Pyrosequencing Offers Simple, Rapid Detection of Mutations in K-ras Oncogene

HIV-1 Genotyping for Drug Resistance

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ON THE COVER
Comparative Genomic Hybridization Array (ACGH) demonstrating genomic gains (green) and losses (red).
**Pyrosequencing Offers Simple, Rapid Detection Of Mutations in K-ras Oncogene**

*By Manjula K. Gupta, Ph.D., and Rose Arciaga, B.S., M.T.*

K-ras (K-ras) protooncogene belongs to a highly conserved family of GTPases involved in signal transduction process. It codes for a 21 kDa protein. Binding of growth factors to receptor tyrosine kinases activates ras protein, initiating downstream cascade, leading to the phosphorylation of key transcription factors.

Oncogenic forms of K-ras are constitutively activated proteins that derive from point mutation of codon 12, 13 or 61. Such activating somatic mutations of K-ras exhibit considerable tumor specificity and are frequently found in a number of human cancers, including nearly 90 percent of pancreatic, 50 percent of colon carcinomas and 30 percent of lung adenocarcinomas, although rarely in breast cancer.

K-ras mutation occurs in early stages of tumor progression, and several studies have suggested that the analysis of these K-ras mutations has both diagnostic and prognostic value. K-ras mutations occur exclusively in codon 12, 13 and 61 (>90 percent in codon 12 and 13). Therefore, K-ras status has been suggested as a candidate test for screening high-risk populations.

A number of techniques have been described for the detection of K-ras mutations. These include gel electrophoresis-based techniques such as temporal temperature gradient electrophoresis, denaturing gradient gel electrophoresis, constant denaturant capillary electrophoresis, or restriction endonuclease-mediated selective polymerase chain reaction (PCR), or by direct sequencing of the PCR product. These techniques are laborious and time-consuming, thus limiting their utility in most clinical laboratories. A simple and rapid assay allowing simultaneous analysis of multiple samples is desirable for routine clinical use.

The Cleveland Clinic Division of Pathology and Laboratory Medicine has developed a simple and rapid non-gel-based assay using pyrosequencing, a sequence-by-synthesis technology, allowing high-throughput screening for both codons 12 and 13 K-ras mutations. For assay, primer pair was designed to amplify a 119 bp segment in exon 1. The reverse primer was biotinylated for easy isolation of PCR product with use of solid-phase sepharose coated with strepavidin. For analysis of point mutation by pyrosequencing, a sequence primer was designed to hybridize at one base to the 5’-side of the codon 12. The 96-well plate format allows analysis of K-ras mutations in large sample sets, providing a simple and cost-effective method for large-scale screening.

The assay has been validated with use of five cell lines carrying known homozygous or heterozygous mutations at codon 12 and 13. The pyrosequencing readouts (programs) for mutation analysis detected all of these various genotypes and gave a specific pattern for allelic combination (homozygous or heterozygous). Figure 1A shows the representative pyrograms generated for the CCL 243 showing wild type sequence at both codon 12 and 13 (GGT; GGC), CCI 185 and Calu-1 showing homozygous (AGT) and heterozygous (TGT/GGC) mutations at codon 12, respectively. CCL247 had heterozygous mutation at codon 13 (GAC/GGC).

At present, in collaboration with Matthew Walsh, M.D., of the Cleveland Clinic’s Department of General Surgery, the Division of Pathology and Laboratory Medicine is using this method to determine the malignant potential of cystic pancreatic tumors before surgery.

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Detecting the presence of drug resistance-associated mutations in HIV-infected patients treated with antiretroviral drugs

By Belinda Yen-Lieberman, Ph.D.

Development of viral resistance to antiretroviral drugs used for treatment of human immunodeficiency virus type-1 (HIV-1) infection is an important cause of treatment failure and limits options for alternative antiretroviral regimens (1). The primary goal of HIV-1 drug-resistance testing is to determine which drugs should not be used when treating HIV-1 infected patients. Therefore, the HIV-1 genotyping test has the potential to become the central diagnostic element in the treatment and long-term care of patients at risk of therapeutic failures.

