Chronic myeloproliferative disorders (CMPDs), are clonal hematological malignancies characterized by abnormal proliferation of myeloid lineages.

One of these disorders, chronic myelogenous leukemia (CML), is defined by the presence of a BCR-ABL1 fusion, but recurring diagnostic molecular genetic abnormalities for the other typical CMPDs (chronic idiopathic myelofibrosis, essential thrombocytopenia, and polycythemia vera) have not been identified. Therefore, diagnosis of these malignancies historically has relied on a constellation of clinical and pathologic features, as well as exclusion of other specific genetic abnormalities such as BCR-ABL1 fusion.

Recent studies have shown that a significant number of patients diagnosed with non-CML CMPDs have an acquired mutation in JAK2 (JAK2 V617F; nucleotide G>T). Furthermore, this mutation has been shown to augment the survival and proliferation of cells expressing JAK2 V617F and to promote cytokine hypersensitivity, suggesting that it can play an important role in the pathogenesis and manifestations of non-CML CMPDs.

Due to the diagnostic significance of the JAK2 V617F mutation, the development of molecular assays to identify it in patients with CMPDs is attracting considerable attention.

Commercially available assays use polymerase chain reaction (PCR) to amplify target DNA, followed by differential enzyme digestion of the PCR product to identify mutant and wild-type. These assays are labor-intensive and time-consuming, however, and a rapid and reliable assay is required for routine clinical use.

We have developed such an assay employing fluorescence probes and melting curve analysis. Carried out in a closed-tube system, it does not require post-PCR processing and can be scaled to high- (LightTyper®, Roche Applied Sciences, Indianapolis, Ind.) or low- (LightCycler®, Roche) throughput format. The assay is applicable in both peripheral blood granulocytes and formalin-fixed, paraffin-embedded bone marrow clot sections and can be used as an ancillary diagnostic test.

Experimental Approach

Genomic DNA from 161 patients were isolated for genotyping, which included formalin-fixed bone marrow clot sections (115) and blood granulocytes (46). Patients were broadly classified into two groups: those with non-CML CMPDs (54 patients) and those with either CML or without CMPD (107 patients). Genotypes of these patients were established either by allele-specific PCR (110) or by direct sequencing (51) of DNA prior to genotyping by melting curve analysis of fluorescent probes. Human erythroleukemia cell line (HEL) DNA, a known homozygous mutant, was used as a positive control.

In a typical melting curve analysis, a fluorescent probe (~ 20 bases long) pair binds to an amplified target DNA sequence in solution to form a duplex. One probe contains an excitation dye (fluorescein) and the other a receptor dye (LCR640). This receptor dye fluoresces when close to the excitation dye due to fluorescence resonance energy transfer (FRET). Upon heating, the probe separates from the duplex at its melting temperature (Tm), resulting in the loss of fluorescent signal. This change can be captured as a melting curve and converted into a melting peak. If the probe pair sequences and the target DNA sequence are perfectly complementary, the Tm will be relatively high. For a mismatched (variant/ mutant) duplex, the Tm will be relatively low. This discrimination forms the basis for assigning genotypes that differ by a single base pair at a certain locus. The melting temperature (Tm) of each sample is an indicator of its classification as wild-type, homozygote mutant, or heterozygote.

Development of a Clinical Assay for JAK2 V617F Genotyping in Chronic Myeloproliferative Disorders

By Gurunathan Murugesan, Ph.D., and Eric D. Hsi, M.D.
Assay Development

Fluorescent probes and the PCR primers were designed in-house using the software provided with the LightTyper. An amplicon of 177 base-pair length was generated using a pair of PCR primers, and melting curve analysis was performed using FRET hybridization probes in a 384-well PCR plate for the LightTyper assay (high-throughput) and in capillaries for the LightCycler assay (low-throughput; routine clinical use).

