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BRAF and FBXW7 (CDC4, FBW7, AGO, SEL10) Mutations in Distinct Subsets of Pancreatic Cancer

Potential Therapeutic Targets

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The recognition of biologically distinct tumor subsets is fundamental to understanding tumorigenesis. This study investigated the mutational status of the serine/threonine kinase *BRAF* and the cyclin E regulator *FBXW7* (*CDC4*, *FBW7*, *AGO*, *SEL10*) related to two distinct pancreatic carcinoma subsets: the medullary *KRAS2*-wild-type and the cyclin E overexpressing tumors, respectively. Among *KRAS2*-wild-type carcinomas, 33% (3 of 9) contained *BRAF* V599E mutations; one of which was identified in the pancreatic cancer cell line COLO357. Among 74 *KRAS2*-mutant carcinomas, no *BRAF* mutations were identified. Among the *KRAS2*/*BRAF* wild-type carcinomas, no mutations within pathway members *MEK1*, *MEK2*, *ERK1*, *ERK2*, *RAP1B*, or *BAD* were found. Using pancreatic cancer microarrays and immunohistochemistry, we determined that 6% (4 of 46 and 5 of 100 in two independent panels) of pancreatic adenocarcinomas overexpress cyclin E. We identified two potential mechanisms for this overexpression including the amplification/gain of *CCNE1* gene copies in the Panc-1 and Su86.86 cell lines and a novel somatic homozygous mutation (H460R, in one of 11 pancreatic cancer xenografts having allelic loss) in *FBXW7*, which was accompanied by cyclin E overexpression by immunohistochemistry. Both *BRAF* and *FBXW7* mutations functionally activate kinase effectors important in pancreatic cancer and extend the potential options for therapeutic targeting of kinases in the treatment of phenotypically distinct pancreatic adenocarcinoma subsets. ***(Am J Pathol 2003, 163:1255–1260)***

Some mutations in carcinomas are highly patterned. As one example, the simultaneous accumulation of mutations within different members of a particular linear signaling pathway is seldom seen within the same neoplasm, perhaps because multiple mutations are unlikely to yield further selective advantages. Studies of the mutational status of *RB1*, cyclin-dependent kinase 4 (*CDK4*) and the Cdk4 inhibitor p16 (*CDKN2A*) support this theory. These three genes lie within a well-described cell cycle control pathway and exhibit mutually exclusive mutations, that is, tumors with mutant forms of one of these genes invariably retain wild-type copies of the others.[1–3] Similar findings have been reported for the genes *TP53* and *MDM2*[4] in the Tp53 suppressive pathway, platelet-derived growth factor receptor α (*PDGFRA*), and the tyrosine kinase receptor *KIT*[5] in the platelet-derived growth factor pathway, β-catenin (*CTNNB1*) and its regulator *APC*[6] in the Wnt signaling pathway, and most recently for *KRAS2* and *BRAF*[7,8] in what is presumably a major regulatory system for mitogen-activated protein kinases. As another example, subsets of neoplasms with unique phenotypic characteristics often harbor specific mutational patterns. A medullary histology in pancreatic cancer is often associated with DNA mismatch repair abnormalities as well as with wild-type *KRAS2* status.[9,10] In ovarian cancer, phenotypic subsets defined by cyclin E overexpression result from cyclin E amplification or from mutations of the F box and tryptophan aspartic acid repeat unit (WD) domain-containing gene, *FBXW7*.[11] The Fbxw7 protein is the ubiquitin ligase that targets cyclin E for degradation[12] after cyclin E catalyzes the transition from the G1 to S phase of the cell cycle.

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This study investigated the relationship of two potential mutational targets, *BRAF* and *FBXW7*, which may associate with distinct subsets of pancreas carcinomas. Both of these genes may be particularly attractive for therapeutic targeting using small molecule inhibitors. To date, the role of kinase oncogene pathways in pancreas cancer is relatively underexplored, with studies mainly focusing on *AKT2* and epidermal growth factor receptor signaling.[13,14] Examination of other kinase signaling pathways may help elucidate novel mechanisms of tumorigenesis in pancreatic cancer and attractive therapeutic targets. Here, we report the mutational status of *BRAF* and *FBXW7*, as well as the genomic amplification of cyclin E (*CCNE1*), in relation to two distinct subsets of pancreas carcinomas having unique histological and immunohistologic phenotypes.

