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TITLE: Targeted Mutation Analysis

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Slide 1:

Hello, my name is Helio Costa. I am an Instructor at Stanford University School of Medicine in the Department of Pathology and an Assistant Director for the Molecular Genetic Pathology Laboratory at Stanford Health Care. Welcome to this Pearl of Laboratory Medicine on "Targeted Mutation Analysis."

Slide 2:

This pearl will focus on the common experimental methodologies used to perform targeted nucleic acid analysis for Mendelian germline disorders and for acquired somatic malignancies. The scope of this presentation will focus on four main topics of targeted nucleic acid testing. First, we will review the rationale and indications for mutation characterization in a clinical setting. Then we will discuss the types of genetic disorders amenable for nucleic acid analysis. Next, we will address input specimen types and points for consideration when analyzing nucleic acids from a diversity of specimen types. Lastly, the bulk of this pearl will focus on the specific mutation testing modalities and their underlying mechanisms.

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Targeted mutation analysis in a clinical setting is performed for a broad range of clinical indications. Some of the reasons genetic tests are performed include confirming a suspected diagnosis (diagnostic testing), determining the likelihood of developing a particular disease (predisposition testing), detecting the presence of carrier mutations in an asymptomatic

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individual (carrier testing), predicting response to therapy (predictive testing), forecasting the course of a disease (prognostic testing), and detecting mutations in a fetus (prenatal testing) or embryo (preimplantation testing).

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Targeted mutation analysis is generally performed for both inherited disorders and acquired somatic malignancies. Inherited disorder testing is commonly referred to as ‘germline’ testing because mutations often are transmitted to offspring via the gametes. Inherited disorders include cystic fibrosis, autism, Fragile-X, congenital hearing loss, achondroplasia, familial cancer syndromes, and many others. Acquired somatic disorders occur in non-gamete tissue and thus are not inherited from parent to child. Common somatic disorders include hematological and solid tumor cancers.

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The input specimen types for targeted mutation testing differ between different disorders. For inherited disorders, DNA extracted from peripheral blood, saliva, amniocytes, and skin are commonly used. For acquired disorders, Formalin-Fixed Paraffin-Embedded tissue (FFPE) and fresh frozen tissue are commonly used for solid tumor malignancies. However, other specimen types such as cell-free DNA (cfDNA), circulating tumor DNA (ctDNA), bone marrow, fluids, and peripheral blood are also used.

Sample collection can also introduce potential issues worth mentioning. When collecting peripheral blood for mutation analysis it is important to use EDTA or ACD collection tubes as other types of anticoagulants inhibit common enzymes used in molecular testing assays. Additionally, when genotyping tissue for somatic disorders it is crucial to note and take into consideration the tumor percentage and heterogeneity of the sample as tumor specimens will contain some amount of normal cells. Lastly, the FFPE fixation process used to preserve tumor tissue causes nucleic acid degradation and artifacts. This will often result in highly fragmented and low molecular weight DNA.

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Four classes of genetic alterations can be detected when performing targeted mutation analysis. Each of these variant types differ on the ideal testing methodology. Single nucleotide variants (SNVs), and small insertion and deletion events (INDELs) are detectable by Polymerase Chain Reaction (PCR) methods, next-generation sequencing (NGS) or Sanger sequencing. For large INDELs spanning hundreds or thousands of base pairs, multiplex ligation-dependent probe amplification (MLPA), Southern blot hybridization, nucleic acid microarrays, and NGS are used. Lastly, large structural variation events like chromosomal translocations and inversions can be detected by MLPA, reverse-transcription PCR, microarrays, and NGS.

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The remainder of this presentation will provide an overview of common testing methods. The described assays include PCR and PCR variants, southern blot hybridization, microarray technology, traditional Sanger sequencing, and next-generation sequencing.

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PCR is the backbone of molecular mutation analysis. This process is crucial for amplifying nucleic acid of interest. The required reagents include the nucleic acid template obtained as DNA or reverse-transcribed cDNA from the specimen, oligonucleotide primers flanking the region of interest, dNTPs used for DNA synthesis, and the Taq DNA polymerase enzyme used to synthesize DNA amplicons. The process begins by denaturation of the template DNA which breaks the hydrogen bonds between the base pairs of double-stranded DNA and produces single-stranded DNA. Primers specific to the region of interest then anneal to the single-stranded DNA. The primer extension occurs with the Taq polymerase incorporating dNTPs and produces a final elongated double-stranded DNA product. This process repeats 25 to 35 times to produce adequate copies of genetic material for downstream analysis.

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Allele-Specific PCR is a variant of PCR utilizing mutation-specific primers. A common primer flanks one side of the amplicon, and two primers—one with homology to the wildtype nucleic acid, and another primer with homology to the mutant allele—will flank the other side of the amplicon. Only primer pairs that anneal with perfect complementation will produce amplicons. The 3' mismatch of the incorrect primer-template pair will prevent DNA polymerase extension.

