindles are commonly seen, and centrosomal anomalies, such as supernumerary centrosomes, centrosomes of abnormal size and shape, and aberrantly phosphorylated centrosomal proteins, as well as prematurely split centrosomes, have been reported (13–19). It is possible that such abnormalities could disrupt normal chromosomal segregation, producing aneuploid cells.

The molecular pathways through which centrosomes regulate segregation of chromosomes remain to be elucidated. Recent cloning of *STK15/BTAK/Aurora A* (20) kinase encoding gene, implicated in the regulation of centrosome function and reported to be amplified/overexpressed frequently in human tumors, raises the possibility that abnormal elevated expression of this regulatory component of chromosomal segregation can cause aneuploidy and transformation (20, 21).

STK15 kinase is a member of the serine/threonine kinase family that includes the prototypic yeast *ipl1* and *Drosophila* aurora kinases, as well as other members of the kinase family involved in regulation of chromosomal segregation (22, 23). In yeast, temperature sensitive *ipl1* mutants missegregate chromosomes, resulting in polyploidy (24). Loss of function of *aurora* in *Drosophila* inhibits separation of centrosomes and leads to the formation of abnormal mitotic spindles (23). The high ho-

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[3] The abbreviation used is: CIN, chromosomal instability.

Table 1 Characteristics of the patients and tumors[a]

Sex	Age	Tumor size (cm)	Differentiation	Lymph node metastasis	Local metastasis	Perineural invasion	Lymphovascular invasion
M	65		Poor	No	Yes		
M	69	3	Well	No		Yes	No
M	72		Moderate	No	Yes	Yes	
M	68	2.9	Poor				
M	63	3	Well	No	No	No	No
M	69	3	Moderate	Yes	Yes		
M	65	4	Well	No	Yes	Yes	
M	73	4	Moderate	No	Yes	No	No
F	79	4	Poor	Yes	Yes		
M	74	1	Poor	Yes	Yes	Yes	Yes
F	80	3	Well	No	Yes	Yes	Yes
M	70	4	Poor	Yes	Yes	Yes	
M	70	2.7	Moderate	Yes	Yes	Yes	Yes
F	73	2		No			
F	58	4		Yes			
F	65		Well	Yes	Yes		
F	73	1	Poor	No	Yes	Yes	No
F	65	5	Moderate	Yes	Yes	Yes	No
M	73	5	Poor	No	No		No
M	63	4.5	Moderate	No	Yes	Yes	Yes
M	59	2.5	Moderate	Yes	Yes	Yes	
M	65	2.5	Moderate	No	No	Yes	No
M	77	3.5	Well	Yes	Yes		
F	65	0.8	Moderate	Yes	Yes	Yes	
M	77	3	Moderate	No	Yes	Yes	
M	76	2.5	Well	No	Yes	Yes	No
F	60	3	Poor	No	Yes	Yes	No
F	51	2.5	Moderate	Yes	Yes		No
F	74	6		No	Yes		No
M	59	1.8	Poor	Yes	No	Yes	No
M	59	1.3	Well	No	Yes	Yes	No
M	56	3.5	Moderate	Yes		Yes	No
M	79	1.3	Moderate	Yes	Yes		
F	67	2.5	Moderate	No	Yes	Yes	No
F	77	2.5	Moderate	No	Yes	Yes	No
F	62	2		No	No	No	No
F	72	2		Yes		Yes	No
F	50	2.5	Moderate	Yes	Yes		Yes

[a] Blank cells indicate information unavailable from the medical record.

mology among human *STK15* and other aurora kinases indicates that these genes have been structurally and functionally conserved through evolution (25).

Pancreatic cancer is one of the most deadly human cancers. Cytogenetic and molecular studies (26, 27) have shown that many human pancreatic cancers exhibit chromosome abnormalities and gain of chromosome 20q, where the *STK15* gene is localized. A recent study has found that inhibition of *STK15* gene expression by antisense oligonucleotides resulted in the arrest of cell growth in the G_2-M phase of the cell cycle and increased apoptosis in pancreatic carcinoma cell lines (28). This finding suggests that Aurora A kinase is a potential molecular target for antitumor activity. In the current study, we examined the expression and possible copy number alteration of *STK15* in pancreatic carcinoma cells and primary tumors, as well as their association with the size, degree of differentiation, and metastasis status of the tumors.

