

Molecular biology techniques for loss of heterozygosity detection: the glioma example

Técnicas de biologia molecular para avaliação da perda de heterozigosidade: o exemplo em glioma

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ABSTRACT

In recent years, many oncology institutions have implemented the use of molecular approaches to assess and manage cancer patients. One commonly observed type of genetic alteration in cancer is the loss of heterozygosity (LOH). In the clinical setting, this molecular genetic marker is an important tool for disease prognosis, diagnosis and treatment. For example, the loss of 1p/19q is a classical molecular marker for oligodendroglioma assessment. In addition, this marker is associated with a favorable prognosis and chemosensitivity in oligodendroglial tumors. Interpretation of the clinical significance of molecular markers requires that health professionals and biomedical scientists understand the basic theoretical fundamentals of molecular diagnostic techniques. Although there are different methodologies to assess LOH, including high-performance techniques, this review aims to describe the polymerase chain reaction (PCR)-based LOH assays and fluorescence *in situ* hybridization (FISH), which are the molecular techniques most used for evaluation of 1p/19q status in pathology laboratories.

Key words: oligodendroglioma; polymerase chain reaction; fluorescence *in situ* hybridization; loss of heterozygosity.

INTRODUCTION

The development of cancer is a multistep process that involves genetic changes in multiple genes or chromosomes. These genes have demonstrated to play an essential role in tumorigenesis and were classified as oncogenes and tumor suppressor genes⁽¹⁾. A commonly observed type of genetic alteration in cancer is the loss of heterozygosity (LOH), in which the wild-type allele of a gene is inactivated in a first hit, and the remaining functional allele is deleted in the second hit. High frequency of allelic loss on a certain chromosome region is known to indicate the presence of a tumor suppressor gene. A variety of genetic mechanisms have been considered to account for LOH: mitotic nondisjunction, loss of a segment of chromosome resulting from a deletion event, mitotic recombination between two homologous chromosomes, break-induced replication, recombination between two nonhomologous chromosomes (translocation), and gene conversion. The mechanisms of LOH are remarkably

chromosome specific. Some chromosomes display complete loss. However, more than half of the losses are associated with the loss of only a part of the chromosome rather than the whole chromosome⁽²⁾. Due to the high level of specificity, LOH is used to characterize different tumor types, pathological stages, and progression^(3, 4), being invaluable as a marker for diagnosis and prognosis of cancer^(5, 6).

MOLECULAR MARKERS IN GLIOMA AND 1p/19q LOSS

Glioma is the most common primary brain tumor in adults and represents 2% of human malignancies⁽⁷⁾. There is a variety of different histological glioma types and malignancy grades. According to the criteria of the World Health Organization (WHO) classification established in 2007⁽⁸⁾, gliomas are divided into astrocytic, oligodendroglial, mixed oligoastrocytic and ependymal tumors. Among these, astrocytic tumors represent the

most typical gliomas and include a very aggressive type, termed “glioblastoma”^(7,8).

Over the last decade, the development of molecular markers has allowed better management of patients with malignant adulthood gliomas. At present, there are three classical molecular genetic markers with clinical importance, namely, the 1p/19q loss, the methylation of the O⁶-methylguanine-deoxyribonucleic acid (DNA) methyltransferase (*MGMT*) gene promoter, and the isocitrate dehydrogenase 1 (*IDH1*)/isocitrate dehydrogenase 2 (*IDH2*) mutations. These molecular markers are currently used in prognostic and diagnostic neuro-oncology^(6,9). In 2012, long-term follow-up trials demonstrated an overall survival benefit from the addition to radiotherapy of chemotherapy with procarbazine/lomustine/vincristine in patients with anaplastic oligodendroglial tumors with (vs. without) 1p/19q co-deletion⁽⁶⁾. Since treatment decisions concerning malignant gliomas can be based on such molecular signatures, molecular testing has been implemented in routine clinical practice. This, however, requires that health professionals and biomedical scientists have theoretical knowledge of molecular diagnostic approaches for detection of genetic markers.

Therefore, the aim of this review is to describe the polymerase chain reaction (PCR)-based LOH analysis and fluorescence *in situ* hybridization (FISH), the main molecular techniques commonly used in the assessment of 1p/19q loss in glioma patients^(6,9).