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Reverse transcription PCR is used to convert RNA into complementary DNA (cDNA). The process uses a viral reverse transcriptase to catalyze the conversion. There are three common primer approaches that will produce different RNA populations. Gene specific priming uses primers to target a specific RNA transcript and preferentially amplifies the target transcript. Oligo-dT priming uses poly-T primers that bind to the poly-A tail of mRNA molecules. This process will only enrich transcribed mRNA molecules. Random hexamer priming is used to amplify total RNA which includes mRNA molecules and regulatory non-coding RNA molecules.

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Real-time PCR is also known as quantitative PCR (qPCR). qPCR is used to detect and quantify nucleic acid levels by measuring fluorescence emitted from a reporter molecule in real time using special thermocyclers. The detection occurs during the amplification of the PCR product each cycle, and allows monitoring of the PCR reaction during early and exponential phases where the first major increase in PCR product correlates to the initial amount of target template. Two common methods exist for this qPCR approach: non-specific dyes that intercalate with DNA such as SYBR green and sequence-specific fluorescently-labelled nucleic acid probes such as the TaqMan assay.

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Melting curve analysis utilizes the principle that single-stranded DNA strands with perfect sequence similarity will anneal to form double-stranded DNA and denature at a higher temperature than those that contain single base pair mismatches caused by single nucleotide variants. Typically, a DNA locus is amplified by PCR, and DNA probes with complementarity to different nucleotide sequences will anneal to and denature from the DNA template molecule at different rates as the temperature is increased.

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Methylation-sensitive PCR is used to detect the presence or absence of methylated DNA in a sample. DNA methylation occurs when cytosine residues contain an additional methyl group. When treated with sodium bisulfite, unmethylated cytocines will be converted to uracil, while

methylated cytocines will remain unchanged. The presence or absence of DNA methylation can then be detected by performing a downstream PCR reaction using primers containing uracils in lieu of cytosine.

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Multiplex Ligation-Dependent Probe Amplification (MLPA) is used to detect large insertions, deletions, and structural variants. MLPA is a variant of PCR that utilizes adjacent oligonucleotide probes that are hybridized to regions of interest in the template DNA. After hybridization, the probes are ligated and PCR amplified using fluorescently labelled universal primers. Regions that contain only one probe are not amplified and no signal is detected.

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Southern blot hybridization is used when a DNA sequence variant is too large to be detected by conventional PCR. This method requires a very large amount of high molecular weight DNA as the starting input. The DNA is initially digested with restriction endonucleases, and the digested fragments are run on an agarose gel. The DNA gel is placed into an alkaline solution to denature the double-stranded DNA, which is then transferred to a membrane. The denatured single-stranded DNA is then bound to the membrane by a heat or UV source. The membrane is subsequently hybridized with sequence specific probes, washed, and exposed to film for imaging.

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DNA microarrays consist of known sequence specific probes covalently bonded to a microarray chip. The input sample nucleic acid is first fluorescently labelled and then hybridized to the chip. Probes that bind to sample nucleic acid emit fluorescence and effectively genotype the nucleic acid for that sequence. One of the most common clinical assays using microarrays is ‘array-Based Comparative Genomic Hybridization’ (aCGH). This method is used to detect copy number differences in a sample of interest. A copy-number neutral reference and a patient sample are fluorescently labelled with two different dyes and hybridized to the chip. Regions

that are a 1:1 ratio of the two colors denote copy-neutral regions. Regions that diverge from the 1:1 ratio suggest deletions or duplications in the tested sample.

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Sanger sequencing is a method of determining nucleic acid sequence composition. A PCR reaction with unlabelled ddNTPs and fluorescently-labelled ddNTPs terminators produces a range of premature and fully amplified PCR fragments. These fragments are run on capillary gel electrophoresis to size-separate the smaller fragments from the larger fragments. The smallest PCR fragments cross a florescence detecting laser first and correspond to the earliest nucleic acid sequences. This information is then converted to a chromatogram that lists the sequence composition of the template. While effective, this method has low-throughput and is costly for large-scale genome analysis.

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Next-generation sequencing provides a high-throughput method to efficiently and cost-effectively sequence a large amount of nucleic acid. There are several prevailing sequencing platforms. However, the most common of these methods is Illumina sequencing by synthesis which produces short (~100-250 base pair) reads. In brief, sample DNA is fragmented to a specified length, prepared into sequencing libraries and randomly hybridized onto a sequencing flow cell. Each unique DNA library fragment undergoes bridge-amplification and results in millions of unique DNA clusters containing identical DNA copies adjacent to the original molecule. Each clonal cluster is then sequenced with a laser to detect the incorporation of fluorescent dNTPs corresponding to the underlying nucleic acid sequence in real time. Post-sequencing analysis includes short read sequence mapping to the human reference genome, quantification of sequence read depth for every nucleic acid position, and variant calling.

Slide 19: References

Slide 20: Disclosures

I have no disclosures.

Slide 21: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on “Targeted Mutation Analysis.”