MATERIALS AND METHODS

Cell Culture. Nine pancreatic carcinoma cell lines were analyzed in this study. HS-766T, MIAPaCa-2, Panc-1, Panc-3, Panc-28, and Panc-48 cells were cultured in DMEM. HPAF II cells were cultured in MEM. Aspc-1 and Bxpc-3 cells were cultured in RPMI 1640 medium. All media were supplemented with 10% fetal bovine serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine (Life Technologies, Inc.). The cell lines were maintained at 37°C under 5% CO_2 and saturated moisture.

Clinical Tissue Sample Collections. Fresh tumor and normal adjacent tissues and paraffin-embedded tumor sections were collected from patients with a pathologically confirmed adenocarcinoma of the pancreas undergoing surgical treatment at the University of Texas M. D. Anderson Cancer Center. Normal pancreatic tissues were collected from organ donors at an organ transplant center at the University of Bern, Switzerland, as controls. Frozen tissue samples were stored at −80°C before processing for protein, RNA, and DNA extraction. Information on patient demographics and pathologic characteristics of the tumors was retrieved from their medical records (Table 1).

Western Blot Analysis. Cellular lysates were prepared with O'Farrell lysis buffer (1.25 mM β-glycerophosphate,

0.5 mM EGTA, 5 mM Na fluoride, 1 mM *N*-ethylmaleimide, 0.25 mM *p*-hydroxymercuribenzoate, and 0.25 mM phenylmethylsulfonylfluoride). Tissue samples were homogenized and sonicated on ice in 1.5 ml of elution buffer (80 mM β-glycerophosphate, 20 mM EDTA, 15 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 2.5 mM Microcystin LR, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin). Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Proteins (50 μg) were separated by 12% SDS-PAGE containing 0.1% SDS and transferred to Hybond-C nitrocellulose membranes (Amersham Life Science) by electroblotting. The membranes were sequentially incubated with rabbit antihuman STK15 antibody as primary antibody (29) and HRP-conjugated mouse antirabbit IgG as a secondary antibody. The target protein was detected with enhanced chemiluminescence Western blotting detection reagents (Amersham Life Science). To confirm equivalent loading of total protein in all lanes, the membranes were reprobed with β-actin antibody. Mammary carcinoma cell line BT-474 was included in all experiments as a positive control for STK15 expression. The membranes were also probed for STK15-associated protein cdc20, and cell cycle regulatory dual specificity protein phosphatase cdc25, using antibodies p55 CDC H-175 and C-20, respectively (Santa Cruz Biotechnology).

Northern Blot Analysis. RNA was prepared using Tri Reagent (Molecular Research Center, Inc.). In brief, 10 μg of RNA were separated on a 1% agarose gel and transferred to a Hybond-N+ positively charged nylon membrane. A total of 1×10^7 cpm of a 1-kb STK15 cDNA probe was mixed with 10 ml of Rapid-hyb buffer and used to probe the membrane at 65°C overnight. The membrane was then stripped and reprobed with the 550-bp cDNA probe 36B4 encoding human acidic ribosomal phosphoprotein PO (30) to confirm equal loading of RNA in all lanes.

Southern Blot Analysis. Genomic DNA was isolated using the phenol/chloroform method. Estimation of *STK15* gene copy numbers was done by Southern blot hybridization analysis according to standard procedures as described earlier (20). Briefly, 10 μg of *Bam*HI-digested total cellular DNA were Southern blot hybridized with a *STK15* cDNA probe. Signal intensity of a STK15-specific *Bam*HI fragment in each sample was measured against the signal intensity of a single copy fragment to estimate the relative copy number of the *STK15* gene.

