Translational Implications of Molecular Genetics for Early Diagnosis of Pancreatic Cancer

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Abstract This chapter discusses the potential applications of molecular genetics to the early diagnosis of pancreatic cancer. The current state of the field is discussed in general terms with an emphasis on the limitations of current technologies and strategies, and the potential of molecular genetic diagnostics to impact diagnosis and management of pancreatic cancer in the future.

Molecular Genetics of Pancreatic Cancer

The altered genetic landscape of pancreatic cancer has been characterized over the past 25 years and is discussed in detail in previous review articles and in other chapters of this book. It is increasingly accepted that there is morphological progression of premalignant lesions in the pancreas (Pancreatic Intraepithelial Neoplasia or PanIN lesions, graded as I, II, and III, with the latter representing carcinoma in situ) that results from an accumulation of genetic and epigenetic events (Maitra and Hruban 2008). Earlier studies undertaken using hypothesis driven science, genomic discovery methods, and candidate gene approaches identified four genes that are mutated or modified epigenetically in a large percentage of pancreatic cancers: *KRAS2* (>90 %), *p16/CDKN2A* (>90 %), *TP53* (50–75 %), and *SMAD4* (>50 %) (Iacobuzio-Donahue 2012). A number of other genes that are altered in less than 5 % of cancers have been implicated by other studies, including two whole genome sequencing efforts (International Cancer Genome Consortium 2010; Jones et al. 2008). With the possible exception of detecting inherited genes that predispose to cancer in families that have a history of malignancy, these low frequency alleles are not currently of use for purely early diagnostic purposes (Hruban et al. 2010).

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There are currently attempts to classify the low frequency mutations into functional categories that largely fall into biochemical pathways that contribute to malignant progression (Jones et al. 2008). This is highly important to understanding the biology of pancreatic cancer and will undoubtedly contribute the development of new targeted-therapies for the disease. As such diagnostic tests for these may contribute to future clinical decisions regarding therapy; however, direct detection of these mutations are unlikely to be useful for early detection of cancer in the near future.

Though much attention has been paid to whole genome exome (that part of the genome that encodes expressed proteins) sequencing of tumors, this strategy is limited since only 1 % of the human genome encodes expressed proteins. More recent efforts have begun to investigate the role of the remaining 99 % of the genome in cancer progression, including expression and role of multiple types of noncoding RNAs. The analysis of mutations in noncoding RNAs, DNA structural elements, and other features of nucleic acids in tumors is only beginning, and will hopefully provide additional insight and the potential for new molecular diagnostic tools in the future.

Difficulties of Early Detection of Pancreatic Cancer

Early detection for cancer has improved the survival of patients with many types of cancer and is critical for future improvements in effectively treating the disease. The rationale for this is that early detection of cancer allows for cures, usually by surgical resection. Pancreatic cancer, however, presents special challenges. Currently, pancreatic cancer is highly lethal, even when detected in early stages. The 5-year survival rate for surgically resected patients with stage 1 disease (tumors less than 2.5 cm, confined to the pancreas) is less than 30 % (Witkowski et al. 2013). One implication of this fact is that even when there is resection of small tumors with negative margins by pathological examination, some tumor cells have escaped to colonize other organ sites. This undoubtedly results in part from aggressive biological properties of pancreatic cancer. It is proposed by some that pancreatic cancer invades and metastasizes relatively early in disease progression, perhaps even before there is full transformation of the cells (Rhim et al. 2012). There is also evidence that clonal evolution of cancer occurs over a longer period of time. Mathematical modeling of the rate of mutation acquisition (as revealed by exome sequence analysis of spatially distinct and presumably progressive lesions from seven pancreatic cancer cases) suggested that there is as an 11.7-year period of time from an initiating mutation in a pancreatic cell to the acquisition of additional mutations that confer fully transformed growth properties on a cell, and that there is another 6.8 years until the first metastatic subclone is derived. In this model death occurred at about 2.7 years after of the appearance of the putative metastatic subclone (Iacobuzio-Donahue 2012). If this model is correct, it suggests that there is a window of time in which early detection may impact disease outcome.

