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TITLE: Molecular Testing of Solid Tumors

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Hello, my name is **Deepika Sirohi**. I am the **Molecular Genetic Pathology Fellow at University of California, San Francisco**. Welcome to this Pearl of Laboratory Medicine on "**Molecular Testing of Solid Tumors**."

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The past few years have seen significant advancement in the understanding of the molecular basis for many solid tumors. Testing methodologies are becoming more advanced, and more solid tumors are being submitted for genetic analysis. Molecular testing is designed as an adjunct to supplement other testing and can be supportive in establishing a diagnosis in difficult cases, provide information about prognostic and predictive markers and guide therapeutic decisions.

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Like all laboratory tests, pre-analytical variables can significantly impact assay results and therefore, solid tumor testing should begin with correct sample selection. Formalin fixed paraffin embedded (FFPE) tissue is the most frequently available sample type, though it's major disadvantage is that it degrades DNA into fragments that are 500 or fewer base pairs in length and can create in-vitro mutations due to artefactual DNA cross-links. Specimen types other than FFPE include cytology specimens such as cell blocks or even stained smears. Stained smears need to be de-stained for retreival of diagnostic material. Fresh snap-frozen material is an excellent specimen source, but it requires prior knowledge that the specimen will require molecular testing.

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If any molecular testing is being contemplated for a specimen, there are certain fixatives that should be avoided. Such fixatives include decalcifying agents like strong acids that irreversibly damage DNA and heavy metals like mercuric chloride that inhibit DNA amplification.

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The next step in specimen selection is to select an adequate sample significantly enriched for tumor cells. Based on assay sensitivity, the preferred tumor cellularity is 30-40%. Two methods commonly used for tumor enrichment are macro and micro dissection. A block with highest tumor volume is selected. Caution is warranted to avoid including mixed inflammatory cells that can dilute out tumor cells. Crushed, cauterized, and necrotic areas should be avoided because these may be difficult to evaluate reliably and may not have viable cells for analysis.

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In macro-dissection, the area of interest is marked. The image on left shows a tumor admixed with inflammatory cells. An area is marked that would provide the highest tumor yield and minimize inflammatory cells to assure maximal enrichment for tumor. In the image on the right, the tumor is selected avoiding the adjacent normal tissue. The area of interest can then be marked on unstained sections and scraped to obtain sample material for the assay. In micro-dissection, laser-capture techniques are used; however, this is mostly done in research settings.

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In the examples here, the image on the left shows tumor adjacent to an area of necrosis that should not be included in sampling as it can dilute the tumor cells and it is difficult to ascertain the nature of cells in necrotic areas. The image on the right depicts cauterized tumor tissue, that similarly makes morphologic evaluation of the cells difficult.

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Other sample considerations include tumor heterogeneity that is observed in many tumors. The tumor samples should be carefully evaluated by a histopathologist to appropriately select tissue. There may be morphogically different areas, and a decision will have to be made if one or both of those areas should be considered for testing. In the example here, a single tumor shows two morphologically distinct areas. Depending on the test, one may consider analysing them separately or selecting a sample that is equally representative of both morphologies.

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If a patient has both a primary and a metastatic tumor, the preferred specimen would be the metastatic tumor if it is obtainable/available. Because normal tissue often provides a comparative reference, it is also preferable in some assays to perform molecular analysis on both the tumor and the normal tissue. Having selected a specimen, the next step is to select an appropriate assay to answer the clinical question for the given case. The choice of assay is guided by the clinically expected underlying molecular alteration.

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Clinically significant molecular alterations in solid tumors usually can be broadly classified into the following categories: Single base change, Insertions, deletions, duplications, inversions, translocations, copy number changes, methylation, and oncogenic viruses. The assays, therefore, should be designed and optimized to enable detection of these alterations.

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During the remaining portion of this discussion, we will address some commonly employed tests in solid tumor molecular testing such as Fluorescence in situ hybridization (FISH), sanger (dideoxy) sequencing, next generation sequencing, and methylation assays. Other assays are available that can be employed depending on the clinical requirements and the alterations expected to be detected.

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Flourescence in-situ hybridization (FISH) tests use fluorescently tagged DNA or RNA probes to identify regions of interest. These can include gene amplifications, deletions, or structural rearrangements. The advantages of FISH testing is that it can be performed on tissue sections to accurately correlate with morphology and ensure the cells of interest are being targeted for analysis. It is a relatively rapid technique and in emergent cases, results can be available within 1-2 days. The limitation of the assay is that only a limited number of probes can be used in any given test. Some examples where FISH testing is employed include ERBB2 amplification in breast carcinoma and ALK rearrangement in non small cell lung carcinoma.

