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■ REVIEW ■

Molecular Biologic Techniques in Cytopathologic Diagnosis

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Molecular diagnosis is an application of the knowledge on molecular mechanisms of disease to diagnosis, therapeutic decision-making, and prognostication. Basically any molecular diagnostic technique could be used in molecular diagnostic cytopathology. Currently applicable molecular techniques in the cytopathology field include PCR based molecular techniques (SSCP, DHPLC, RFLP, LOH, MSI, RT-PCR, QRT-PCR, allele-specific PCR, sequencing, and methylation analysis), FISH, cDNA microarray, aCGH, and reverse-phase protein microarray, etc. Exfoliative cytology as well as fine needle aspiration cytology specimen can be used for analysis. In order to obtain a successful result, collection of target cells without contamination of the blood cells, inflammatory cells including histiocytes, and stromal cells, and a good DNA yield are most important. Molecular diagosis finds its full meaning when interpreted by those who can combine the clinical background of the disease with morphological, immunocytochemical, and molecular diagnostic results. Therefore, these assays would fulfill their full potential when interpreted by the cytopathologists.

Key Words : Molecular biologic technique; Cytopathologic diagnosis

Molecular diagnosis is an application of the knowledge of the molecular mechanisms of disease and molecular biological techniques to establish an unequivocal diagnosis, provide extra information on the prognosis or therapy of diseases, and provide genetic information on the inherited nature of diseases. While molecular diagnosis has been traditionally linked to the diagnosis of inherited diseases, it is increasingly obvious that many of these tests are applied to solve diagnostic dilemmas and use clinical samples directly generated by the histopathologists or cytopathologists.[1]

Cytopathology is a widely-used diagnostic method that is relatively inexpensive, reliable, and less invasive compared to other approaches. Although the potential of molecular diagnosis applied to cytopathology specimens was predicted years ago,[2,3] this technique has been used relatively little in routine molecular diagnostic practice.[4] Exfoliative cytology as well as fine needle aspiration cytology specimens can be used for analysis. Basically any molecular diagnostic technique can be used in molecular diagnostic cytopathology. However, some are established and others are still emerging, and comprise four main diagnostic areas: infectious diseases, inherited diseases, solid tumors, and hematologic neoplasms.

Currently applicable molecular techniques in cytopathology field are numerous and include PCR-based molecular techniques, single strand conformational polymorphism (SSCP), denaturing high performance liquid chromatography (DHPLC), restriction fragment length polymorphism (RFLP), loss of heterozygosity (LOH), microsatellite instability (MSI), reverse transcriptase-PCR (RT-PCR), quantitative real time-PCR (QRT-PCR), allele-specific PCR, sequencing, methylation analysis, and others: FISH, complimentary DNA (cDNA) microarray, array comparative genomic hybridization (aCGH), and reverse-phase protein microarray. Among these, the best known and most widely used molecular test in solid tumors is the determination of human epidermal growth factor receptor (HER-2/neu) gene amplification in breast cancer.[2]

In this review, the principles and applications of basic molecular techniques to cytopathology and the basic technical tips are discussed. A present major challenge is the molecular cytology diagnosis of solid tumors using a smear slide, and the main target is mutation analysis. Most of the methods presented in this review are currently used in our molecular pathology laboratory.

MAIN TEXT

Specimens

Any cytology specimen can be used for the mutation analysis. Those used most commonly are sputum, bronchial wash samples, bronchial brush samples, pleural fluid, pericardial fluid, peritoneal fluid, cervical smear, and fine needle aspiration (FNA) specimens.

DNA extraction

Using a pen, the areas of the FNA slide containing the target cells of interest are marked by the cytopathologist. A diamond-tipped pencil is then used to mark the underside of the slide. After removing the cover slip, target cells are scraped with a 26-gauge needle. Briefly, 20-50 μL of DNA extraction buffer solution (50 mM Tris buffer, pH 8.3; 1 mM EDTA, pH 8.0; 5% Tween-20, and 100 μg/mL proteinase K) with 10% resin is added to the scraped cells. After incubation at 56℃ for at least 1 h, each tubes is heated to 100℃ for 20 min followed by centrifugation at 12,000 rpm for 10 min at 4℃ to pellet the debris. The recovered supernatant is used for the PCR reaction.

Detection of mutations

DHPLC, mutant allele-specific PCR, PCR-RFLP, and DNA sequencing analysis are the most frequently used techniques for the mutation analysis.

