Technical Advance

A Tumor Sorting Protocol that Enables Enrichment of Pancreatic Adenocarcinoma Cells and Facilitation of Genetic Analyses

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Molecular profiling of human cancer is complicated by both stromal contamination and cellular heterogeneity within samples from tumor biopsies. In this study, we developed a tissue-processing protocol using mechanical dissociation and flow cytometric sorting that resulted in the respective enrichment of stromal and tumor fractions from frozen pancreatic adenocarcinoma samples. Molecular profiling of DNA from the sorted populations using high-density single nucleotide polymorphism arrays revealed widespread chromosomal loss of heterozygosity in tumor fractions but not in either the stromal fraction or unsorted tissue specimens from the same sample. Similarly, a combination of KRAS mutations and chromosomal copy number changes at key pancreatic cancer loci, such as CDK2NA and TP53, was detected in a substantial proportion of the tumor fractions but not in matched stromal fractions from the same sample. This approach to tissue processing could greatly expand the amount of archived tissue that is available for molecular profiling of human cancer and enable a more accurate diagnosis of genetic alterations in patient samples. (J Mol Diagn 2009; 11:290–297; DOI: 10.2353/jmoldx.2009.080124)

Pancreatic adenocarcinoma is among the 10 most common forms of cancer and is associated with particularly poor prognosis, with median survival from time of diagnosis of 3 to 6 months and an overall 5-year survival rate of less than 5%.¹ A primary reason for such poor outcomes is lack of early detection, mainly because of relatively symptom-free early stages of the disease.² As with all cancer, pancreatic adenocarcinoma is a genetic dis-

ease driven by a step-wise accumulation of somatic alterations, including point mutations, chromosome translocations, loss of heterozygosity (LOH), and changes in gene copy number, resulting in the inactivation of tumor suppressor genes and activation of proto-oncogenes.² The role of a number of known cancer genes in the development of pancreatic adenocarcinoma has been well documented.^{2,3} Activating mutations of the KRAS oncogene are the most common genetic aberrations and increase in frequency with disease progression, from 30% in early stage disease to 80 to 90% of pancreatic adenocarcinomas.^{4,5} Cyclin-dependent kinase inhibitor 2A (CDKN2A) encodes two tumor suppressor genes, INK4a (p16) and ARF (p14) that appear to play an important role in pancreatic cancer, as loss of function of CDKN2A either by mutation, deletion or promoter hypermethylation occurs in 80 to 90% of pancreatic adenocarcinomas.^{2,5} The tumor suppressor gene TP53 has been shown to be either deleted or mutated in 50 to 75% of the pancreatic tumors,^{6,7} and although not commonly lost in pancreatic cancer, the tumor suppressor PTEN has been shown to exhibit reduced expression⁸ and to undergo LOH⁹ in pancreatic adenocarcinoma in some instances. The transforming growth factor- β pathway has also been implicated in the pathogenesis of pancreatic cancer, primarily through loss of the transcriptional regulator SMAD4/DPC4, an event that occurs in ~55% of pancreatic cancers and seems to be a later event in the progression of pancreatic cancer.¹⁰

In addition to the involvement of known cancer genes, pancreatic cancer is characterized by high levels of aneuploidy and extensive chromosomal instability.¹¹ The molecular basis of such instability is not well understood but a number of studies have implicated mutations in

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genes associated with kinetochore structure, centrosome formation, and mitotic spindle checkpoint, in contributing to instability of chromosomes.¹² The BRCA2 locus, which plays a crucial role in maintenance of genomic stability by regulating homologous recombination-linked DNA repair processes, is occasionally lost in later stage pancreatic adenocarcinomas and may impact the overall level of chromosomal instability.¹³