Immunohistochemistry. Expression of the STK15 protein in tissue sections was measured by immunohistochemistry using the ABC Kit (Vector Laboratories, Burlingame, CA). Briefly, the sections were heated at 65°C overnight, deparaffinized in xylene, and rehydrated in graded alcohol. The primary *STK15* polyclonal antibody (29) was incubated with the sections at 4°C overnight in a humid chamber, at a 1:200 dilution. The biotinylated secondary antibody was incubated with the sections at 37°C for 30 min, at a 1:200 dilution. Nonspecific binding sites were blocked with 3% BSA and 1.5% goat serum. The antibody complex was detected by incubation with an avidin-biotin-peroxidase complex solution and visualized by 3,3′-diaminobenzidine (Zymed Laboratories, Inc., San Francisco, CA). Tris-Nacl-Tween 20 buffer [0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20] was used to wash the sections. The sections were counterstained with hematoxylin for 5 min and mounted with Eukit (Calibrated Instruments, Hawthorne, NY). The primary antibody was replaced with Tris-Nacl-Tween 20 buffer as a negative control. Specimens were observed under a light microscope for immunostaining. Specimens were then rated for staining intensity and staining extent. Staining intensity was rated as: (*a*) negative (0); (*b*) borderline (1); (*c*) weak (2); or (*d*) strong (3). Staining extent was rated according to the percentage of positive cells seen. Samples in which ≥50% of the cells showed positive staining were rated 2, and those with <50% of cells stained were rated 1. The product of staining intensity and staining extent gave an overall staining score.

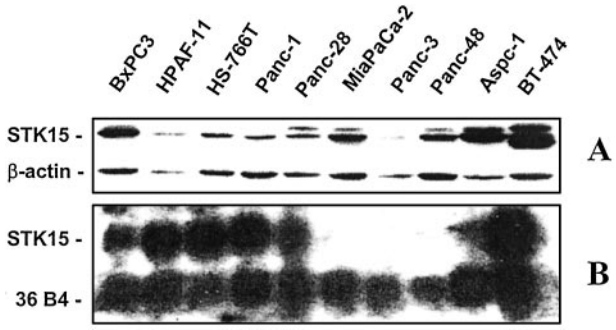

Fig. 1 Protein, mRNA expression of *STK15* in pancreatic carcinoma cell lines. *A*, Western blotting. Equal amounts of total protein (50 μg) were loaded in all lanes. Immunoblots were probed with anti-STK15 polyclonal antibody (*top panel*) and reprobed with anti-β-actin polyclonal antibody (*bottom panel*) to confirm the equivalent loading of total protein. Mammary carcinoma cell line BT474 was included as a positive control of *STK15* expression. *B*, Northern blotting. 36B4 RNA was used to normalize the loading of total RNA.

RESULTS

STK15 Expression and Copy Number in Cell Lines. Western blotting analysis showed *STK15* protein expression in all nine pancreatic carcinoma cell lines (Fig. 1*A*). Positive control mammary epithelial cell line BT-474, reported previously to express >5-fold elevated level of STK15, was included in the same analysis. The highest level of STK15 protein expression (~5-fold) was seen in Aspc-1 and BxPc-3 cells. The lowest level of expression (~1.5-fold) was seen in Panc-1 and Panc-3 cells. The slower mobility band detected in some of the lanes of Western blot (Fig. 1*A*) likely represents the hyperphosphorylated form of STK15, as described in our recent publication (31). Northern blot analysis (Fig. 1*B*) showed that *STK15* mRNA was highly overexpressed in HPAF-II (~10-fold) and HS-766T cells (~8-fold); moderately overexpressed (~5–7-fold) in BxPc3, Panc-1, and Panc-28 cells; and weakly expressed (<2-fold) in the remaining cell lines. Altered amounts of STK15 mRNA detected in these cells indicate that the gene is regulated at transcriptional level. Indeed, our unpublished data have revealed that the minimal promoter of this gene is differentially regulated in different cell lines. It was interesting that in MiaPaca-2 and Panc48 cell lines, the level of protein expression was elevated without detectable mRNA expression. The result suggests that post-transcriptional and post-translational

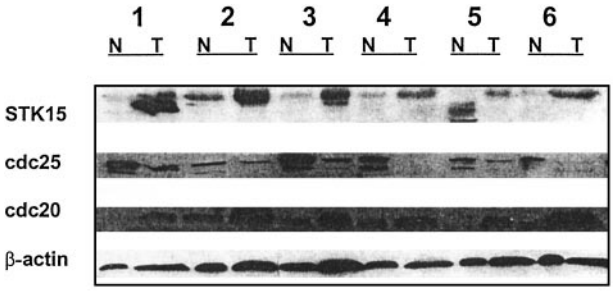

Fig. 2 Western blot analysis of STK15, cdc20, and cdc25B expression in paired normal (*N*) and tumor (*T*) tissues from six representative patients with pancreatic carcinoma. Equal amounts of total protein (50 μg) were loaded in all lanes. STK15 and cdc20 were overexpressed, and cdc25B was underexpressed in tumors.