Denaturing high performance liquid chromatography (DHPLC)

This technique discriminates between wild-type and variant alleles by their distinctive chromatographic properties at partially denaturing temperatures and pH. DHPLC detects mutations on the basis of mismatches between amplified chromosomal fragments that result in the formation of heteroduplexes. Variants are detected by differential binding of the homo- and heteroduplexes to the column. The separation of DNA molecules is achieved mainly by the electrostatic interactions between the positive surface potential generated by triethylammonium ions and the negative surface potential generated by the dissociated phosphodiester groups of DNA. Therefore, double-stranded DNA (dsDNA) molecules are retained according to their chain length. Partially denatured heteroduplexes elute faster than non-denatured homoduplexes and the eluting DNA fragments are typically detected by UV absorbance at 254 nm. This technique enables screening of unknown single nucleotide polymorphisms (SNPs) and mutation detection without sequencing in partially denaturing condition at 55-72℃.

Comparative studies have shown pDHPLC to be one of the most sensitive DNA screening techniques available.[4] pDHPLC can detect variants in mixtures with the wild type allele at ratios as low as 1:100.[6] Detection of sequence variations using DHPLC in numerous disease-related genes (e.g., *CFTR, RET,* and *PTEN*) has been reported.[7] In genes such as *BRCA1/2, TSC1/2, NF-1*, and *APC*, the large size and presence of few hot spots makes DHPLC a preferred method for rapid mutation screening and directed molecular analysis.[8-11]

Mutant allele-specific PCR

Allele-specific PCR is a commonly applied method for the speedy detection of known single-base polymorphisms in DNA utilizing specially designed oligonucleotides. It requires the design of a specific oligonucleotide that selectively amplifies one allele. Specificity is achieved by designing the oligonucleotide to match the desired allele but mismatch other allele near the 3′ end of the allele-specific oligonucleotide. The mismatch between the template DNA and the oligonucleotide results in specific amplification of the desired allele and little or no amplification of the undesired allele by preventing elongation at the 3′ end by the enzyme. This technique can be used to detect hot-spot mutations such as *BRAF, K-RAS, EGFR*, as three examples. Only a predefined mutation can be detected by this method, whereas new mutations remain hidden.

Detection of *BRAF T1799A* mutation using mutant allele-specific PCR

Two different forward primers with substitution of a single base at the end of the primer (5′- GTGATTTTGGTCTAGCTACAGT-3′ and 5′-GTGATTTTGGTCTAGCTACAGA-3′) are used to amplify the wild-type allele or *BRAF T1799A* mutated allele, respectively. The sequence of the reverse primer is 5′-TCCACTGATTAAATTTTGGCC-3′. Each PCR mixture contains forward and reverse primers (each 0.4 pmoL), 0.2 mmoL of each dNTP, 1.5 mmol/L of MgCl2, 1× PCR buffer, 1.5 U of immolase DNA polymerase, and 2 μL of genomic DNA in a total volume of 20 μL. The PCR reactions are performed separately with initial denaturation for 10 min at 94℃ followed by 35 cycles (30 s at 94℃, 30 s at 58℃, and 30 s at 72℃). The amplification products are resolved on 2.0% agarose gel and the presence of a 129 bp size product is confirmed.[12] Mutant allele-

specific PCR is a specific, sensitive, and reliable method to detect point mutations such as *BRAF* and *K-RAS*,[12,13] but non-specific priming is still problematic.

Even after the requirements of rigid primer search parameters and optimization of PCR conditions are satisfied, a high risk of non-specific priming is inevitable. Large scaled comparative study regarding the specificity and sensitivity of the mutant allele-specific PCR and other methods in detecting *BRAF T1799A* mutation has not yet been published.

PCR-RFLP

RFLP relies on a difference in homologous sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific endonuclease/restriction enzymes. RFLP of genomic DNA is a vital tool in genome mapping and genetic disease analysis. RFLP analysis is also the basis for early methods of genetic fingerprinting, forensic identification of samples retrieved from crime scenes, determination of paternity, and characterization of genetic diversity or breeding patterns in animal populations. The technique for RFLP analysis is, however, slow and cumbersome. It requires a large amount of sample DNA, and combines the processes of probe labeling, DNA fragmentation, electrophoresis, blotting, hybridization, washing, and autoradiography. However, the sequence changes directly involved with RFLP can also be analyzed more quickly by PCR amplification (PCR-RFLP) directed across the altered restriction site, which is followed by restriction enzyme digestion.