Nonetheless, at this time, early detection of pancreatic cancer followed by surgical resection will not be curative for most patients. This has led some to suggest that early detection of pancreatic cancer will not be useful for improving survival. Many clinicians believe that surgical resection should be accompanied by neoadjuvant or adjuvant therapy; however, the currently available therapies are also generally not curative. Part of the reason for this deficiency is the conundrum presented by the fact that we cannot identify early cancers at a rate that is sufficient to undertake clinical trials of large numbers of these patients so that we can identify curative therapies. It is anticipated that early detection will provide more opportunities for clinical trials in the future. Moreover, the advent and anticipated improvement of cancer therapies that target molecular defects that arise from genetic mutations, epigenetic alterations, and other factors will provide treatment options in the future. Thus, diagnostic tests of the future should attempt to identify the presence of malignancy and characterize the molecular defects that are responsible for driving the biological properties of each malignancy.

Assays for Early Detection of Pancreatic Cancer

Several factors must be taken into account regarding the development of diagnostic tests for pancreatic cancer. These include the development of accurate and molecularly sensitive tests for appropriate clinical samples that show performance characteristics (diagnostic sensitivity and specificity) that will be helpful in making clinical decisions. The assays must also be economically feasible. A starting point for most diagnostic tests that are currently under development include assay of bloods—serum, plasma, or cellular content. A second source of diagnostic samples includes stool, or samples obtained by endoscopic sampling (pancreatic juice, fine needle aspirate, or biopsy). A third type of diagnostic test would be an imaging test, presumably that included a targeted imaging agent that improved discrimination of malignant cells from benign conditions. One problem for early detection is that of the molecular or cellular sensitivity of the test. Early small lesions are unlikely to produce a sufficient amount of material or cells to be detected when diluted into the large volume of blood that is in circulation. This is further complicated in pancreatic cancer by the fact that up to 90 % of each pancreatic tumor is comprised of a desmoplastic reaction, which does not include tumor cells that are the targets of molecular diagnostic tests.

Regarding assays for blood or other body fluids, there are efforts to develop tests to detect nucleic acids (DNA and RNA) and protein in blood, stool or other clinical samples, and there are assays to capture circulating tumor cells. There are practical positives and negatives for translational application of all of these tests. There is evidence that nucleic acids derived from tumors can be detected in blood, although the molecular forms of these are not well established. Possibilities include free nucleic acids or forms bound to proteins, exosomes, cellular debris, or material carried by immune cells such as macrophages or dendritic cells. One problem

encountered in analyzing nucleic acids in blood or other body fluids is the issue of identifying mutated sequences against a background of normal sequence. Newly developed economical technologies such as ICE-COLD PCR that selectively amplify mutated sequences (Milbury et al. 2011) may increase the sensitivity of these assays and enable practical diagnostic tests in the future.

Circulating tumor cells represent an important source of potential diagnostic material. Unfortunately, by definition, the presence of circulating tumor cells implies that the tumor is metastatic, and so it is anticipated that analysis of these cells will not aid early detection, but instead will provide a potential source of a "peripheral biopsy" that will allow for molecular characterization of the genotype and phenotype of the parental tumor (Yu et al. 2012). Whether circulating tumor cells are representative of the parental tumor and all metastatic deposits remains to be determined.

An important component of diagnosis of early stage pancreatic cancer is the analysis of fine needle aspirate (FNA) samples of pancreatic lesions obtained by endoscopic ultrasound. Unfortunately, cytopathological analysis of these samples is often difficult and results in indeterminate findings (Payne et al. 2009). The addition of molecular genetic analysis to these samples should enhance the accuracy of diagnosis, and in the future may aid in directing therapeutic approaches; however, this area of diagnostic endeavor requires further development.

The imaging of pancreatic lesions has improved with the development of pancreas specific CT protocols; however, the detection of small lesions in the pancreas remains a problem that could be improved by the addition of imaging techniques that identify alterations associated with malignancy (Fisher et al. 2008). Efforts to develop agents that target molecules expressed as a consequence of malignant transformation are underway, but are nascent at this point in time (Bausch et al. 2011). Ultimately, early diagnosis of pancreatic cancer will need to include improved imaging techniques.

Molecular Discrimination of Disease by Molecular Genetics

If an acceptable assay is developed that will accurately and sensitively detect mutated, methylated or expressed nucleic acid sequences, it is likely there will be problems with the performance characteristics of these tests with respect to sensitivity and specificity for detecting pancreatic cancer. Consider the genes known to be commonly affected in pancreatic cancer. There are mutations in *KRAS2* in virtually all pancreatic cancers, but similar mutations have been observed in patients with pancreatitis and in normal individuals (Lohr et al. 2000, 2005). In fact, 50 % of the relatively common PanIN 1 lesions are predicted to contain *KRAS2* mutations (Feldmann et al. 2007). This suggests that detection of *KRAS2* mutations alone does not predict the presence of cancer and would lead to numerous false positives. Thus, it would be desirable to add another test to detection of mutations in *KRAS2*. *p16/CDKN2A* is inactivated in almost all pancreatic cancers, and is apparent in PanIn 2