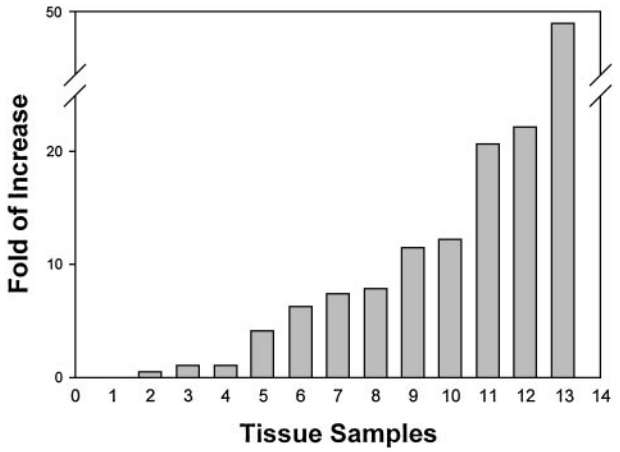

Fig. 3 Fold of increase in intensity ratios of STK15 over β-actin in 12 tumors (*Lanes 2–13*) compared with the mean ratio in 20 normal pancreatic tissues from organ donors (*Lane 1*).

mechanisms also regulate the steady-state STK15 mRNA and protein levels. Southern blot analysis revealed that *STK15* gene amplification was infrequent in the pancreatic carcinoma cell lines, and only Panc-1 cells showed ~2-fold amplification of the gene (data not shown).

STK15 Protein Expression in Tumor Tissues. The expression of STK15 protein was examined in 20 pancreatic tumors and 11 normal adjacent tissues from pancreatic cancer patients and 20 normal pancreatic tissues from organ donors by Western blot analysis and in paraffin sections of 38 pancreatic tumors by immunohistochemistry. STK15 was found overexpressed in tumors compared with normal adjacent tissues by Western blot analysis as shown in paired samples from individual patients (Fig. 2). Additional faster migrating bands were detected in some of the sample lanes. Whether these represent isoforms of the protein or its degradation product is not known at this time. Using the mean level of STK15 expression in 20 normal pancreatic tissues as the baseline (*Lane 1* of Fig. 3), STK15 protein expression was highly elevated in 12 of the 20 tumors (60%) examined with 5 tumors displaying a >10-fold increase (Fig. 3). Reprobing of the same membranes revealed overexpression of the STK15-associated protein cdc20 and clearly reduced expression of the mitosis-activating protein phosphatase cdc25 in tumor *versus* normal adjacent tissues (Fig. 2). The expression of STK15 protein was also examined in 38 paraffin-embedded pancreatic cancer tissues by immunohistochemistry (Fig. 4). Strong cytoplasmic staining (score ≥3) of STK15 protein was detected in 28 tumors (74%). Weak to moderate staining (scores 1–2) of STK15 was detected in 7 tumors (18%). STK15 expression was not detectable in three tumors.

STK15 Expression and Pathological Characteristics of the Tumors. The association between *STK15* expression and the size of the tumor, degree of tumor differentiation, status of lymph node metastasis, presence of perineural or lymphovascular invasion, and local metastasis to peripancreatic adipose tissues or soft tissues and adjacent organs was explored. It appeared that none of these clinical and pathological characteristics of the tumors were significantly associated with the extent of *STK15* expression (Table 2).

DISCUSSION

Pancreatic cancer is the fifth leading cause of cancer death in the United States.[4] Because the diagnosis is usually made in the advanced stage of the disease when distant metastasis and/or invasion of surrounding tissues has occurred, the prognosis of pancreatic cancer patients is very poor. Novel approaches to the early diagnosis and treatment of this deadly disease are needed, and understanding the molecular mechanism of pancreatic cancer is the key to any such approach.