Three DNAs of known genotype determined earlier by sequencing, one each for wild-type (GG), heterozygote (GT), and homozygote mutant (TT; HEL cell DNA), were used to develop the assay (Figure 1). The sensor probe was designed to match with the wild-type sequence (G) in the target DNA; therefore, it resulted in a higher Tm (~61°C). A mismatched mutant sequence (T) in the target DNA provided less stability to the wild-type-matched sensor, which melted away at a lower Tm (~53°C). As expected, a heterozygote sample yielded a melt profile corresponding with wild-type (G, ~61°C) and mutant (T, ~53°C). Melt peaks were highly reproducible and consistently distinguished each genotype.

Validation

A total of 161 DNAs, 114 wild types and 47 mutants, previously genotyped for the JAK2 V617F mutation by allele-specific PCR (110) or by sequencing (51) in the laboratory of Dr. Jarek Maciewski were used for validation. Representative melting peaks corresponding to wild type, heterozygote and homozygote mutant are shown.

Figure 2. JAK2 V617F genotype validation of 161 patients was performed by melting analysis using the LightTyper. Representative melting peaks corresponding to wild type, heterozygote and homozygote mutant are shown.

Figure 1. Genotyping of JAK2 V617F with the LightTyper. One DNA representing each genotype was used to optimize PCR conditions and melting curve analysis. Melting curves (upper panel) and derivative melting peaks (lower panel) characteristic of wild type (GG), heterozygote (GT) and homozygote mutant (TT) are shown.

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LightCycler Assay

The LightTyper assay, detailed above, has been adapted for the real-time PCR platform LightCycler because of its widespread use in clinical laboratories. DNA from three samples (wild-type, heterozygote, and homozygote mutant) were subjected to real-time PCR and melting analysis (Figure 3). Melting peaks generated with the LightCycler were similar to those obtained with the LightTyper (Figure 1), displaying a distinct Tm for the wild type (~ 60°C) and the mutant (~ 52°C). Heterozygote DNA had two peaks, one each corresponding to the wild type (~ 60°C) and the mutant (~ 52°C). For validation, more than 30 patient DNA samples representing three different genotypes previously genotyped by AS-PCR, sequencing and the LightTyper were analyzed in two separate experiments with consistent reproducibility and 100% concordance and an analytical sensitivity of approximately 5 to 10% for the mutant DNA.

Clinical Significance of JAK2 V617F Mutation

The exact role that JAK2 V617F mutation plays in the pathogenesis of myeloid disease is poorly understood. It clearly is not specific for any single, currently recognized disease, although it appears limited to myeloid disease. The JAK2 V617F likely is involved in the pathogenesis of myeloid diseases, but it may not by itself be the causative genetic defect in the disorders in which it is found. Regardless of these uncertainties, detection of this mutation will be useful diagnostically. A positive result will definitively exclude a reactive process. Because the mutation is not specific for any one disease, interpretation of the results will require knowledge of the clinicopathologic setting. From the therapeutic standpoint, demonstration of this mutation might, in the near future, be required for enrollment into trials of small molecule tyrosine kinase inhibitors as they become available.

References


Figure 3. Genotyping of JAK2 V617F with the LightCycler. One DNA representing each genotype was used to optimize PCR and melting conditions for the LightCycler. Melting peaks characteristic of wild type (GG), heterozygote (GT) and homozygote mutant (TT) are shown. Melting temperatures for the wild type (~60°C) and mutant (~52°C) are similar with this LightCycler assay compared to the LightTyper assay shown in Figure 1.
The human genome is made up of many discrete and functional units of DNA, the genes, and each gene has two parental copies or alleles. Certain genes are highly polymorphic, which means that among the human population many different allelic variations exist. Polymorphic genes are extremely useful when performing identity testing, as two individuals are likely to be different at any given polymorphic gene. If differences are detected, the tested samples derive from different people.