Background

Viral genotyping is defined as the nucleotide sequence of a virus from which the amino acid sequences of a protein can be inferred. The ability to analyze viral nucleic acid sequences allows us to distinguish viral genes in their natural setting from those that are genetic variants or mutants due to drug pressure. Thus, DNA sequencing is the gold standard for identifying critical changes in the nucleotides that can lead to a dysfunctional protein associated with a particular disease or condition (2). For example, a change in a single base in a viral protease gene can lead to amino acid substitutions that can disrupt the function of the viral enzyme, preventing binding to a drug and leading to drug-resistant virus in the patient.

Sequencing methods also are the most accurate way to type and subtype viruses. In the cases of hepatitis C virus and HIV-1, genotyping has become a necessity for patient management.

In the clinical application of genotyping, the nucleotide sequence of interest must be compared to a reference wild-type viral sequence. Any change from the wild-type sequence is usually reported as a change in amino acid at a specific residue (codon) of the nucleotides. For example, in HIV-1 genotyping, a change in the nucleotide sequence from ATG to GTG at codon 184 would be reported as a change from methionine to valine at residue 184, or M184V (3). The presence of M184V usually confers resistance to several antiretroviral drugs including lamivudine (Epivir, GlaxoSmithKline) and abacavir (Ziagen, GlaxoSmithKline).

Clinical Significance

Currently, three classes of FDA-approved antiretroviral drugs, plus a fourth class that is in clinical trials, are used in clinical care: nucleoside reverse transcriptase inhibitors, non-nucleosides reverse transcriptase inhibitors, protease inhibitors, and entry-inhibitors (experimental). Viral resistance can occur with each of these drug classes, particularly when viral replication is not maximally suppressed during therapy. Resistance is more likely to occur with monotherapy than with combination therapy, which is the reason combination multi-drug therapy has become the standard of care in managing HIV-1-infected individuals.

Treatment of HIV-infected individuals with highly active antiretroviral drugs has resulted in multiple drug-resistant forms of virus (4). Drug mutations that arise during treatment have significantly affected patient management and the long-term effectiveness of antiretroviral therapy (5), and testing for these types of mutations has become an important factor in developing treatment strategies for individual AIDS patients (6). HIV genotype testing is playing an increasing role in improving clinical outcomes for HIV-infected patients by identifying these mutations.

An International AIDS Society-USA panel of experts has established clinical guidelines for the use of HIV-1 resistance testing in adults (6). The panel suggests that clinicians should consider resistance testing prior to initiating therapy on patients with primary HIV infection or in treatment-naive persons with established HIV infection. The guidelines also recommend resistance testing (either genotypic or phenotypic) for patients who fail either an initial antiretroviral regimen or numerous regimens. A recent study reported that genotypic resistance testing to determine the next regimen following treatment failure is cost-effective (7). Finally, the guidelines recommend resistance testing in pregnant women to optimize treatment and maximize the chances of preventing vertical transmission of HIV-1.

Methodology

DNA sequencing generally requires a commitment of time, training and resources beyond what is required by most other clinical laboratory methods currently Continued on page 4
The last decade has brought a virtual explosion of microarray technologies in the medical sciences and biotechnology industry. The aim of this new technical development has centered on providing a platform for simultaneous determination of the genetic profiles of hundreds to tens of thousands of genes in a single experiment. The rapid and global adoption of this technology can be attributed to its unprecedented high-throughput nature, which allows it to generate large quantities of complex data in a previously unimagined short period of time. The information provided by these approaches remarkably facilitates defining the key molecular components of cellular pathways regulating cancer cell proliferation, and many of the identified changes will become clinically useful as diagnostic and prognostic markers or therapeutic targets.

Microarrays have been exploited most extensively for gene expression studies, but other applications recently have been developed, including the use of arrays for the detection of gains and losses of genomic DNA. As understanding of the sequence, structure and function of the human genome increases, fluctuations in DNA sequence copy number with concomitant microscopic or cryptic chromosomal aberrations are becoming increasingly correlated with phenotypic abnormalities (1). Gene amplification and deletion are common alterations occurring in cancer cells, and, like other structural changes, are associated with genomic instability and contribute to the process of carcinogenesis. The development of most human neoplasms follows a defined series of histopathological stages, a process that involves multiple genetic changes such as translocations, deletions, duplications and

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available. However, the use of polymerase chain reaction (PCR) amplification in conjunction with DNA sequencing and the availability of automated sequencing systems have greatly facilitated this testing in clinical laboratories.