In the present study, we demonstrated overexpression of *STK15* in pancreatic carcinoma cell lines and pancreatic tumors. Moreover, we also noted a possible association between *STK15* expression and two other cell cycle regulating proteins, *i.e.*, cdc20 and Cdc25. Even though we did not find any significant association between *STK15* expression and several pathological features of the pancreatic tumors, the recent finding that inhibition of *STK15* gene expression by antisense oligonucleotides resulted in the arrest of cell growth and increased apoptosis in pancreatic carcinoma cell lines (28) suggests that *STK15* overexpression may play a role in the development of pancreatic cancer and may serve as a novel molecular target for diagnosis and treatment of pancreatic cancers.

Our finding of *STK15* mRNA overexpression in 5 of the 9 pancreatic cancer cell lines studied is supported by several previous studies. *STK15* mRNA has been reported to be overexpressed in breast, colon, prostate, ovarian, neuroblastoma, cervical, lung, renal, gastric, and melanoma tumor cell lines (20, 21, 32). Another study found *STK15* RNA overexpression in 54% (22 of 35) of primary human colorectal carcinomas *versus* matched normal tissues (21). In primary gastric carcinoma, reverse transcription-PCR showed that 51% (18 of 35) of tumors had *STK15* RNA overexpression compared with normal gastric mucosa (32). The highly elevated level of STK15 protein in pancreatic tumors compared with that in normal tissues further

[4] Internet address: http://www.cancer.org/eprise/main/docroot/stt/stt_0.

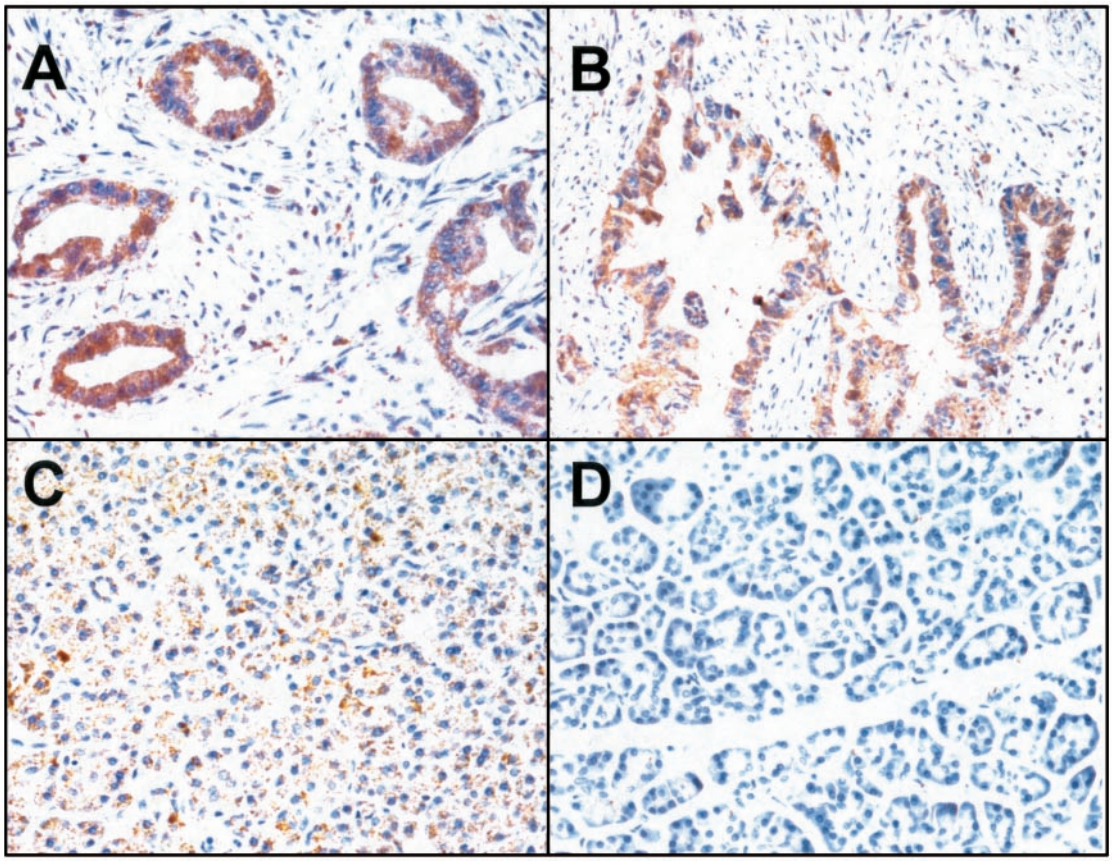

Fig. 4 STK15 protein expression in pancreatic tumors and normal tissues detected by immunohistochemistry. Pancreatic carcinomas showed strong cytoplasmic staining of STK15 (*A* and *B*), and normal adjacent tissue displayed weak staining (*C*). *D*, negative control of normal pancreatic tissue. Original magnification, ×100.