Materials and Methods

Tissues

Primary adenocarcinomas of the pancreas and non-neoplastic tissues were collected from surgical specimens obtained from patients treated at The Johns Hopkins Medical Institute. Surgically resected cancers were either implanted and propagated in mice as described previously[15] or formalin-fixed and paraffin-embedded for use in the construction of a tissue microarray (array 1). A second cancer tissue array was also created from samples resected at Wayne State University, Detroit, Michigan (V. A., array 2). Sample collection and tissue studies were approved by the Institutional Review Boards.

Cell Lines

The pancreatic cancer cell lines Su86.86, AsPC-1, and Panc-1 were purchased from the American Type Culture Collection (Manassas, VA). The COLO357 pancreatic cell line was obtained from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). The *KRAS2* mutational status was confirmed as wild-type at codons 12, 13, and 61 in COLO357 cell lines by direct sequencing.[10] Genomic DNA was isolated from cell lines, as well as from harvested xenografts and non-neoplastic tissues for the sequencing studies.

Gene Sequencing

PCR primers for *BRAF*, *MEK1*, *MEK2*, *ERK1*, *ERK2*, *RAP1B*, *BAD*, and *FBXW7* were designed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) from reference sequences at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The exons of *FBXW7* were amplified by PCR from 11 samples known to have loss of heterozygosity (Iacobuzio-Donahue et al, unpublished data) near 4q31.3 while the exons of *BRAF*, *MEK1*, *MEK2*, *ERK1*, *ERK2*, *RAP1B*, and *BAD* were amplified from nine samples having only wild-type *RAS* genes. Automated sequencing was performed on amplified fragments and all sequence variants identified were confirmed by the sequencing of independent PCR products. Primer sequences used in this study are available on request.

Fluorescence in Situ *Hybridization (FISH)*

FISH was performed as described previously.[16] *AKT2* (19q13.2) amplification was evaluated using the BAC clone 127D1. *CCNE1* (19q12) amplification was evaluated using either the BAC clone 246K7 or phage clones 25 or 26 (described elsewhere[17]). Signals were evaluated with respect to the control gene, *TCF3* (19p13.3, P1 clone 8542). Lymphocytes from a normal donor served as a control.

Southern Blot Analysis

Cyclin E genomic amplification was evaluated by standard Southern blot technologies. The cyclin E hybridization probe was created from a *Not*I digested fragment of the IMAGE clone, 357807. The blots were then stripped and probed with Clone WI-12306 (19q) as a loading control.

Immunohistochemistry

Immunolabeling was performed as previously described[18] using an anti-cyclin E primary antibody (clone CYE05; Lab Vision, Fremont, CA) and the DAKO (Carpinteria, CA) EnVision+ peroxidase-linked secondary antibody. Optimal cyclin E antibody dilutions were predetermined using known positive control tissues included in each run. Immunopositivity was evaluated by two observers (S. E. K. and R. H. H.).

Results

KRAS2 Signaling Pathway

Exons 11 and 15 of *BRAF* were sequenced from a unique collection of rare pancreatic adenocarcinomas ($n = 9$) retaining only wild-type copies of the *KRAS2*, *NRAS*, and *HRAS* genes. Two xenografted pancreatic tumors and the COLO357 cell line were each found to harbor the *BRAF* codon V599E mutation (Figure 1; Table 1) previously shown to stimulate the kinase activity of Braf.[19] Sequencing of *BRAF* in the two available constitutional DNA samples from these patients, as well as in an additional 74 typical *KRAS2*-mutant xenografted pancreatic carcinomas, revealed no genetic alterations within exons 11 and 15 (Figure 1; data not shown). The coding sequences of *MEK1*, *MEK2*, *ERK1*, *ERK2*, *RAP1B*, and *BAD* were sequenced in the *KRAS/BRAF* wild-type pancreatic cancer cases. These genes are proposed to play a role in the effector arms of Ras and Raf signaling, and might be additional targets of oncogenic disruption in tumors. We, however, failed to identify any mutations in these genes.