To conduct HIV genotyping, the patient’s HIV-1 RNA is isolated, and the protease gene and reverse transcriptase gene are amplified. The amplified products are analyzed using the automated dideoxynucleotide terminator cycle sequencing method. The TRUGENE HIV Genotyping Assay (Visible Genetics, Toronto, Ontario, Canada) utilizes bi-directional sequencing (CLIP) of the entire protease gene and up to codon 244 of the RT gene of the virus. The CLIP technology uses two fluorescent-labeled primers for each of the four sequencing reactions. Samples then are analyzed on the Open Gene sequencing system (Visible Genetics).

Sophisticated software is used to analyze sequence data, including sequence alignment and editing, to identify mutations and to generate reports with clinical interpretations. ♦

References


alterations in chromosomal copy number changes. Importantly, the DNA amplifications or losses frequently involve oncogenes and tumor suppression genes that play an important role in the cell cycle control, and their alteration affects cancer growth.

It is now possible to screen tumors for genomic changes, as an alternative to the examination of the transcriptional activity of the tumor cells. Analysis of the genome represents a different level of molecular profiling as compared to the examination of mRNA levels, and each of these approaches has its advantages. Although the mRNA expression profiles provide more complete information about the actual gene activity in the studied cells, many of the detected changes may represent “innocent bystanders” or transient fluctuations unrelated to the neoplastic process itself. In contrast, the genomic changes are less variable and are less likely to be subject to transient changes in the tissue environment (2). This reduced complexity greatly simplifies the identification of causal genetic changes in individual tumors.

A second advantage is that the dynamic range of the genomic changes is more manageable (the locus-to-locus variation of DNA is small, including either allele loss or locus amplification), while messenger RNA levels present in cells typically vary over several orders of magnitude.

The knowledge of genetic changes common to specific tumor types may facilitate the development of markers for early detection, diagnosis and monitoring during clinical intervention. Furthermore, the identification of these genomic alterations may help to define the causal molecular changes leading to more targeted therapeutic approaches to disease. The best illustration of the power of this approach is the identification of the Her-2/neu gene amplification in breast cancer, which has not only provided a prognostic criterion but also led to the development of targeted therapy (3).

Classical cytogenetics has been used for decades to karyotype cells, but it is limited due to the need to culture the cells to produce metaphase chromosomes, and it requires expertise in interpreting the chromosomal spreads. In addition, cytogenetics provides a relatively crude analysis of the chromosome number and has limited sensitivity in identifying deletions and amplifications.

The introduction of comparative genomic hybridization to metaphase chromosomes (M-CGH) revolutionized clinical cytogenetics by permitting a genome-wide analysis of cancer specimens with chromosomal aberrations that were either too many or too complex to be fully characterized by routine cytogenetics (4).

Moreover, since CGH requires only genomic DNA from the sample, it permits the analysis of specimens from which chromosomal preparations are impossible to obtain due to poor cell growth. In this technique, the genomic DNA from the tumor is labeled with a fluorescent dye in one color while a normal reference DNA sample is labeled in a different color. These differentially labeled samples are then co-hybridized to normal metaphase chromosome spreads. Chromosomal imbalances across the genome in the tumor DNA are then quantified and positionally defined by analyzing the ratio of fluorescence of the two different colors along the target metaphase chromosomes (5).

However, the resolution of CGH applied to the metaphase spreads is limited by cytogenetic resolution of approximately 5 megabases (Mb), and considerable cytogenetics expertise is required to accomplish such analysis. Therefore, M-CGH never has become a widely utilized technique, and its use remains limited to specialist research applications. The inherently low resolution associated with metaphase chromosomes banding, along with the labor-intensive nature of this procedure, make M-CGH largely incapable of accomplishing genome-wide screens for chromosomal aberrations that are less than 5 Mb in size. This limitation makes this approach unsuitable for a high-throughput study of human neoplasms.