Although a number of mutational and chromosomal alterations have been characterized, molecular analyses of pancreatic cancer have been hindered by the low cancer cellularity of this neoplasm and the contaminating influence of euploid stromal cells that make up the complex anatomy and heterogeneous cellularity of pancreatic tissue.¹⁴ Indeed, the later stages of pancreatic cancer are typically characterized by a vigorous, nonneoplastic stromal response with malignant cancer cells representing only 25% of the cells in the tumor on average.^{15,16} The relatively low percentage of neoplastic cells may pose less of a problem for detecting mutated and dominantly acting oncogenes (eg, KRAS), because highly sensitive methods can be deployed to identify lesions in heterogeneous material with very low tumor content,¹⁷ but may be a prohibitive issue for detecting patterns of allelic loss in tumor cells. A number of methods for genome-wide screening of genetic aberrations in cancer are now available, and new innovations and methodological improvements have further enhanced the sensitivity of these technologies and have led to the identification of numerous chromosomal regions that may harbor novel genes important for pancreatic cancer pathogenesis.¹⁸ The majority of this work has been accomplished *in vitro*, using pancreatic cell line models, or through laborious methods such as laser capture microdissection (LCM).¹⁶ To provide a facile method to allow characterization of genomic aberrations in isolated pancreatic neoplasms, we developed a neoplastic cell enrichment protocol from snap-frozen pancreatic adenocarcinoma resection specimens. A fluorescence-activated cell sorting-based approach has previously been reported to enrich for neoplastic cells using disaggregated breast tumor samples.¹⁹ Molecular profiling of enriched neoplastic cells from whole adenocarcinoma tissue samples was used to confirm specific mutation of KRAS in neoplastic but not stromal fractions and has also revealed several regions of LOH that were undetectable in unsorted whole tissue. This method should have applications to the identification of novel genetic alterations in pancreatic cancer and molecular diagnostic applications in terms of providing a means of accurately assessing chromosomal abnormalities in pancreatic tumor samples.

Materials and Methods

Snap-frozen surgically resected samples from patients diagnosed with pancreatic adenocarcinoma were obtained from a commercial vendor and archived in the Genentech Tumor Bank. All samples were subjected to review by a board-certified pathologist (D.A.E.) to confirm diagnosis and assess overall tumor content (Table 1).

Fourteen of the sixteen samples were confirmed as adenocarcinoma or ductal adenocarcinoma, although one sample was found to exhibit neuroendocrine differentiation and one sample found to exhibit primarily inflammation and fibrosis with no evident neoplastic cells (Table 1). Before sorting, tissue samples were slowly thawed on ice and then cut into ~5-mm³ pieces with tweezers and scalpel in a Petri dish on ice. Cut pieces were loaded with 500 ml of phosphate-buffered saline (PBS) into a sterile Medicon chamber, a component of the MediMachine (BD Biosciences, San Jose, CA). Tissue was disaggregated within the chamber by microblades spinning at constant 100 rpm. The optimal time for disaggregation of pancreatic tissue was determined to be 3 minutes. Minced tissue was then aspirated out of the chamber and forced into single cell suspension using a 70- μ m syringe filter (BD Biosciences). Cell suspensions were slide-mounted via cytospin to evaluate cellular integrity after tissue dissociation.

Cytokeratin expression is a common marker of adenocarcinomas and so a pan-cytokeratin antibody was chosen to define tumor cell populations. In preparation for flow cytometric sorting, cell suspensions were permeabilized by 0.2% Triton X in PBS, and then fixed with 80% ethanol (-20°C). Cells were stained with fluorescein isothiocyanate-conjugated C-11, a pan-cytokeratin mAb (AbCam, Cambridge, MA), then counterstained with DNA-binding 4,6-diamidino-2-phenylindole (DAPI) (Roche, Indianapolis, IN). Sorting of tumor cells [high-fluorescein isothiocyanate (+), high-DAPI (+)] from somatic cells [fluorescein isothiocyanate (-), DAPI (-)] was conducted on a MoFlo cell sorter system (DAKO, Carpinteria, CA).