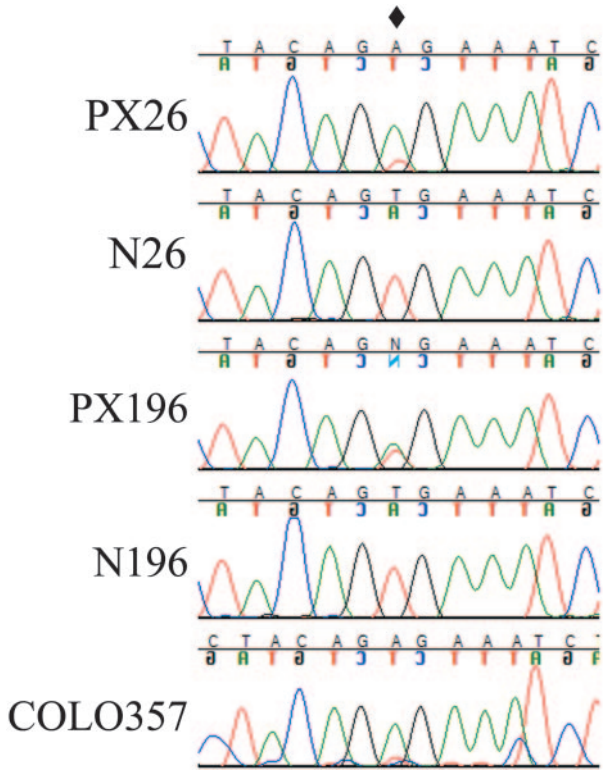

Figure 1. BRAF mutations in pancreatic cancer. Tumors (PX) and the COLO357 cell line display the V599E mutation (**diamond**). Constitutional DNA samples (N) verify mutation is somatic.

Frequency and Mechanisms of Cyclin E Overexpression

To estimate the frequency of cyclin E overexpression, two pancreatic adenocarcinoma tissue microarrays, created from tissues collected from two separate institutions, were studied by immunohistochemistry. We found that 6% (4 of 46 and 5 of 100) of the pancreatic carcinomas had immunohistochemically detectable levels of nuclear cyclin E when compared to normal cells within the same tissue cores (Figure 2). To investigate the mechanism of this overexpression, Southern blot analysis was performed on the pancreatic cancer cell lines Panc-1, AsPC-1, and Su86.86. The results suggest an increase in *CCNE1* copy number (Figure 3E) when compared to levels in the normal N57. FISH analysis confirmed the Southern data showing a low-level amplification of *CCNE1* (separate from AKT2 amplification) in Panc-1 cells while ectopic copies of *CCNE1* were identified in the Su86.86 cell line (Figure 3).

To investigate another potential mechanism for cyclin E overexpression, the exons of *FBXW7* were amplified and sequenced from pancreatic cancer xenografts (n = 11) that had known loss of heterozygosity at 4q31.3. We identified a novel, single nucleotide somatic alteration that resulted in a histidine to arginine missense mutation at codon 460 (H460R) in tumor PX221 (Figure 2E; Table 1). The formalin-fixed, paraffin-embedded carcinoma of this patient was immunohistochemically studied, and strong immunopositivity was observed for cyclin E specifically in the neoplastic cell nuclei (Figure 2F). This confirmed the H460R mutant to be functionally inactive.

Discussion

As many as 90% to 95% of ductal adenocarcinomas of the pancreas have *KRAS2* mutations, a finding suggestive of a virtually necessary role in the development of pancreatic cancer. By reverse analogy, however, this suggests that a relatively large percentage (as much as 10%) of pancreatic carcinomas might use alternative methods to stimulate this pathway. Recent reports by Davies et al[19] and Rajagopalan et al[8] have shown that Braf, a serine/threonine kinase located immediately downstream in Ras signaling, is a frequent mutational target in several cell lines and primary cancers including 66% of melanomas and 10% of colorectal carcinomas. In

Table 1. *BRAF* and *FBXW7* Mutations in Subsets of Pancreatic Cancer

Subsets and samples	Prevalence	Result	Note
Cyclin E overexpression subset			
Tissue array 1	4/46 (9%)	Overexpression by IHC	Array created at Johns Hopkins
Tissue array 2	5/100 (5%)	Overexpression by IHC	Array created at Harper Hospital, Wayne State University
PX221		*FBWX7* mutation (H460R, CAT to CGT, homozygous)	Overexpression of cyclin E subsequently determined by IHC
KRAS2 wild-type Subset			
Tumor panel	7/77 (9%)*	Mutations of K-, N-, and H-ras excluded by sequencing	
PX26		*BRAF* mutation (V599E, GTG to GAG, homozygous)	Known MSI, medullary histology
PX196		*BRAF* mutation (V599E, GTG to GAG, heterozygous)	Known MSI, medullary histology
COLO357		*BRAF* mutation (V599E, GTG to GAG, homozygous)	Not MSI, commercial cell line

*From consecutive xenografted pancreatic adenocarcinomas, previously reported.[10]
IHC, immunohistochemistry; MSI, microsatellite instability.