lesions. As such *p16/CDKN2A* would be a good candidate as a marker, except that in 40 % of cases there is homozygous deletion of the allele. Another 40 % have a mutant allele that is accompanied by deletion or methylation of the second allele and 10–15 % show methylation of the promoter. A similar scenario is evident for *SMAD4*, which is lost by homozygous deletion in 30 % of pancreatic cancers and is mutated and inactivated by loss or methylation in 25 % of cases (Iacobuzio-Donahue et al. 2012). Detection of loss of alleles is feasible if not optimal for tissue biopsies, but assaying for loss of alleles is not practical as a test for circulating DNA derived from cancer. Inactivation of *TP53* may be more promising as a test, as mutant alleles can be found in up to 75 % of pancreatic cancers and this is usually accompanied by loss of the second allele directly or through methylation. Mutations in *TP53* are found in PanIN 3 lesions, which is an appropriate diagnostic target. Thus, one early detection strategy that should be evaluated carefully would be to detect the presence of mutations in both *KRAS2* and *TP53* in blood products (plasma or cells). One limitation of this approach that has prevented a comprehensive analysis to date is the fact that there are numerous sites of mutation in TP53, which complicates the development of economical detection assays for many clinical samples.

Besides detecting mutated genes directly, it should be possible to develop diagnostic tests to detect consequences of mutated genotypes. This is being evaluated by detecting mutated proteins directly, or by detecting alterations in expression of cellular products (or biochemical pathways) whose levels or features (e.g., posttranslational processing) are altered as a consequence of the mutated genome. There are a number of efforts to develop biomarkers based on this approach; however, none have achieved performance characteristics that warrant deployment as an early diagnostic test to date.

Another possibility for early detection that is currently being explored is analysis of the specificity of autoantibodies that are produced in cancer patients (Raedle et al. 1996). The rationale is that many individuals develop autoantibodies to mutated proteins, overexpressed proteins, or altered forms of proteins that arise during malignant progression of cancer. An advantage of this strategy, at least in theory, is that it may allow for detection of alterations that occur in very early lesions. For example, a small focus of malignant cells may not make a sufficient level of any compound to be detected in blood or body fluids. However, the development of antibodies to proteins in those cells would be amplified and detectable as stable compounds in serum.

An increasing clinical dilemma with respect to diagnosis of pancreatic cancer is the increased incidental findings of pancreatic cystic lesions that result from increased use of imaging techniques (CT and MRI). It is often difficult to discriminate malignant precursors (intraductal papillary mucinous neoplasms, mucinous cystadenomas) from other benign cystic lesions. Molecular diagnostic strategies that could evaluate FNA samples or other tissue samples from these lesions or blood based assays of that would discriminate those lesions with aggressive biological properties would impact disease management. Initial exome sequencing studies of several cystic lesions has begun to reveal candidate mutations that should be further explored (Wu et al. 2011).

Summary and Future Directions

Molecular genetic studies have provided a great deal of insight into the biochemical underpinnings of pancreatic cancer; however, this characterization is far from over, as only a fraction of the genome has been analyzed and the results obtained so far have not yet translated into improved diagnostics or therapeutics. The early detection of pancreatic cancer remains possible if the premalignant lesions are present for several years; however, the small size and relatively inaccessible location of these lesions present a significant barrier to many diagnostic modalities. It would be desirable to develop economical screening assays that detect the presence of mutations and could be deployed annually in at risk or aging populations. An example of this would be tests that detect mutated genes, their products, or autoantibodies to mutated gene products or products that are altered or uniquely expressed as consequences of the acquisition of mutations. It is unlikely that a defined set of mutations will be solely diagnostic for pancreatic cancer, but these tests may indicate the presence of premalignant or malignant cells in certain organ types. It should be possible to develop secondary screening protocols (such as cancer-specific imaging) to detect the locations of the neoplasms. Prohibitive costs associated with endoscopic and imaging studies will preclude their use as early diagnostic tools, though these modalities should be used and enhanced as secondary screening protocols. Molecular diagnostic tests should be developed to aid in detecting malignancies in the small quantities of clinical samples obtained by FNA or biopsy. Ultimately, molecular characterization of tumors should be used to direct appropriate targeted therapies at the time of diagnosis, or to direct prevention strategies aimed at blocking the progression of premalignant lesions.

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