Genomic DNA (gDNA) was extracted from sorted fractions by the DNEasy kit (Qiagen, Valencia, CA). Amplification of exons 2 to 3 of KRAS was accomplished using nested primers (KRAS exon 2, 1^o: sense 5'-GCAGTCAACT-GGAATTTTCATG-3', antisense 5'-ACTCATGAAAATGGT-CAGAGAAACC-3', 2^o: sense 5'-TGTAACACGACGGC-CAGTGTACTGGTGGAGTATTTGATAGTGT-3', antisense 5'-CAGGAAACAGCTATGACCTGGTCAGAGAAACCTTTAT-CTG-3', KRAS exon 3, 1^o: sense 5'-AACGCATCGATAGCTC-TGCC-3', antisense 5'-AGGAAGGAAGGGTTCCCCGTC-3', 2^o: sense 5'-TGTAACACGACGGCCAGTTCGATAGCTC-TGCCCTCTGCG-3', antisense 5'-CAGGAAACAGCTAT-GACCGGGACCCCTAATTCATTCAC-3'). Amplicons were sequenced in sense and antisense directions using dye-terminator chemistry (Applied Biosystems, Foster City, CA). Subsequent rounds of independent PCR and sequencing reactions confirmed detected KRAS mutations.

The GeneChip mapping assay (Affymetrix, Santa Clara, CA) was performed according to the manufacturer's protocol by the Genentech Microarray Facility on genomic DNA from unsorted tissue, postsort tumor tissue, and postsort stromal tissue from five tumor specimens. Restriction enzyme-digested genomic DNA is PCR-amplified in triplicate, fragmented, and then fluorescent-labeled before loading on Affymetrix Human Map100k array chips. Single nucleotide polymorphism (SNP) data were analyzed using the Affymetrix Chromosome Copy Number Analysis Tool (CNAT) and The Loss

Table 1. Characteristics of Pancreatic Cancer Samples Used in This Study

Sample number	Profiled on SNP arrays?	Tissue	Tissue diagnosis	Tumor content (%)	Patient diagnosis	Stage	Age (years)	Sex
HF-18411	Yes	Pancreas	Adenocarcinoma	20	Pancreatic cancer	T4N1M0 (IVA)	48	Female
HF-18996	Yes	Pancreas	Adenocarcinoma	30	Pancreatic cancer	T2N0M0	49	Female
HF-18998	Yes	Pancreas	Adenocarcinoma	30	Pancreatic cancer	T2N0M0	50	Male
HF-19000	Yes	Pancreas	Adenocarcinoma	10	Pancreatic cancer	T2N0M0	59	Female
HF-16979	No	Pancreas	Adenocarcinoma ductal	20	Pancreatic cancer	pT3pN1pMX (IIB)	60	Female
HF-18764	No	Pancreas	Adenocarcinoma	30	Pancreatic cancer	T3N0M0 (II)		
HF-16976	No	Pancreas	Adenocarcinoma ductal	20	Pancreatic cancer	pT3pN1bpMX (III)	64	Male
HF-2035	No	Pancreas	Adenocarcinoma	75	Adenocarcinoma	72		Female
HF-18409	No	Pancreas	Adenocarcinoma	30	Pancreatic cancer	T3N0M0 (II)	63	Female
HF-16359	No	Pancreas	Adenocarcinoma ductal	10	Pancreatic cancer	T 3N0M0R1 (II)	66	Female
HF-16349	No	Pancreas	Adenocarcinoma ductal	10	Pancreatic cancer	T3N1M0R 0 (III)	50	Male
HF-16345	Yes	Pancreas	Adenocarcinoma ductal	20	Pancreatic cancer	T4N 1bM0R0 (IVA)	75	Female
HF-16975	No	Pancreas	Adenocarcinoma ductal	10	Pancreatic cancer	pT3pN1pMX (IIB)	69	Female
HF-16980	Yes	Pancreas	Adenocarcinoma ductal	10	Pancreatic cancer	pT3pN1pMX (IIB)	68	Female
HF-17928	No	Pancreas	Carcinoma, neuroendocrine	90	Pancreatic cancer	T3N0M0 (II)	79	Male
HF-17700	No	Pancreas	Inflammation and fibrosis	<5%	Pancreatic cancer	T4N0M0 (IVa)		Male

of Heterozygosity (LOH) Algorithm in Genotyping Console 2.0, and copy number data were processed using the Affymetrix GSA_CN algorithm. Data were visualized using Spotfire Software (TIBCO, Palo Alto, CA). All SNP array data from pancreatic tumors described in this study have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE14579.