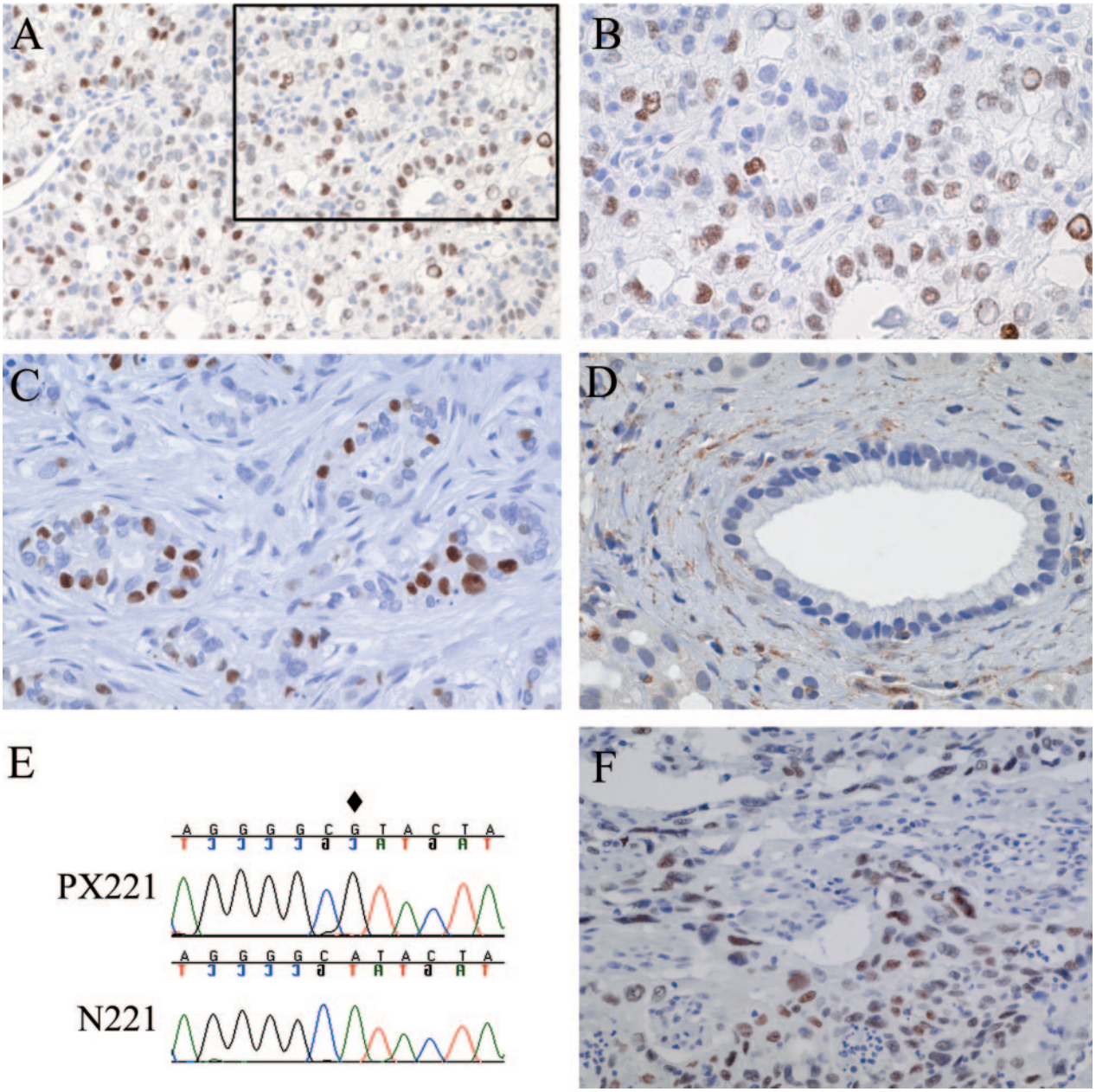

Figure 2. Cyclin E overexpression in pancreatic adenocarcinoma. Pancreatic carcinoma tissue arrays 1 (**A** and **B**) and 2 (**C**) studied for cyclin E expression. **B:** Higher magnification of **A inset**. Normal ductal structures (**D**) failed to express nuclear cyclin E. **E:** The pancreatic tumor xenograft, PX221, carries a homozygous CAT to CGT somatic missense mutation within exon 9 (**diamond**). **F:** Immunohistochemistry confirms the predicted overexpression of cyclin E in PX221. Magnifications: **A**, ×40; **B**-**D** and **F**, ×100.

the colorectal carcinomas studied, *BRAF* mutations were exclusively found in neoplasms having wild-type *RAS* (half of which also displayed abnormalities in DNA mismatch repair). The current study provides the first evidence of *BRAF* mutations in pancreatic cancer and reaffirms the mutually exclusive nature of *KRAS2*/*BRAF* mutations as well as the apparent requirement for *KRAS2*-related signal activation during most instances of pancreatic ductal carcinogenesis.

The second signaling pathway investigated in this report focuses on the cell cycle regulator cyclin E. Cyclin E is a known protooncogene that is overexpressed in a variety of cancers. The mechanism of overexpression is reportedly due to amplification at 19q13.1 in some neoplasms.[20–23] Recently, however, several groups[11,24,25] have described mutations in *FBXW7*, which codes for the cyclin E/ubiquitin ligase conjugating protein, which appear to impair the ability of cells to degrade cyclin E in ovarian, endometrial, and breast cancer. Such observations provide a mechanism for the overexpression of cyclin E in these cancers and for the consequential effects that would disregulate cell cycle control.

For pancreatic cancer, however, the frequency of cyclin E overexpression and therefore its role in tumorigenesis remained uncharacterized. Using two separate tissue microarrays created from geographically distinct

