Molecular Techniques and Methods Applied in Cytology

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Introduction

The recent increase in the discovery and clinical implementation of biomarkers has led to the use of a large number of diagnostic molecular assays for identification of individuals eligible for target therapies. Mutations in genes such as *EGFR*, *K-RAS*, and *BRAF* have been investigated in a large number of solid tumors, with the successful use of targeted therapies as alternatives or primary treatment for a great number of patients. Cytological samples stand as an important alternative to tissue specimens, especially in impediment clinical scenarios.

The molecular assays employed for the detection of genomic alterations depend on the alteration to be detected and the type of preparation or specimen used. PCR based methodologies are the most common used for the detection of abnormalities at gene level, such as point mutations. Abnormalities in chromosomal

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structure and number are commonly identified by conventional cytogenetics and/or fluorescence in situ hybridization (FISH). Main challenges for the application of molecular technologies in cytology comprise selecting the appropriate test for that type of specimen and validating a methodology specifically for cytology, even if the method has already been used for histological samples, with the use of negative and positive controls and whenever possible, correlation of cytological and histological results.

In the present chapter, the most common molecular tests currently used in routine clinical practice applied to cytology, the array of cytological specimens that can be potentially used for molecular analysis, and the main issues related to biorepository for cytological materials are discussed.

Polymerase Chain Reaction Based Assays

Polymerase chain reaction (PCR) based assays are the methods of choice for procurement of mutations in several neoplasms. They have well-established protocols and are relatively cheap assays and widely available.

The traditional PCR assay involves DNA denaturation, annealing, and extension, with the use of primers (sequence of nucleotides complementary to the target DNA). A forward and reverse primer flank the designated area containing the desired sequence of DNA to be amplified, and multiple copies (amplicons) of the target DNA (known sequence to verify the presence of mutations) are generated. Variation on the optimal temperatures for annealing, number of cycles and reagents involved vary depending on the primers involved. The amplified DNA can then be sequenced for the verification of any mutations. Direct sequencing is a simple technique that uses chemically modified nucleotides labeled with distinct fluorescent dyes, and the order of incorporation of these fluorescent nucleotides into the amplified DNA will appear as a sequence electropherogram. In case a mutation is present, two different overlapping peaks will be seen (from the wild-type and mutant cells). Similarly, a deletion will be seen as a “truncation” of the peak signals.

The main advantage of classic PCR followed by direct sequencing is that it is an exploratory technique per se, meaning it can not only find mutations known to be commonly present in that specific type of tumor, but also any additional mutation that might be present. It is a robust and popular technique, reproducible in laboratories worldwide. Its main limitation when used in cytological samples is its low sensitivity, usually requiring a high percentage of tumor cells.

Various alternatives to classic PCR followed by direct sequencing have been used for cytological samples, and include, among others, real time-PCR, high-resolution melting analysis (HRMA), restriction fragment length analysis, COLD (co-amplification at lower denaturation temperature)-PCR, scorpion amplification refractory mutation system (S/ARMS), and peptide nucleic acid-locked PCR (PNA-LCA PCR). Real time PCR uses oligonucleotide primers that bind specifically to flank regions of the most common mutations. It is a very sensitive technique and allows a quantification of the percentage of mutated cells, since as the reactions occur a curve shows the number of amplified DNA regions in that sample. HRMA is a rapid and cost-effective method that relies on the combination of real time PCR and evaluation of DNA melting curves to accurately detect mutations, comparing the patterns of obtained curves to preset curves from non-mutated sample. Restriction fragment length analysis, on the other hand, uses mutation-specific restriction endonucleases, and amplification is only possible in the mutated sites. Although more sensitive than conventional PCR followed by direct sequencing, these allele-specific assays only analyze mutations already known and any additional mutations possibly present are not detected. Therefore, they are mostly recommended for small or paucicellular samples where sensitivity is compromised and in clinical instances where finding of additional mutations will not change therapy or prognosis.

More recently high-throughput techniques capable of detecting multiple mutations at the same time have been employed and include multiplex mutation analysis. It consists of multiple primer sets targeting multiple genes at once, saving time and material. It is of especial interest in cytology, since limited specimens may harbor a small number of tumor cells and specific panels of genes

that optimize the use of nucleic acids can increase the usefulness of such specimens. MASS-Array spectrometry using multiplex mutation analysis with preset commercial panels, such as the Oncocarta Panel, have been successfully used in FNA specimens, with reliable results. Customized panels can also be created for analysis of multiple mutations of interest and more commercially available panels specifically for each group of tumors have been recently released. When DNA extracted from fresh cells suspensions retrieved by FNA and stored on FTA cards were used for multiplex mutation analysis, rate of satisfactory results was similar to frozen samples and higher than FFPE tissue, showing that nucleic acids obtained from cytological samples were suitable for this technology.

PCR based assays are the methods of choice for procurement of mutations in *EGFR* in lung cancer, *BRAF* in melanomas and papillary thyroid carcinomas and *KRAS* in colon cancer, for example. All sorts of cytological samples, including fresh cell suspensions, smears (stained and unstained), cytospins and FFPE cell blocks can be used to extract DNA for PCR based assays. Recent CAP and IASLC molecular testing guidelines have recommended that laboratories may use any validated *EGFR* testing method. The minimal number or percentage of tumor cells present in the sample should be established at the discretion of each laboratory at the time of validation, with strong encouragement to use more sensitive tests, with accuracy of detecting mutations in samples with as little as 10 % of tumor cells. Several studies have reported successful results in finding *EGFR* mutations in cytology samples with as little as 1 % of tumor cells, showing that the overall quality of the material is more important than the percentage of tumor cells in the sample, and that very special attention should be paid to the preanalytical steps of cytology specimens collection and handling.