Recently, the advent of bacterial artificial chromosome (BAC) array technology has again drawn attention to the applicability of CGH to the study of genomic alterations in human disease. BACs are large-insert DNA clones that have been cytogenetically and physically mapped to the human genome. Currently, more than 8,800 of such clones are known, with at least one clone on average per Mb available on each chromosome.

This resource affords the opportunity to generate an ordered array of DNA segments at a very high genomic resolution and to replace the metaphase spreads as the hybridization template. Such array-based CGH (A-CGH) circumvents the considerable limitations associated with the use of chromosome spreads.

A high-resolution fluorescent scanner is utilized to capture the fluorescent intensity of each spot in these arrays and converts them into intensity ratios. The fluorescence ratio of the two colors can be compared between different spots representing different genomic regions (Fig. 1). This provides a genome-wide molecular profile of the sample with respect to the regions of the genome that are deleted or amplified.

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The resolution level of this approach is dependent on a combination of the number, size and map positions of the DNA elements within the array (6). BAC arrays provide an opportunity to perform high resolution genomic scans (currently at 1 Mb) in a rapid, highly reproducible fashion.

Researchers in the Cleveland Clinic Division of Pathology and Laboratory Medicine have begun exploring the suitability of the A-CGH approach with commercially available human genome arrays containing probes to most known human oncogenes and tumor suppressor genes, along with other structurally important regions on all human chromosomes (Vysis Inc., Downer’s Grove, Ill.). To date, a number of clinical entities, as well as cell lines, with a broad spectrum of genetic aberrations has been studied. The results demonstrate the potential of microarray-based genome profiling as an effective tool for identification of new diagnostic and prognostic markers.

Despite the original assumptions that the primary use of CGH would be in the field of solid tumors, as many of these neoplasms remain intractable to conventional cytogenetic analysis, CGH has already contributed significantly to the analysis of hematological malignancies. The important novel discoveries in this area included the identification of previously unrecognized gene amplifications in lymphoproliferative disorders such as chronic lymphocytic leukemia or diffuse large B-cell lymphoma, as well as the discovery of secondary genetic changes associated with progression of several hematologic neoplasms (7).

In many instances, CGH provided further evidence confirming the rationale for combining molecular genetic data with morphologic and immunophenotypic criteria in the classification of hematolymphoid neoplasms. For example, CGH demonstrated a high prevalence of specific genomic gains in a primary mediastinal B-cell lymphoma (PMBCL), which are extremely rare in any other type of lymphoma. The abundance of genomic data available in the literature for this entity prompted the selection of PMBCL as the subject of the initial studies in the Division using the newly available array-based CGH. It has been our experience that, when compared with the wealth of the metaphase CGH data in the literature, the unique genetic features of this lymphoma can be detected even more frequently using the array-based modification of this technique. Our results compare favorably to those generated by highly sensitive assays such as fluorescence in situ hybridization (FISH) (8).

In the solid tumor field the focus in the Division is currently on gastrointestinal neoplasia, including colorectal, pancreatic, and esophageal carcinomas. Preliminary results from the study of uniformly staged and treated patients with microsatellite-stable colonic adenocarcinoma show that important differences exist at the genomic level between patients with excellent 10-year prognoses in contrast to those who develop tumor recurrence or metastasis within five years after surgery. Besides the generally higher extent of chromosomal instability in the tumors associated with the worst prognosis, genomic changes of several important oncogenes appear to correlate with the diverse clinical outcomes of these patients (unpublished data).

Other areas of study at The Cleveland Clinic in the field of gastrointestinal pathology currently include the identification of the most common DNA alterations in esophageal and pancreatic adenocarcinomas, as well as those occurring in patients with a long-standing inflammatory bowel disease. These findings subsequently will be utilized for the development of novel FISH-based diagnostic and prognostic assays.

With the advent of microarray-based CGH technology, analyzing the genomic changes in human neoplasms at an unprecedented level of resolution is now possible. In addition to the oncogene/tumor suppressor gene-oriented platform, which is currently utilized in the Division, substantially larger whole-genome arrays will soon become available from commercial sources. In addition, the recently described CGH to cDNA microarrays provide a promising platform for the study of both genomic and gene expression profiles in the same sample. This will have a profound impact on studying how changes in the genome can affect gene expression and phenotype, and ultimately will provide a rationale for the development of novel molecular diagnostic assays aimed at clinically important diagnostic, prognostic and therapeutic targets.