PCR-RFLP analysis is used to detect mutations in amplified DNA sequences by PCR that are recognized by a specific restriction enzyme.[14] The mutation site of the template DNA may not typically be the target of restriction enzyme activity. This technique can be used to detect known hot-spot mutations such as *BRAF, K-RAS, EGFR*, as three examples.

Detection of *BRAF T1799A* mutation using PCR-RFLP

The target area for the analysis of the *BRAF T1799A* mutation is amplified using a specific primer followed by digestion with the restriction enzyme *XbaI*. To create a *XbaI* restriction site in amplicons derived from the mutant DNA template, the forward PCR primer is designed with two base mismatch as follows: *BRAF* exon 15; 5′-TAAAAATAGGTGATTTTG-GTCTAGCTCTAG-3′ (F, mismatched nucleotides are underlined), 5′-ACTATGAAAATACTATAGTTGAGA- 3′ (R). PCR is then performed with an initial denaturation for 5 min at 95 ˚C followed by 35 cycles (30 s at 94˚C , 30 s at 58˚C , and 30 s at 72˚C) with a 10 min incubation at 70˚C to produce a PCR product of 195 bp. The PCR product is then digested with 5 U of *XbaI* for 1-2 h and electrophoresed in a 7% acrylamide gel followed by ethidium bromide staining. The mutant DNA digested with *XbaI* produces 166 bp and 29 bp products.[15]

BRAF T1799A mutation analysis by PCR-RFLP has only been recently approved for use by the non-National Healthcare Service (NHS). However, the sensitivity and the specificity is considerably lower than the sequencing analysis that recently received non-NHS approval.[15-18]

Dideoxysequencing analysis

In dideoxysequencing (Sanger sequencing), the extension is initiated at a specific site on the template DNA by using a short oligonucleotide primer complementary to the template at that region. The oligonucleotide primer is extended using DNA polymerase, an enzyme that replicates DNA. Included with the primer and DNA polymerase are the four deoxynucleotide bases (DNA building blocks), along with low concentration of the chain terminating nucleotide (most commonly a dideoxynucleotide). Limited incorporation of the chain terminating nucleotide by DNA polymerase results in a series of related DNA fragments that are terminated only at positions where that particular nucleotide is used.[18] The fragments are then size-separated by electrophoresis in a slab polyacrylamide gel, or more commonly now, in a narrow glass tube (capillary) filled with a viscous polymer.

BRAF T1799A mutation analysis using dideoxysequencing

PCR primer sequences used for the amplification of *BRAF T1799A* mutation site are 5′-biotin-CTTCATAATGCTTGC-TCTGATAGG-3′ (F) and 5′- GGCCAAAAATTTAATCA-GTGG AA-3′ (R). PCR mixture contains forward and reverse primers (each 0.4 pmoL), 0.2 mmoL each of dNTP, 1.5 mmol/L of MgCl2, 1 × PCR buffer, 1.5 U of immolase DNA polymerase, and 5 μL of genomic DNA in a total volume of 50 μL. PCR is done using an initial denaturation for 5 min at 95˚C followed by 50 cycles (30 s at 95˚C , 30 s at 55˚C , and 30 s at 72˚C) with a10 min incubation at 72˚C . After amplification, the PCR products are resolved by 2% agarose gel electrophoresis to confirm successful amplification. Sequencing of the PCR products is done using a reverse primer described above and commercially available sequencing reagents using an initial denaturation for 30 s at 95˚C followed by 35 cycles (15 s at 95˚C , 15 s at 50˚C , and 4 min at 60˚C . The DNA sequence can then be determined using automated equipment.[18]

Dideoxy sequencing is a more reliable method than PCR-RFLP for detecting *BRAF T1799A* mutation with a high sensitivity and specificity.[16] As well, dideoxysequencing has an advantage over the aforementioned methods in detecting unpredicted or non-hot-spot mutations such as *p53* or *beta-catenin*.

Pyrosequencing analysis

The method relies on the sequential addition and incorporation of nucleotides in a primer-directed polymerase extension reaction. The pyrophosphate released on incorporation of a base-paired nucleotide is coupled with the enzymes ATP sulfurylase and luciferase to generate detectable light. The light intensity is proportional to the number of nucleotides incorporated, allowing the sequence to be determined in real-time. The resulting peak pattern will differ among wild-type, and both heterozygous and homozygous mutations. Therefore, the type, exact localization, and amount of the mutations can be determined. In a rapid, simple, and cost-effective manner, short stretches of sequence from a large number of samples are analyzed in parallel. The above-mentioned features, in addition to the ability to distinguish between different alleles, make this method especially suitable for applications such as single-nucleotide polymorphism analysis and hotspot mutation detection.