MOLECULAR TESTING FOR 1p/19q LOSS

The combined loss of the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q), known as 1p/19q co-deletion, became a genetic hallmark of oligodendroglial tumors^(10,11). This genetic alteration might be the consequence of an unbalanced centromeric translocation of 1p and 19q^(12,13). Some reports estimated a frequency of the 1p/19q loss in 80%-90% of grade II oligodendrogliomas, 50%-70% in grade III oligodendrogliomas⁽¹⁴⁾ and 20%-30% in mixed oligoastrocytomas⁽¹⁵⁾. As a rule, this genetic signature is a favorable prognostic marker in grade II or III oligodendrogliomas, pure oligodendrogliomas and mixed oligoastrocytomas. Since 1p/19q loss also has diagnostic and predictive values, it should help neuropathologists refine the histomolecular classification of gliomas⁽¹⁶⁾.

In order to evaluate 1p/19q loss, there are two typical molecular methods: PCR-based LOH assays and FISH.

THE PCR-BASED LOSS OF HETEROZYGOSITY

Also known as PCR-based microsatellite analysis, this technique is often employed to detect allelic losses. Essentially, it is based on a comparison of microsatellites or short tandem repeat (STR) markers between tumor and normal DNA. These markers are polymorphic DNA loci that contain a repeated sequence ranging from two to six nucleotides in length⁽¹⁷⁾. The number of repeat nucleotide units is the same for the majority of repeats within a microsatellite locus. Moreover, microsatellite markers have been extensively used in LOH screening^(18,19). Regarding technical details, LOH testing can be divided into four steps, namely, DNA extraction from the tumor and non-tumor tissue, then the PCR, followed by electrophoretic separation and, finally, data analysis.

First, DNA is extracted from the tumor tissue, usually using a paraffin block of formalin-fixed tissue, although fresh or frozen tumor samples obtained from surgery or biopsy can also be used. As a 1p/19q loss negative control, DNA from normal tissue, usually blood, is required for comparison with glioma DNA. This requirement is one of the drawbacks of LOH assays. Some clinical studies have used histologically normal areas of the brain and nail clippings as alternative negative controls^(20,21). Following isolation of genetic material, a molecular technique, named multiplex PCR, is run (**Figure 1**). This approach is employed for simultaneously amplifying multiple microsatellite regions by one million times or more using several primers, which are located in the lost chromosomal regions. Due to the fact that not all patients are heterozygous at all tested loci, LOH assays need many microsatellite regions to make sure that sufficient informative loci will be available for LOH detection. In addition to the primers and genetic material, multiplex PCR also needs other components, including deoxyribonucleotide triphosphate (dNTPs), a thermostable DNA polymerase, a buffer solution and magnesium ions. For an effective amplification, many repeated reaction cycles are required, hence the term “polymerase chain reaction”. Each cycle consists of three following steps: denaturation, annealing and extension. In denaturation, the double-strand DNA is separated into two single-strands at a high temperature. In annealing, primers hybridize to complementary sequences flanking the microsatellite region. Finally, in extension, two new double-strands are synthesized from the annealed primers. As this procedure is repeated many times, the newly synthesized DNA molecules are used as templates. In general, each cycle doubles the number of copies of the target region and this results in an exponential amplification of the specific gene target⁽²²⁾.

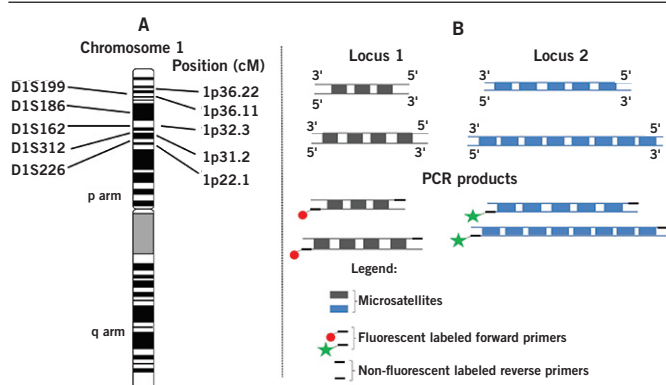


FIGURE 1 – Amplification of microsatellite regions using the multiplex PCR technique
PCR: polymerase chain reaction; LOH: loss of heterozygosity.

This molecular procedure consists of a series of repeated cycles for amplification of microsatellite regions of 1p and 19q chromosomes using microsatellite primers. A) In the illustrated example, microsatellite loci located in chromosome 1p were used for the loss of heterozygosity (LOH) assay; B) fluorescent labeled forward and non-fluorescent reverse primers are used in PCR amplification producing amplicons at each PCR cycle, so that after 20-30 cycles, greater than 1 million-fold amplification is reached. At the end of PCR, the majority of generated fragments in the loci 1 and 2 are fluorescent.