This assay uses PCR amplification of short, repetitive DNA sequences, short tandem repeats (STRs), that are selected because they are highly polymorphic. Each STR locus has two alleles, and each allele has a specific length that is stably inherited from each parent. After PCR amplification, the resultant products are separated by high-resolution capillary gel electrophoresis that reveals the size of each allele in a given tissue sample (Figure 1).

For each STR examined, the length of the known “control” DNA derived from either archival tissue or a current blood sample known to come from the patient is compared to that of the unknown identity or “test” specimen. Using a total of 16 highly polymorphic loci in the DNA identity test applied at Cleveland Clinic, two specimens deriving from unrelated individuals have a 99.9998% likelihood that they will differ in one or more loci. A difference in even one locus indicates non-identity to 100% certainty.

DNA Fingerprinting Analysis for Specimen Identification

By Mary P. Bronner, M.D.

Background
DNA fingerprinting analysis by polymerase chain reaction (PCR) is a highly accurate technique for determining patient identity for a tissue sample. This same test is used in forensic pathology for criminal identification, but it is equally useful in surgical and autopsy pathology when the patient identity of tissue samples is in question.
Clinical Indications

Assessment of tissue identity by DNA fingerprinting is useful for confirming possible tissue contaminants identified on histologic slides and for determining specimen identity in the case of suspected mislabeling or unlabeled samples. A common example of such testing regards the finding of a cancer fragment on a patient’s slide when no cancer is suspected clinically. A specific example would be a fragment of cancer that appears on an otherwise normal 12 year-old child’s benign appendectomy specimen. Initially, the pathologist determines whether the cancer fragment is on only one profile of the multiple slices of tissue on the slide, in which case it is easily dismissed as a so-called “floater” or fragment left behind in an improperly cleaned water bath used to prepare slides. In the event that the cancer fragment is on all of the different slices from a given tissue block, DNA fingerprinting can be used to determine whether it belongs to this hypothetical 12 year-old patient undergoing appendectomy.

If the DNA from the cancer fragment differs from the benign appendix tissue on the slide, the cancer fragment is an inadvertent contaminant. The certainty involved in knowing that a given patient does not have cancer is of obvious and great importance.

Sources of Tissue Contaminants

Considering the numerous medical professionals involved in processing a tissue sample from the patient to the pathologist reviewing it microscopically, it is truly a marvel that so few errors occur. Consider that from the patient, a biopsy sample or surgical resection specimen is initially handled by the treating physician using carefully cleaned biopsy and surgical tools that have been used previously to obtain many other patients’ samples. One or more nurses or other assistants assist the physician in getting the tissue sample into a properly labeled specimen container. The specimen container is batched with many other containers and transported to the pathology laboratory. There, the specimen is accessioned into the pathology computer system and is assigned a unique surgical pathology identifier.

Next, the paperwork and specimen container are processed by a pathology assistant, resident, or pathologist who removes the specimen from the labeled container and examines and describes the tissue grossly. The tissue is dissected by carefully cleaned instruments that also are used to dissect many other patients’ samples. The specimen is divided among a number of tissue cassettes, small plastic containers, which are labeled individually with the surgical pathology number and a unique block number. The tissue cassettes have holes in them to permit flow of the various processing fluids required to process the tissue chemically into a final wax tissue block. Many hundreds of different patients’ cassettes are placed into a common chemical bath for this processing stage. Rarely, a tissue fragment from one patient can exit its cassette and enter through the cassette holes of another patient’s block to become part of this second patient’s block.

A histotechnologist uses clean forceps, which are cleaned and used subsequently on many other patients’ specimens, to pick up the wax-infused tissue fragments from the processing cassettes and place them into the final wax tissue block that will be used to section the actual histologic slides. Another histotechnologist sections 5 µm slices on a razor blade affixed to a microtome. These thin wax slices are floated onto a carefully cleaned water bath, which has had many other patients’ wax slices previously floating in it.