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FISH test usually employ two fluorescent probes of different colors that hybridize to 2 different regions on a chromosome, one for the reference and one for the region of interest. Fusion of the 2 probes generates a third color. Break apart probes are commonly employed to detect

structural rearrangements. The two probes are designed such that they map proximal and distal to the breakpoints in the gene of interest. When the probes are fused, it indicates a normal gene; if the probes are apart, it indicates a break in the gene involved in translocation, irrespective of the fusion partner. Fusion probes are used to identify structural rearrangements with predictable fusion partners. The two probes are designed for the 2 regions of interest that are fused when there is a fusion between the two genes.

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Probes can also be designed to detect amplifications or deletions. In these cases, two probes of different colors are used, one for a normal control that is usually directed to the centromeric region and the other for the gene of interest. Signal differences between the two probes are compared to identify deletions or amplifications as illustrated on these slide images. The image on the left illustrates an assay for 1p deletion that is common in gliomas. Most cells in this illustration show 2 green reference probes and only one red probe that is directed to 1p locus, implying a deletion. In the image on the right, amplification of EGFR is illustrated with numerous yellow signals is seen in each cell.

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An alternative modification to FISH testing is the Chromogenic in situ hybridization or CISH. In this assay, the probes are colorimetric instead of fluorescent. This difference obviates the requirement of fluorescent microscopy, and the probes do not fade over time.

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Sequencing methods are used for identification of single nucleotide variants as well as for small and medium insertions and deletions. Sanger sequencing remains the gold standard for variant detection and has an analytical sensitivity of 20%. Hence, to detect a heterozygous mutation, a minimum tumor cellularity of 40% would be required. A starting DNA quantity of 10ng is usually required. This chromatograph shows a heterozygous substitution of Adenine for Guanine at codon 172 of the IDH1 gene. Single genes commonly sequenced include EGFR, BRAF and c-KIT

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In recent years, next generation sequencing (NGS) has become available and enables evaluation of multiple genes simultaneously. There are different platforms with diverse sequencing biochemistry but conceptually similar workflows. They provide a cost-effective means of detecting single base changes; Insertions, deletions, duplications, inversions,

translocations; as well as copy number changes. Compared to Sanger sequencing, NGS assays can detect variants with an allele frequency as low as 5%, but requires approximately 100ng of DNA. Additionally, the read lengths are much shorter, about 100 to 150 bp in length.

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This image shows alignment of sequences to reference genome for normal and tumor tissue sequenced using NGS technology. The tumor shows an adenine to guanine change that is not seen in the normal tissue.

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This image shows an example of a fusion involving TMPRSS2 and ERG genes in a case of prostatic adenocarcinoma. Breakpoints are identified in the intronic region of the two genes that pair with the reciprocal gene when aligned to the reference in the tumor in the bottom panel while being absent in the normal tissue.

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This image illustrates that target reads from the sequencing that can be used to identify copy number changes across the genome compared to a pool of normal samples. The data in this image depicts the log 2 ratio in the top panel and the allele frequency in the lower panel. This allows for identification of copy number gains and losses as well as focal amplifications and deletions.

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Another important test for solid tumors is gene methylation assays. The 5' promoter regions of most genes contain CpG dinucleotides that are prone to spontaneous methylation of cytosine to methylcytosine that results in silencing of the gene. Methylation of certain tumor suppressor genes is a frequent mechanism of gene inactivation in many cancers.

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Methylation of CpG residues can be detected by treatment with sodium metabisulfite that converts unmethylated cytosine to uracil, leaving methylated cytosine intact. Cytosine and uracil can then be distinguished by different methods such as sequencing, methylation specific PCR, or restriction endonuclease digestion with methylation-sensitive enzymes. MLH1 hypermethylation and MGMT promoter methylation are 2 common methylation tests for solid tumors.

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To summarize, in recent years, molecular testing of solid tumors has made significant progress that has improved our understanding tumor molecular biology. Correct sample selection and careful evaluation by a histopathologist is crucial to enable accurate results. One important point that I would like to leave you with is that assays need to be selected and designed based on the molecular alterations in consideration.

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Slide 25: Disclosures

I have no disclosures to make or conflicts of interest.

Slide 26: Thank You from www.TraineeCouncil.org

Thank you for joining me on this Pearl of Laboratory Medicine on "**Molecular Testing of Solid Tumors**."