Initially, PCR products were analyzed on a traditional denaturing polyacrylamide gel electrophoresis (PAGE) by manual reading (**Figure 2**). After electrophoretic separation, the DNA bands could be visualized by autoradiography, using predominantly ^{32}P -labelled-dNTPs. This traditional method is based on the exposure of the gel to an X-ray film. The emission decay of the radioactive-labeled PCR amplicons produces dark bands on the film at the position of the amplified fragments in the gel, and the sequences are manually read by determining the

order of the fragment sizes and presence/intensity of the bands compared with non-tumor DNA. Due to the use of isotopes, this traditional methodology obligates researchers to receive radiation safety training. This could be avoided by the use of silver staining to visualize DNA bands⁽²³⁾. Nowadays, as radioactivity is less used because of the laser-based DNA sequencers and fluorescently labeled primers development, the automated reading by capillary electrophoresis of DNA sequencers became more common than the manual reading. This technology provides a more precise estimation of allelic loss. Furthermore, capillary gel electrophoresis accomplished with fluorescently labeled primers gives a high sensitivity for amplified microsatellite loci⁽²⁴⁻²⁶⁾. Thus, after PCR amplification, fluorescently labeled PCR products are detected by capillary electrophoresis, performed on an automated DNA sequencer (**Figure 3**). Inside this device (Figure 3B), amplified fluorescent PCR products and internal-lane size standards are co-injected into thin capillaries filled with polymer, acting as an electrophoresis gel, which is used as a sieve to separate the DNA fragments by size. When a voltage is applied across all capillaries, the samples begin to migrate at the same time. The DNA fragments move towards the other end of the capillaries, with the shorter fragments moving faster than the longer fragments. As the fragments travel through the polymer in the capillary, they enter the detection cell, a component of capillary electrophoresis system. In the detection cell, the laser beam excites the dye attached to the DNA fragments causing them to fluoresce. The fluorescence is then captured by a charge-coupled device (CCD) camera, which converts the incident fluorescence into electronic information for transfer to a computer workstation for processing by a data collection software. The processed data is automatically extracted from the instrument database and analyzed by analysis software (Figure 3C). In summary, this software generates a plot, called electropherogram, which expresses amplicons sizes and fluorescence intensity peaks on x and y axis, respectively. The analysis software uses internal-lane standards to reduce band-shift artefacts and run-run variability. Based on migration of the internal standards, the software plots a sizing curve and calculates peak sizes of each sample, comparing the migration of the sample to the internal standards in the sizing curve. In addition, the software algorithms automatically identify and size peak areas and peak height information. Utilizing electropherograms in LOH assays, the allelic status from glioma DNA is compared to normal tissue DNA. The alleles of each locus are separated by size. The alleles of a different locus are automatically shown in the different-colored fluorescence associated with each forward primer and corresponding PCR product. In an electropherogram, a locus with one allele (one peak) could indicate either homozygosity or LOH, while two alleles (two peaks of different sizes) could

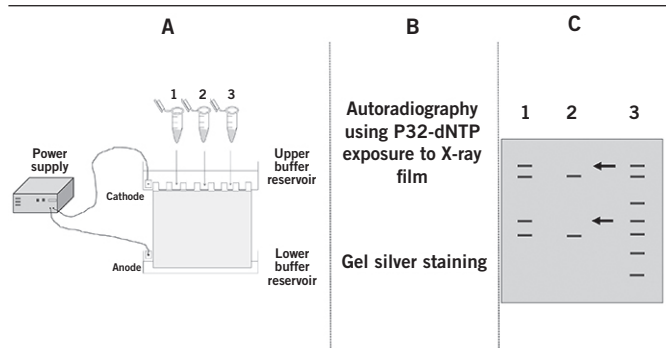


FIGURE 2 – Manual reading of LOH screening using denaturing PAGE
LOH: loss of heterozygosity; PAGE: polyacrylamide gel electrophoresis; PCR: polymerase chain reaction; DNA: deoxyribonucleic acid.

A) PCR products from normal DNA (1), tumor DNA (2) and molecular weight standard (3) are applied to polyacrylamide gel; B) after electrophoretic separation, the DNA bands are visualized by autoradiography or silver staining; C) data analysis of PAGE. The normal tissue DNA (lane 1) shows heterozygosity for locus 1 and locus 2 in 1p chromosomal arm, tumor tissue DNA (lane 2) shows loss of heterozygosity for both loci (arrows). Molecular weight standard is shown in lane 3.