Pyrosequencing is a real-time nonelectrophoretic sequencing method that is rapidly gaining popularity in a large variety of applications.[19-22] This technique could be used to detect known hot-spot mutations such as *BRAF, EGFR, K-RAS, CFTR, BRCA1, BRCA2,* and *PIK3CA*.[19,23-28] It can also be applied to quantitative CpG island methylation analysis.[29]

BRAF T1799A mutation analysis using pyrosequencing

PCR primer sequences for the amplification of *BRAF T1799A* mutation site are 5′-biotin- CTTCATAATGCTTGCTCTGATAGG-3′ (F) and 5′-GGCCAAAAATTTAATCAGTGGA A-3′ (R). In the technique, 5 μL of DNA is added to produced 50 μL of PCR solution mixture containing 0.2 mmol each of dNTP, 1.5 mmol/L MgCl2, 1× PCR buffer, 1.5 U of Immolase DNA taq polymerase, and 20 pmol of each primer. PCR is carried out with an initial denaturation for 5 min at 95℃ followed by 50 cycles (30 s at 95℃, 30 s at 55℃, and 30 s at 72℃) and incubation for 10 min at 72℃. The PCR products are resolved by agarose gel electrophoresis to confirm successful amplification. The biotinylated products are then immobilized to streptavidin-coated beads using solution from a commerical PSQTM 96 sample preparation kit. Three microliters of beads are diluted in binding buffer with 10 μL biotinylated PCR products and incubated for 10 min at room temperature. The beads are then transferred to a filter probe and the liquid is removed by vacuum filtration. The DNA in the denaturation solution is separated, templates are washed with washing buffer, and are transferred to a PSQ 96 SNP plate and annealed with sequencing primer, 5′-CCACTCCATCGAGATTT-3′, in annealing buffer at room temperature. Finally, the samples are analyzed using a PyroMark ID System with SNP reagent kit for sequence analysis.[17]

In our experience, among the variable mutation analysis methods, pyrosequencing analysis has a better sensitivity and specificity over PCR-RFLP and dideoxysequencing in detecting *BRAF, EGFR*, and *K-RAS* mutations. Others also reported pyrosequencing method as superior to dideoxy sequencing in detecting *BRAF* mutations.[23,30]

EGFR mutation analysis using pyrosequencing

PCR primer sequences for the amplification of *EGFR* mutation sites are exon 18, 5′-biotin- GCTCCCAACCAAGCTCTCTT-3′ (F) and 5′-TATACACCGTGCCGAACGC-3′ (R); exon 19, 5′- GCATGTGGCACCATCTCA-3′ (F) and 5′-biotin-AAAAGGTGGGCCTGA GGTT-3′ (R); exon 20, 5′- biotin-ATGGCCAGCGTGGACAAC-3′ (F) and 5′-TTTGTGTTCCCGGACATAGTC-3′ (R); exon 21, 5′- ACCGCAGCATGTCAAGATCAC-3′ (F) and 5′-biotin- TCCGCACCCAGCAGTTTG- 3′ (R). Briefly, add 5 μL of DNA to reach 50 μL of PCR solution mixture containing 0.2 mmol each of dNTP, 1.5 mmol/L MgCl2, 1× PCR buffer, 1.5 U of Immolase DNA Taq. Polymerase (Bioline, London, UK), and 20 pmol of each primer. Perform PCR with intial denaturation for 5 min at 95℃ followed by 50 cycles (30 s at 95℃, 30 s at 55℃, 30 s at 72℃) and incubate 10 min at 72℃. Electrophores PCR products in an agarose gel to confirm a successful amplification. Immobilize biotinylated PCR products to streptavidin-coated beads (GE Health Care, Uppsala, Sweden) using solution from the PSQTM 96 sample Preparation Kit (Biotage, Uppsala, Sweden) by following a standard protocol. Dilute 3 μL of beads in binding buffer with 10 μL biotinylated PCR products and incubate for 10 min at room temperature. Transfer the beeds to filter the probes and remove liquid by vacuum filtration. Separate DNA in denaturation solution for 2 min. Wash the templates with washing buffer and then transfer to a PSQ 96 SNP plate and anneal with sequencing primers exon 18 E709K: 5′-TGATCTTTTTGAATTCAGTT-3′ and G719A: 5′-CCGAACGCACCGGAG-3′, exon 19 deletion: 5′- ATTCCCGTCGCTATC-3′, exon 20 T790M: 5′-GATGCCCAGCAGGCG-3′, exon 21 L858R and A859T: 5′-AAGATCACAGATTTTGG-

3′ in annealing buffer at room temperature. Analyse samples using a PyroMark ID System (Biotage) with SNP reagent kit (Biotage) to determine the sequence.