LCM and DNA Extraction

Snap-frozen pancreatic tumor samples were embedded in the cryoprotectant OCT and serial 7- μ m sections were cut and mounted on microscope slides. The sections were maintained at -80°C until used for LCM. At the time of processing, frozen sections immediately fixed in 75% ethanol for 30 seconds, nuclease-free water for 30 seconds, stained with HistoGene stain (MDS Analytical Technologies, Sunnyvale, CA) for 20 seconds, washed in nuclease-free water for 30 seconds, followed by sequential dehydration in 70, 95, and 100% ethanol, and a final 5-minute dehydration in xylene. Once air-dried, neoplastic cells and normal cells were identified by a pathologist and microdissected using the Veritas microdissection instrument (MDS Analytical Technologies, Sunnyvale, CA) on to separate CapSure Macro LCM caps using instrument settings recommended by the manufacturer. After microdissection, each cap was incubated overnight with 50 μ l of proteinase K using the PicoPure DNA Ex-

traction Kit (MDS Analytical Technologies). After proteinase K inactivation by heating at 95°C for 10 minutes, samples were collected and directly used for PCR and sequencing.

Results

Selection of Tumors and Sorting

Histopathological review and analysis of neoplastic cell content was conducted on a large number of samples representing a diverse group of solid tumor malignancies (Figure 1A). Overall, the pancreatic adenocarcinomas had the lowest percent tumor cell content and thus were selected for tumor enrichment sorting studies. Figure 1 shows examples of samples with high (Figure 1B) and low (Figure 1C) tumor cell content. The majority of pancreatic cancer samples used in this study had an overall neoplastic cell content of less than 30% (Table 1).

A key step to sorting tumor from stromal tissue was the selection of a pan-cytokeratin marker specifically expressed in tumor cells but not in surrounding tissues. The C-11 antibody has been shown to have broad specificity for keratins 7, 8, and 18 and hence was ideal for this purpose because keratin 7 is the mostly commonly expressed cytokeratin in pancreatic cancer.²⁰ Immunofluorescence and histopathological analyses were used to

Figure 1. Analysis of tumor content in a range of solid tumor malignancies. **A:** Histopathological review was conducted on at least 10 samples from a range of tumor types and the percentage of tumors with at least 80% tumor cell content is shown. Gastric and pancreatic cancers were found to have substantially lower tumor content than other tumor types. H&E-stained sections showing relatively high (**B**) and low (**C**) tumor cell content sections. Samples similar to that shown in **C** were used for sorting experiments described in this study.

show that the pan-cytokeratin antibody C-11 specifically labels neoplastic pancreatic cells but not cells in the surrounding stroma (Figure 2A).

Samples were then mechanically dissociated as described in the *Materials and Methods* and immunofluorescence was used to confirm the presence of intact cells with DAPI-positive nuclei as well as cytokeratin-positive cytoplasmic staining (Figure 2A). Dissociated samples were fixed, stained, and then flow cytometrically sorted into high and low cytokeratin (CK)-staining populations as shown in Figure 2B with the idea that the high CK population comprises neoplasm-enriched tissue and the low CK population comprises stromal-enriched tissue.

Validation of the Sorting Protocol

We next performed a variety of genomic analyses to demonstrate that the sorting protocol was having the desired effect in terms of enriching the population of neoplastic cells relative to stromal cells. First, we profiled genomic DNA from unsorted starting tumor material as well as fractions enriched for tumor or stromal cells on Affymetrix high-density SNP arrays. The rationale was to determine whether copy number alterations (ie, chromosomal gains or losses) or copy neutral LOH that are not apparent in unsorted tumors are detectable in the sorted neoplastic cell fraction. LOH is a common mechanism that contributes to tumorigenesis, primarily by inactivation of tumor suppressor genes, and may be assessed using SNP arrays by identification of stretches of homozygous SNPs unlikely to arise strictly by chance.²¹ LOH is difficult to evaluate in tumors in the absence of patient-matched nontumor DNA because stromal DNA compromises genotyping accuracy. The LOH scores we used reflect the lengths of contiguous regions of homozygosity

Figure 2. Fluorescence-activated cell sorting tumor sorting data. **A:** Example of a solid tumor section stained with DAPI (blue) and anti-pan-cytokeratin antibody (C-11) (green) in the **left** panel and after mechanical dissociation in the **right** panel. **B:** Representative flow cytometric sorting data from a pancreatic adenocarcinoma sample. The y axis indicates DAPI staining intensity and the x axis anti-pan-cytokeratin staining. Boxes indicate subpopulations enriched for stromal or tumor cells, as indicated.