Floating allows slices to flatten and be transferred onto a glass slide, which has been hand-labeled by the histotechnologist. The slide later receives a permanent computer-generated slide label that is affixed to the slide by another technician. Finally, all of the

Normal liver needle biopsy (upper inset at higher magnification to the left of the figure) with an unexpected fragment of adenocarcinoma (lower inset at higher magnification to the left of the figure). DNA fingerprinting was used to prove that the adenocarcinoma does not belong to the patient, based on the differing genetic alleles at all 16 of 16 tested loci in this example.
slides on any given patient’s procedure are assembled with the accompanying paperwork and delivered to the pathologist for diagnostic interpretation.

A mistake leading to inadvertent tissue contamination can occur at any one of the above logistically complex processes. DNA fingerprinting now can solve the great majority of the questions arising from mistakes in this intricate process.

Results Reporting

The results of DNA fingerprinting are reported as the number of non-identical alleles between the known and unknown samples. An interpretation as to the identity of the unknown specimen is provided if the suspected source tissue is also included.

Specimen Requirements

Requirements for DNA fingerprinting on known and unknown specimens vary and are detailed here.

Known specimen: The formalin-fixed paraffin block containing representative tissue or 5 to 10 unstained 5 µm formalin-fixed, paraffin-embedded tissue sections of a “known” tissue sample certain to derive from the patient are required. When sections are submitted in lieu of a block, they should be placed onto charged, unbaked glass slides. A corresponding H&E slide should be marked by the submitting pathologist to indicate the tissue of interest for testing.

To reduce the risk of contamination in the event that microdissection is required, a separate, known-identity block from the block with the unknown tissue is requested. Alternatively, overnight shipment of 4 mL of EDTA anti-coagulated whole blood (at room/ambient temperature and not refrigerated) may also be submitted for analysis.

Unknown specimen: Either the formalin-fixed paraffin block with the tissue of questionable identity is required or 5-10 charged slides with unstained 5 µm unbaked sections containing the tissue of unknown identity. A corresponding H&E also is required, marked by the submitting pathologist to indicate the tissue for identity testing. If deeper slide levels have exhausted the tissue of interest from the block and the only sample remaining for testing is tissue on the original stained slides, removal of the coverslip to use the stained tissues for DNA testing can be attempted. This has been accomplished successfully on many occasions. Analysis can be attempted from any fragment size, but success rates are lower for samples smaller than 2 mm in size.

Methodology

DNA is extracted from a sample of known identity and from the unknown sample. The suspected source of a possible contaminant may comprise a third specimen. Microdissection is performed as needed, in which a pathologist microscopically assesses the tissues on the slides and marks the slides with a permanent marker under microscopic guidance. A technologist uses the markings to dissect the indicated tissues, also under microscopic guidance. PCR amplification of 16 highly polymorphic short tandem repeat loci \(\text{[D3S1358, THO1, D21S11, PentaE, D5S818, D13S317, D7S820, D16S539, CSF1PO, PentaD, vWA, D8S1179, TPOX, D18S51, FG, and a sex chromosome specific locus amelogenin]}\) is performed (1). Fluorescently labeled PCR products are detected, analyzed, and quantified by capillary gel electrophoresis. Positive and negative external controls are included with every assay.

References


![Figure 1. Capillary gel electropherogram of DNA fingerprinting results, comparing known patient DNA (top graph), to DNA from a suspected cancer contaminant (bottom graph). This result displays six tested genetic loci, which are listed along the top of the figure (D5S818, etc.). The numbers in boxes listed along the base of each fluorescent signal peak indicates the base-pair size of each amplicon. Note that differently sized alleles are present for all six genetic loci tested, genetically confirming tissue non-identity. For example, for the D5S818 locus, the patient DNA (top graph of patient area A) has two alleles that are 11 and 13 base-pairs in size each, in comparison to the confirmed tissue contaminant (lower graph of suspect area B) that has different fragment sizes of 9 and 11 base-pairs each.](image-url)
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