Fluorescence In Situ Hybridization (FISH)

FISH (fluorescence in situ hybridization) is a popular molecular technique used in routine diagnostic specimens, using fluorochrome-labeled sequences of nucleic acids (called probes), that

hybridize to a complementary sequence in the tested sample, identifying or quantifying the target. It was first described using cytological samples, and the most common types of probe clinically used are chromosome enumeration probes (CEP) and locus-specific identifier (LSI).

CEP probes are employed for copy number enumeration of a given chromosome. LSI probes are applied to detect unique sequences of genes and determine amplifications, deletions, and translocations. Other probes such as break-apart and fusion probes are also used for the detection of translocations.

FISH is a technique that involves the following steps: pre-hybridization, hybridization, pos-hybridization washes, nuclear DNA counterstaining anti-fade, slide examination under fluorescence microscope, and slide storage in the dark. Pre-hybridization is critical and its main objective is to prepare the cells for probe penetration and efficient hybridization. Diverse adjustments in the protocols depend on the type of sample used and will vary on different types of cytological preparations.

The limitations of the technique include its high cost due to the use of specialized equipment (fluorescence microscope) and the limited number of alterations that can be detected at the same time. Alternative techniques such as chromogenic in situ hybridization (CISH) and silver-enhanced in situ hybridization (SISH) use, instead of fluorescent probes, chromogenic and sliver probes and have the advantage of using bright-field microscopes.

Cytological samples are particularly superior to FFPE cell block sections or FFPE tissue sections for in situ hybridization. Different from using tissue specimens, the problem of nuclear truncation, which can lead to inaccuracy in signal visualization and counting, is avoided since the probe hybridizes directly to intact cells on a smear or cytospin. Air-dried unstained and Romanowsky stained smears and then destained are suitable for FISH analysis. Results for the analysis of HER-2 amplification by FISH using breast carcinoma fine needle aspirates have shown to be comparable and sometimes better than using tissue sections. The analysis of HER-2 status for introduction of therapy with trastuzumab in metastatic samples, usually sampled by FNA, have made this application even more popular. FISH has also been

successfully used in sarcomas and hematologic malignancies sampled by FNA, with demonstration of its characteristic chromosomal translocations which allows subtyping of those neoplasms. The use of FISH in cytological samples has also increased recently due to the description of the *ALK* rearrangement in lung adenocarcinomas, with good results on the use of Crizotinib for patients harboring this specific translocation.

Issues Related to Biorepository of Cytological Materials

Most biomarkers studies and original papers describing molecular methodology have been validated with the use of formalin-fixed, paraffin-embedded (FFPE) tissue blocks. For that matter, it's just natural that most of the primary studies involving the use of cytological samples for molecular analysis have relied on FFPE cell blocks. Cell blocks do have this clear advantage of being analogous to paraffin tissue blocks, with minimal need of standardization and prompt reliable results. However, since it usually involves fixation with formaldehyde and paraffin embedding, DNA from the cells tend to be more degraded and multiplex PCR analysis or reactions involving primers resulting in larger amplicons may be hampered.

An extensive array of cytological samples has been successfully used for molecular analysis even including samples classically restricted for diagnosis, such as archival stained smears. High-quality DNA could be obtained from smears more than a decade old, good enough for use in high-throughput techniques such as array-comparative genome hybridization, methylation assays, and genotyping, techniques that require high-quality intact DNA, usually obtained only from fresh cell suspensions submitted to cryopreservation. Classic PCR followed by direct sequencing can be routinely performed in DNA extracted from archived smears, with very good results in the analysis of *BRAF* mutation in melanomas and papillary thyroid carcinomas and *EGFR* mutations in lung carcinomas.

Stained smears have a clear advantage over other specimens for their availability and possibility of retrospective studies. Unstained cytospins and liquid based preparations have also been successfully used. For using archived specimens rapid coverslip removal is an important step. The "freezer method" can significantly speed up the time spent with coverslip removal from old slides.

The use of fresh cells obtained from FNA and body fluids warrants a good preservation of nucleic acids and cryopreservation of cytological samples warrants morphology and nucleic acid integrity. More important than the quantity of cells, however, is the quality of the DNA preserved, and one crucial step for ensuring quality of the stored material is proper pre-analytical handling of the specimens.

A novel option for storage of fresh cell suspensions lies on the use of DNA-preserving paper cards, such as the FTA cards. DNA is trapped in the cards and can be preserved for years, at room temperature, being an inexpensive and practical alternative to cryopreservation. Residual material from the needle rinse of fine needle aspirates stored on FTA cards yielded sufficient quantities of DNA for successful Mass ARRAY spectrometry and the quality of DNA harvested from the cards was better than to nucleic material obtained from FFPE cell blocks and similar to the quality of DNA from frozen samples. Furthermore, the cards are easy to store and transport, being an alternative source for biobanking in remote locations.

For any type of sample used, the pre-analytical control has to be accurately performed, guaranteeing the overall quality of the sample. Accurate and consistent results using all these sorts of samples for molecular analysis will rely on standardized protocols for maximizing DNA yield.

High throughput and multiplex technologies including MassArray spectrometry and next generation sequencing have been applied to cytological specimens for mutational profiling simultaneously several genes on different types of tumors. The use of these technologies is rapidly increasing and promises to address the issue of testing multiple genes in minimal volume of tissue/cells.

Suggested Reading

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