indicates that this oncogenic protein is involved in pancreatic carcinogenesis.

It may be proposed that overexpression of *STK15* is involved in pancreatic carcinogenesis by causing centrosome abnormalities and CIN, *e.g.*, it has been demonstrated that pancreatic ductal carcinomas exhibit centrosome abnormality (33) and that such abnormalities correlate with CIN in human pancreatic cancer cell lines (26). It has also been reported that *STK15* mRNA overexpression correlates with CIN values in human breast carcinoma (34). In the light of these observations, a possible correlation between *STK15* overexpression and CIN in pancreatic cancer is now being investigated.

Taking into consideration the complexity of genomic alterations seen in the vast majority of solid tumors, it is unlikely that *STK15* is the sole contributor of CIN. It is most likely that additional genes in the same and/or similar pathways act synergistically with *STK15* in regulating the process of chromosomal segregation. Altered expression levels of cdc20 and Cdc25, observed in the tumor samples included in this study, may be a reflection of this phenomenon.

The concurrent overexpression of cdc20 with STK15 in pancreatic tumors is consistent with a association reported previously between cdc20 and STK15 in HeLa cells (35). Cdc20/Fizzy family proteins are required for activation of the an-

Table 2 Association between STK15 expression and pathological features of the tumor

	Intensity		Area		Score	
	0–1	2–3	<50%	≥50%	0–3	4–6
Differentiation						
Well	1	7	2	6	2	6
Moderate	4	12	5	11	7	9
Poor	1	8	4	5	4	5
P[a]		0.61		0.68		0.92
Lymph node metastasis						
No	4	16	8	12	10	10
Yes	3	14	6	11	6	11
P		0.86		0.77		0.37
Adjacent organ metastasis						
No	2	3	1	4	2	3
Yes	4	23	10	10	11	16
P		0.54		0.80		0.59
Perineural invasion						
No	1	2	1	2	1	2
Yes	4	18	9	13	11	11
P		0.26		0.57		0.96
Lymphovascular invasion						
No	3	15	6	12	7	11
Yes	2	3	1	4	2	3
P		0.19		0.23		0.98

[a] All *P*s are from χ^2 test.

aphase-promoting complex/cyclosome, which catalyzes the ubiquitin-dependent proteolysis of cell cycle regulatory proteins, such as anaphase inhibitors and mitotic cyclins, leading to chromosome segregation and exit from mitosis (36). It has also been speculated that cdc20 can be targeted by STK15 and, alternatively, that STK15 can function by phosphorylating cdc20, thereby influencing the activity of cdc20 (35). Members of the *cdc25* gene family of protein phosphatases function as mitotic activators by dephosphorylating cyclin-dependent kinases, such as cdc2 p34 (37). cdc25B is essential for G_2-M transition in human cells (38), and cdc25B levels apparently remain relatively constant throughout the cell cycle (39). Thus, the reduced expression of cdc25 in pancreatic tumors is intriguing. It is possible that STK15 may antagonize cdc25B from responding to mitotic activation signals. For this reason, the interaction of these proteins needs to be further investigated. It is relevant to mention in this context that protein phosphatase type 1 has been reported recently by us to be acting in a feedback regulatory pathway with STK15 kinase in controlling chromosome segregation during mitosis (31). Abrogation of this pathway was shown to cause anomalous chromosome segregation during mitosis. Although we could not detect altered expression of protein phosphatase type 1 in the tumor tissues analyzed, it is plausible that additional gene products like cdc20 and cdc25 involved in the *STK15/BTAK/Aurora A* pathway play roles in pancreatic carcinogenesis and, therefore, could prove to be relevant novel disease markers and therapeutic targets.

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