and the data are represented in the form of a heatmap with yellow regions indicating contiguous runs of homozygous SNPs. Genome-wide analysis showed multiple contiguous regions of chromosomal LOH across the genome that was detected specifically in the sorted neoplastic fraction but not in the sorted stromal fraction or the starting unsorted tissue (Figure 3). Analysis of specific chromosomal regions clearly showed that LOH in the chromosomal regions spanning the tumor suppressors PTEN (Figure 4, top) and CDK2NA (Figure 4, bottom) can be detected specifically in the tumor fraction but not in the starting tumor material or in the stromal fraction. Recent studies have also shown that high-density SNP arrays can be used to detect genome-wide DNA copy number changes in human cancers,²² including pancreatic ductal adenocarcinomas,²³ suggesting that these arrays have utility in identifying recurrently amplified or deleted chromosomal regions that may harbor oncogenes or tumor suppressor genes, respectively. We compared genome-wide copy number gains and losses in sorted stromal and neoplastic fractions and found that chromosomal gains and losses were substantially more apparent in DNA from tumor fractions. Genome-wide analysis showed evidence of chromosomal loss of key tumor suppressors such as CDK2NA, TP53, and SMAD4 with substantial enrichment in neoplastic compared with

Figure 3. Genome-wide LOH analysis from a single pancreatic adenocarcinoma sample. Heatmap showing regions of normal heterozygosity (red, black) or regions of LOH (yellow) as determined by SNP array analysis. Colors represent the probability that a stretch of homozygous SNPs could arise by chance as indicated in the key. Columns represent data from genomic DNA from unsorted tissue and sorted stromal and tumor (ie, neoplastic) fractions and chromosomal location is indicated to the right. The approximate locations of key tumor suppressor loci in pancreatic cancer are indicated to the right. Widespread LOH is observed specifically in the tumor fraction but not unsorted or stromal fractions.

stromal fractions (Figure 5A). In addition, deletion of a number of other chromosomal regions reported to be altered in pancreatic adenocarcinoma, such as loss of 6q and 4q and gain of 8q and 3q,² were also apparent predominantly in the neoplastic fraction (Figure 5A). Analysis of smaller chromosomal regions (Figure 5, B and C) identified elevated copy numbers of chromosomal regions harboring the microphthalmia-associated transcription factor (MITF) and cyclin E1 (CCNE1)²⁴ that were apparent in neoplastic but not stromal fractions. Because MITF amplification has not been reported in pancreatic cancer previously, we sought independent confirmation of copy number gains at the MITF locus by examination of pancreatic cell line DNA on Affymetrix SNP arrays because cell lines offer a source of neoplastic DNA free of stromal contamination. We found that a number of pancreatic cell lines showed between 2.5 and 3 copies of MITF in this analysis (see Supplemental Figure S1 at <http://jmd.amjpathol.org>). Although somewhat lower in magnitude and frequency than we and others observe in melanoma cell lines (see Supplemental Figure S1 at <http://jmd.amjpathol.org>),²⁴ this finding does support the data obtained from the sorted tumor cells that copy number alterations at the MITF locus may occur in pancreatic adenocarcinomas. Together these results suggest that the sorting protocol can reveal copy number alterations and copy neutral LOH that are not apparent in starting tumor material or surrounding stromal material.

Figure 4. Specific examples of LOH in pancreatic adenocarcinoma samples. **Top:** Chromosomal region of chromosome 10 that harbors the PTEN tumor gene (coordinates indicated to the right) in unsorted and sorted fractions from three separate tumors. Heatmap colors represent the probability that a stretch of homozygous SNPs could arise by chance as indicated in the key. **Bottom:** Region of chromosome 9 that harbors the CDK2NA locus. Tumor 2 shows LOH at the PTEN and CDK2NA loci specifically in the neoplastic fraction but not in unsorted tissue or the stromal fraction.