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References
Barrett’s esophagus (BE) is a complication of chronic gastro-esophageal reflux disease, defined as a change in the esophageal epithelium of any length that can be recognized at endoscopy and is confirmed to have intestinal metaplasia by biopsy. The importance of accurately diagnosing BE is related to its association with the subsequent development of esophageal adenocarcinoma, the frequency of which has rapidly increased over the past several decades for reasons that are not entirely clear. In fact, the incidence of adenocarcinoma of the esophagus has increased at a rate greater than that for any other cancer in Caucasian men in the United States. This is a lethal disease with a five-year survival of only 11 percent.

Current practice guidelines recommend endoscopic surveillance of patients with BE in an attempt to detect cancer at an early and potentially curable stage. However, endoscopic surveillance of patients with BE has numerous shortcomings. Dysplasia and early adenocarcinoma are endoscopically indistinguishable from intestinal metaplasia without dysplasia, and even the most thorough biopsy surveillance program has the potential for sampling error. Furthermore, there is considerable interobserver variability in the histologic interpretation of dysplasia.

Current surveillance programs are labor-intensive, time-consuming and very expensive. Despite the alarming increase in the incidence of esophageal adenocarcinoma, the vast majority of patients with BE will never develop cancer. Thus, it is necessary to make surveillance techniques more effective so that only those patients who are truly at risk for progression to dysplasia and adenocarcinoma undergo intensive surveillance. This goal can be accomplished by either sampling larger areas of the affected mucosa or developing risk stratification tools that allow concentration of effort on individuals at greatest risk for developing adenocarcinoma.

Brush cytology may be complementary to endoscopic biopsies in surveillance programs and has a number of theoretical advantages compared with routine endoscopic biopsy. This technique is much simpler and costs considerably less than traditional endoscopy with biopsy; it also has the ability to sample a much greater area of the involved esophageal epithelium. Although cytology has probable specificity for the detection of high-grade dysplasia and adenocarcinoma in this setting, the specificity for the detection of low-grade dysplasia is low.

A number of clinical and biologic markers for the determination of which patients are at increased risk for the development of adenocarcinoma have been evaluated. However, most of these techniques are labor-intensive and have not yet achieved widespread acceptance.

Fluorescence in situ hybridization (FISH) is a rapid technique that allows for the determination of the numeric changes of entire chromosomes, as well as gains/losses of specific genetic regions in conjunction with cellular morphology. Additionally, it can be applied to both fresh and archival cytology and histology specimens, making it a versatile tool. Studies in bladder cancer suggest that FISH analysis of cytology specimens is more sensitive than cytology alone in detecting biopsy-proven urothelial neoplasia, and abnormalities detectible by FISH may precede evidence of urothelial carcinoma on biopsy. Thus, our group has analyzed the presence of specific genetic alterations in cytologic specimens.
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from BE-associated, high-grade dysplasia/early adenocarcinoma by FISH and compared these changes to genetic alterations in patients with BE without dysplasia.

Archival brush cytology slides from 40 patients with BE were studied. Twenty-one of these patients had biopsy-proven, high-grade dysplasia/early adenocarcinoma, and 19 of these patients had BE without dysplasia, with a minimum of five years of clinical follow-up. The cells were studied by four-color FISH using chromosomal enumeration probes (CEPs) 6, 7, 11 and 12, and locus-specific probes 9p21 (p16 gene) and 17p13.1 (p53 gene), along with their corresponding chromosome enumeration probes.

Using this technique, we found that FISH detected genetic changes in routinely processed brush cytology specimens with high-grade dysplasia/early adenocarcinoma that are not seen in patients with BE without dysplasia. Specifically, 17p13.1 (p53) loss and/or aneusomy of chromosomes 6, 7, 11 and 12 were common in BE patients with high-grade dysplasia/early adenocarcinoma and absent in those without dysplasia. In contrast, 9p21 (p16) loss was not found to be useful since it occurs in both groups of patients.

Based upon these data, this panel of biomarkers merits further evaluation to determine its potential for detection of the studied genetic changes in BE prior to the development of high-grade dysplasia/early adenocarcinoma.

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