K-RAS mutation analysis using pyrosequencing

PCR primer sequences for the amplification of *K-RAS* mutation are codon 12 and 13, 5′- CTGGTGGAGTATTTGATAGTGTA-3′ (F), 5′-biotin-TGGTCCTGCACCAGTAATAT-3′ (R); codon 61, 5′-Biotin-TCCAGACTGTGTTTCTCCCTTC-3′ (F), 5′-TACTGGTCCCTCATTGCACTGT-3′ (R). Briefly, add 5 μL of DNA to reach 50 μL of PCR solution mixture containing 0.2 mmol each of dNTP, 1.5 mmol/L MgCl2, 1 × PCR buffer, 1.5 U of Immolase DNA Taq. Polymerase (Bioline, London), and 20 pmol of each primer. Perform PCR for 5 min at 95℃ followed by 50 cycles (30 s at 95℃, codon 12, 13 and 61 are 30 s at 55℃; 30 s at 72℃) and incubate for 10 min at 72℃. Electrophorese the PCR products in an agarose gel to confirm a successful amplification. Immobilize biotinylated PCR products to streptavidin-coated beads (GE health Care) using solution from the PSQTM 96 sample Preparation Kit (Biotage) by following a standard protocol. Dilute 3 μL of beads in binding buffer with 10 μL biotinylated PCR products and incubate for 10 min at room temperature. Transfer the beads to filter the probes and remove liquid by vacuum filtration. Separate DNA in denaturation solution for 2 min. Wash the templates with washing buffer and then transfer to a PSQ 96 SNP plate and anneal with sequencing primers codon 12 and 13, 5′-ATAAACTTGTGGTAGTTGG-3′; codon 61, 5′-CCTCATTGCACTGTAC-3′ in annealing buffer at room temperature. Analyse samples using a PyroMark ID System (Biotage) with SNP reagent kit (Biotage) to determine the sequence.

CONCLUSIONS

Cytology samples not only allow the application of techniques and technologies available in other areas of diagnosis, but also allow pioneering work due to the relative ease of sampling neoplasms when compared with other, more invasive methods. Moreover, molecular analysis using cytology specimens produces better results than paraffin-embedded tissue, given sufficient target cells.The spectrum of molecular techniques could be used in molecular diagnostic cytopathology when optimum dissection techniques are provided for the target cells, the contrast is enough to dissect the target cells (dripping of the extraction solution onto the uncovered slide provides good contrast to view the target cells), sufficient numbers of target cells are present, and the method of DNA extraction is robust. To obtain a successful result, collection of atypical cells of interest is a prerequisite; the importance of this fact cannot be overemphasized.

Contamination of blood cells, inflammatory cells including histiocytes, and stromal cells should be avoided. The optimum number of cells for successful PCR is about 100, although as few 50 cells has been successful in our experience. Since most cytology specimens have a limited amount of target cells, DNA extraction is another very important step. To obtain a good DNA yield from a very limited number of target cells, avoid DNA loss is crucial. Suggestions to avoid loss include the use of the smallest tubes possible and minimal filtering and transportation from one tube to the other.

Most of the current molecular techniques for mutation analysis are based on PCR. Since development of PCR, a variety of modifications in primer design and reaction conditions have been proposed to enhance and optimize specificity.[2,4] But, a fundamental solution for eliminating non-specific priming still remains a challenge and limits the versatility of PCR in nucleic-acid-based tests. Recently, a novel dual priming oligonucleotide was proposed, in which two separate priming regions are joined by a polydeoxyinosine linker. This structure results in two primer segments with distinct annealing properties; a longer 5′-segment that initiates stable priming, and a short 3′-segment that determines target specific extention.[31]

Since molecular diagnosis have a direct impact on the final cytopathological diagnosis, or prognostic and therapeutic implications, such a diagnosis is profoundly important in clinico-pathological correlations and therapeutic decision-making, when interpreted by those who can combine the molecular basis of the disease with the clinical and pathophysiological presentations.

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