Because the KRAS oncogene is frequently mutated in pancreatic adenocarcinoma, we also sought to determine whether our sorting protocol allowed detection of KRAS mutations in sorted neoplastic cells but not stromal cells. KRAS exon 2 mutations were found in 8 of 16 samples screened, and were exclusively detected in neoplastic fractions (Figure 6, Table 2). Samples that were negative for KRAS mutations were clearly wild type at the locus with clear sequence reads and not scored as negative because of a failure of the sequencing reaction (data not shown). A KRAS exon 3 mutation was detected in both neoplastic and stromal fractions of one sample, although apparent enrichment of mutant DNA was observed in the tumor fraction (Figure 6, Table 2). To confirm these results with a more established method, we performed LCM to isolate both neoplastic and normal cells from four of the samples that had been analyzed using our sorting protocol (Table 2). Notably we found the same KRAS alteration in DNA from the captured neoplastic cell population but not the captured stromal cell population in three of the four samples (Table 2), suggesting reasonably good agreement between genetic analysis on material obtained by these two different methods. The failure to detect a KRAS mutation in the LCM-captured neoplastic tissue from sample HF-19000 might potentially be because of the fact that only a subsection of the sample was captured and analyzed. Overall these results suggest that the tumor sorting protocol is successfully enriching for neoplastic cells, because for the most part

Figure 5. Copy number alterations in sorted tumor and stromal fractions from pancreatic adenocarcinomas. **A:** Genome-wide patterns of chromosomal gains (red) and losses (green) in sorted stromal (S) and tumor (T) fractions from three tumor samples. The key at the bottom indicates copy number in the heatmap. Chromosomal location is indicated to the right of the heatmap. The location of several important tumor suppressors in pancreatic cancer are indicated by arrows. **B:** Analysis of the region on chromosome 3 harboring the MITF transcription factor, with focal amplification apparent in tumor fraction 1 and broader amplification of the region apparent in tumor fraction 2. Chromosomal coordinates are given to the right of the figure. **C:** Analysis of the region on chromosome 19 harboring CCNE1, with high-level amplification apparent specifically in tumor fraction 1.

mutations detected in the neoplastic fraction are not apparent from analysis of the stromal fraction.

Discussion

Tumor cell sorting enabled identification and detailed characterization of genetic changes in pancreatic adenocarcinoma tumors, some of which were difficult or impossible to detect in the original heterogeneous tumor environment. In particular, the method revealed widespread LOH in pancreatic neoplastic fractions that was not apparent in unsorted tissue or sorted stromal material. Whereas previous efforts to separate neoplastic cells from stromal contaminants have required methods such as LCM that require specialized pathology training to identify relevant cell populations,²⁵ the fluorescence-activated cell sorting protocol we describe here is robust, rapid, and provides ample material for genomic characterization.

One somewhat discordant result of our findings compared with previous studies is that we identified a relatively low percentage of KRAS mutations in the samples in our study. Whereas previous reports have identified KRAS mutation frequencies well in excess of 80% of pancreatic adenocarcinomas,^{4,5} we found mutations in only 8 of the 16 samples we analyzed (50%). There are several possible explanations for this observation. First,

as noted in the Results and Table 1, 2 of the 16 samples are not bona fide adenocarcinomas and thus might not be expected to have KRAS mutations. Excluding those samples we identified mutations in 8 of 14 or 57% of samples. This is still low compared with most estimates in the literature, but we note that a January 15, 2009 search of the Sanger COSMIC mutation database <http://www.sanger.ac.uk/genetics/CGP/cosmic/> shows a mutation rate of 71% when the search is restricted to pancreatic ductal adenocarcinoma ($n = 3404$ samples). Thus one interpretation would be that our relatively small sample size has led to sampling error and an underestimate of the true frequency of KRAS mutations. It is also possible that the failure to detect KRAS mutations in some of the samples may be because of the presence of significant amounts of contaminating nonneoplastic epithelial cells in the cyokeratin-positive fraction, because we are sorting based solely on cyokeratin expression and not aneuploidy or some other specific marker of neoplastic cells. Still, the fact that we can identify alterations specifically in the neoplastic fraction but not stromal fraction, and that we have corroborated this finding by using LCM, supports the utility of the fluorescence-activated cell sorting tumor sorting protocol in enrichment of neoplastic tissue for genetic analyses.