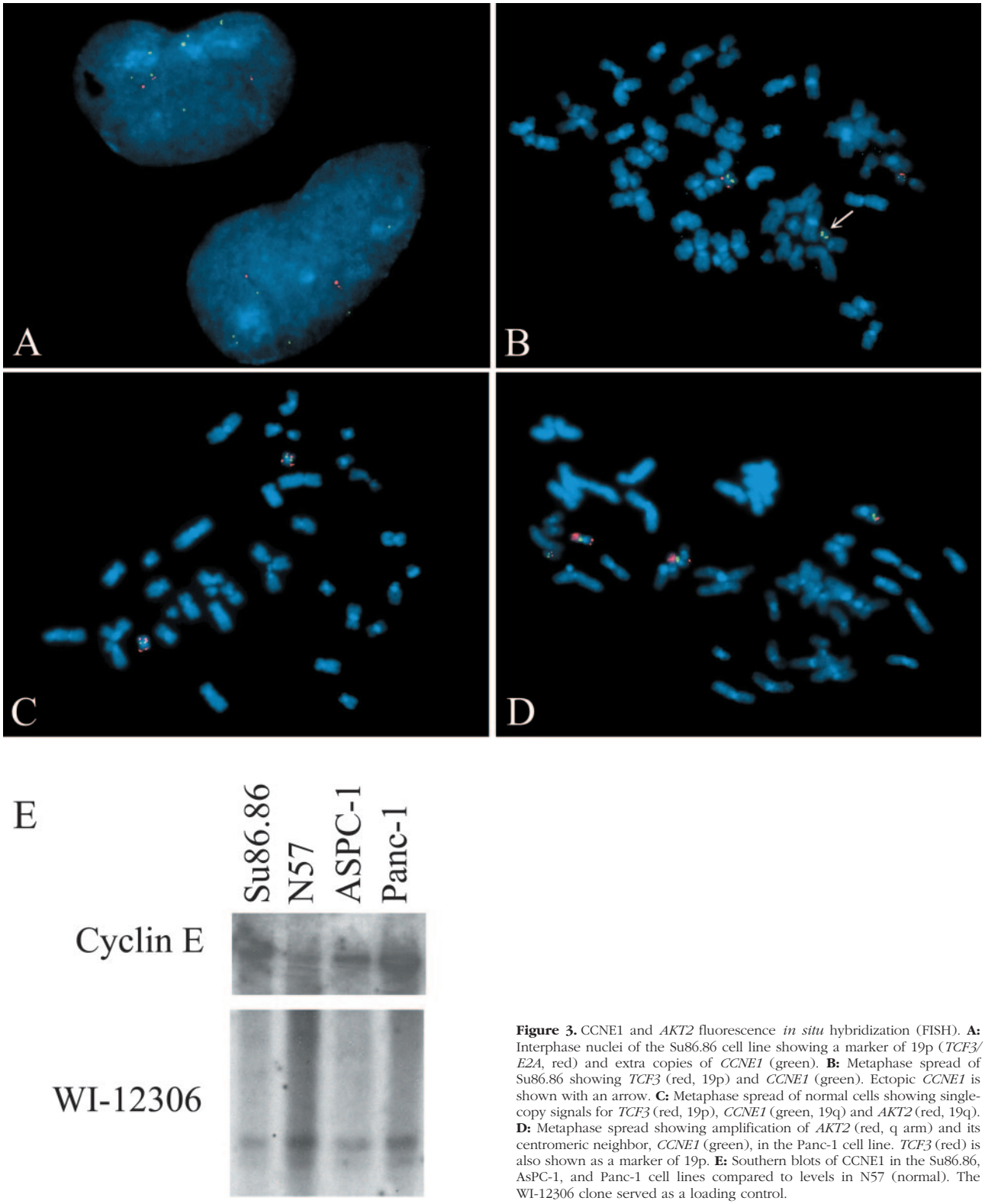

Figure 3. CCNE1 and *AKT2* fluorescence *in situ* hybridization (FISH). **A:** Interphase nuclei of the Su86.86 cell line showing a marker of 19p (*TCF3/E2A*, red) and extra copies of *CCNE1* (green). **B:** Metaphase spread of Su86.86 showing *TCF3* (red, 19p) and *CCNE1* (green). Ectopic *CCNE1* is shown with an arrow. **C:** Metaphase spread of normal cells showing single-copy signals for *TCF3* (red, 19p), *CCNE1* (green, 19q) and *AKT2* (red, 19q). **D:** Metaphase spread showing amplification of *AKT2* (red, q arm) and its centromeric neighbor, *CCNE1* (green), in the Panc-1 cell line. *TCF3* (red) is also shown as a marker of 19p. **E:** Southern blots of CCNE1 in the Su86.86, AsPC-1, and Panc-1 cell lines compared to levels in N57 (normal). The WI-12306 clone served as a loading control.

tumor banks, we estimated that the overall frequency of cyclin E overexpression in pancreatic cancer is near 6%. Mechanistically, the overexpression of cyclin E appears to be attributable in part to the amplification of 19q13.1 or the mutation of its negative regulator, *FBXW7*. The *FBXW7* mutation identified in this study occurred near the site of other reported mutations suggesting exons 8 and 9 to be critical hotspots for mutations that inactivate this protein.[11,24,25] Indeed, the site of our mutation occurred within the fifth WD domain and is conserved between *Homo sapi-*

ens, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*.[25]

The current study identified two new mutational targets in pancreatic cancer: the genes encoding the serine/threonine kinase Braf and the cyclin E/ubiquitin ligase conjugating protein Fbxw7. Their identification contributes to a satisfying orderliness of specific mutations in phenotypic subsets of pancreatic cancers as demonstrated by the presence of specific mutations in pancreatic carcinomas with a medullary phenotype and often with microsatellite instability (including *ACVR2*, *TGFBR2*, and *BRAF*[8,26,27]) and in at least some of those that overexpress cyclin E. In addition, these mutations extend the knowledge of kinase and cyclin abnormalities to genes not previously reported in pancreatic cancer. These findings are of potential therapeutic importance as each of the mutations appears to result in the increased activity of effector kinases important for tumor development. As such, they may be sensitive to small molecule inhibitors currently under development. The clinical recognition of qualitatively distinct tumor subsets may in the future dictate more effective treatment strategies against this deadly disease.

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