We also note that the tumor sorting protocol should have utility in the identification of novel targets and ge-

Figure 6. Sequence analysis of the KRAS locus in sorted pancreatic adenocarcinoma tissue. **Upper** sequence traces show analysis of a region of exon 2 in KRAS with nucleotide change predicted to cause a G12D amino acid substitution apparent in the neoplastic fraction but not the stromal fraction. **Lower** sequence traces show analysis of a region of exon 3 in KRAS with nucleotide change predicted to cause a Q61R amino acid substitution apparent in the tumor and stromal fractions but substantially enriched in the tumor fraction.

netic alterations that characterize pancreatic adenocarcinoma that have been obscured by the significant heterogeneity common to this tumor type. Cyclin E1 (CCNE1) is an oncogene that is expressed in the G₁ phase of the cell cycle and is involved in initiating DNA synthesis via activation of cyclin-dependent kinases.²⁶ Although CCNE1 has been shown to be overexpressed in a number of tumor types and in two pancreatic cell lines,^{27,28} amplification has not been reported in pancreatic tumor tissue. We have shown here that amplification of CCNE1 can be detected specifically in sorted tumor material pancreatic adenocarcinoma and suggest that additional confirmatory studies in larger data sets are warranted. Similarly, the microphthalmia-associated transcription factor (MITF) has been shown to be amplified specifically in melanoma and to constitute a lineage-specific oncogene because it can transform primary human melanocytes when transfected in conjunction with BRAF.²⁴ Our data suggest that MITF copy number gains occur in pancreatic tumors and cell lines and thus may

Table 2. KRAS Mutation Detection in Sorted Tumor Samples

Sample number	Fraction*	KRAS status, FACS†	KRAS status, LCM‡	Tissue diagnosis
HF-18411	T	G12D	G12D	Adenocarcinoma
	S	WT	WT	
HF-18996	T	G12D	G12D	Adenocarcinoma
	S	WT	WT	
HF-18998	T	G12D	G12D	Adenocarcinoma
	S	WT	WT	
HF-19000	T	G12R	WT	Adenocarcinoma
	S	WT	WT	
HF-16979	T	G12R	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-18764	T	G12R	ND	Adenocarcinoma
	S	WT	ND	
HF-16976	T	G12V	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-2035	T	Q61R	ND	Adenocarcinoma
	S	Q61R	ND	
HF-18409	T	WT	ND	Adenocarcinoma
	S	WT	ND	
HF-16359	T	WT	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-16349	T	WT	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-16345	T	WT	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-16975	T	WT	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-16980	T	WT	ND	Adenocarcinoma ductal
	S	WT	ND	
HF-17928	T	WT	ND	Carcinoma (neuroendocrine)
	S	WT	ND	
HF-17700	T	WT	ND	Inflammation
	S	WT	ND	

*CK⁺ neoplastic tumor fraction (T) or CK⁻ stromal fraction (S).

†FACS tumor sorting protocol.

‡LCM, laser capture microdissection protocol.

not be unique to the biology of melanoma disease progression.

Understanding the pattern of loss of tumor suppressor genes and activation of oncogenes in a particular patient's tumor may also have utility in understanding patient prognosis or selecting targeted therapies in the future. Indeed, previous studies have shown that overall LOH at 15 loci indicate aggressive tumor biology and correlate strongly with survival in resected pancreatic carcinomas,⁹ and the methods described here offer a facile method for obtaining material for such analyses. In addition, identification of LOH or oncogenic mutations at specific loci may be of key importance in the emerging field of personalized medicine.²⁹ For instance, loss of PTEN function in breast cancer models has recently been shown to predict preclinical response to PI3 kinase inhibitors,³⁰ whereas KRAS mutations have been shown to predict preclinical response to selective inhibitors of

MEK.³¹ Thus the ability to use heterogeneous patient tumor samples to accurately assess chromosomal alterations in PTEN or other tumor suppressor genes, or activating mutations in key oncogenes, may become crucial as novel targeted therapies are clinically validated and associated companion diagnostic tests are used to select patients for therapy. The tumor sorting protocol described herein may facilitate more accurate diagnosis and personalized therapy for patients with pancreatic cancer by providing a clearer picture of the alterations in a given tumor.

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