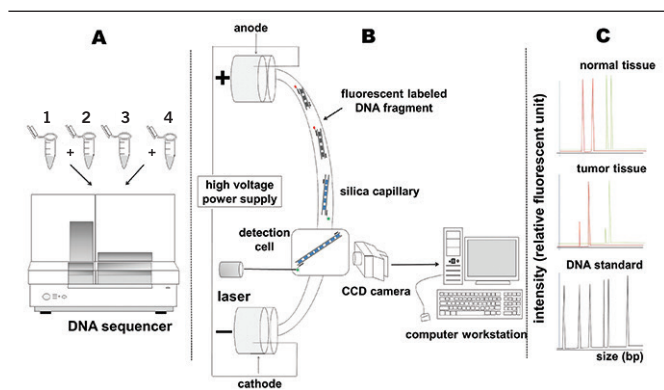


FIGURE 3 – Reading of LOH screening by capillary electrophoresis in automated DNA sequencer

LOH: loss of heterozygosity; DNA: deoxyribonucleic acid; PCR: polymerase chain reaction; CCD: charge-coupled device.

A) After microsatellite amplification, internal-lane size standard (1 and 3), PCR products from normal DNA (2) and tumor DNA (4) are subjected to capillary electrophoresis on an automated DNA sequencer, and DNA fragments of PCR-based LOH assays are separated; B) illustrated scheme of capillary electrophoresis system. This system is composed by cathode (-) and anode (+) electrodes, a high-voltage power supply, silica capillary, detection cell, charge-coupled device (CCD) camera and a computer workstation. When a high voltage is applied, negatively charged DNA fragments begin to migrate through the capillary. In the detection cell, the DNA fragments are intercepted by a laser beam, which excites the attached dye labels making them fluoresce. Following laser excitation, the incident fluorescence is captured by a CCD camera, which converts fluorescence into electronic information. This information is transferred to a computer workstation, where the data are processed and analyzed by specific software; C) data processing and analysis of capillary electrophoresis. Two representative capillary electropherograms of two PCR-amplified microsatellites are shown from normal and tumor tissues. The normal tissue DNA shows heterozygous peaks for locus 1 (red peaks) and locus 2 (green peaks) in the 1p chromosomal arm, while loss of heterozygosity is noted for both markers (red and green peaks) in the tumor tissue DNA. Internal-lane size standard is represented as a black line.

represent heterozygosity. When LOH occurs in a glial tumor, there is a complete absence or a significant decrease in amplification of one allele. Furthermore, LOH can be evaluated through a value obtained by the following formula:

$$\text{LOH} = (\text{height of normal allele 2} / \text{height of normal allele 1}) / (\text{height of tumor allele 2} / \text{height of tumor allele 1}).$$

In general, an LOH value less than 0.5 indicates that a glial tumor shows a loss of the allele 1, while a value greater than 1.5 demonstrates that the tumor has a significant loss of allele 2⁽²⁷⁾.

FISH

The development of chromosomal banding techniques in the early 1970s facilitated the identification of each human chromosome and easily detects gains or losses of whole chromosomes (aneuploidy) and large structural rearrangements (deletions, duplications, inversions, translocations). Banding

techniques, particularly, can detect deletions or duplications of > 10 Mb, and in its high-resolution version an alteration of 3 ± 5 Mb can be detected. Alterations < 3 Mb are extremely difficult to detect⁽²⁸⁾. To address this, additional techniques to detect smaller chromosome abnormalities (submicroscopic) are needed. FISH technology entered clinical cytogenetics in the 1990s and has complemented chromosome studies due to its relatively high resolution. FISH testing uses fluorescently labeled probes that hybridize to a particular position on a chromosome and can be used to detect chromosomal rearrangements as small as 100 kilobases (100,000 base pairs) in routine clinical practice. This technique uses fluorescence-labeled probes to detect chromosomal abnormalities in interphase nuclei, directly at surgery or in biopsy samples⁽⁷⁾, which can be applied to formalin-fixed and paraffin-embedded tissue samples and also to fresh or frozen tissue and cytological preparations⁽²⁹⁾. Compared with PCR-based LOH, FISH does not need normal tissue DNA as a control and provides additional information, including assessment of polysomy, which could predict earlier recurrence in patients with anaplastic oligodendroglioma⁽³⁰⁾. Such predictions with FISH, however, should be carefully analyzed, since they could provide false-negative results when the glioma loses one chromosome in the presence of duplication of another allele. This latter genetic phenomenon, called uniparental disomy, is frequent for 19q in primary glioblastomas^(31,32). Preservation of tissue architecture is a distinct advantage of FISH, because it allows neuropathologists to evaluate the tissue morphology in concert with genetic status. On the other hand, one disadvantage of FISH includes laborious and time-consuming preparation of tissue samples. Moreover, FISH has low scalability, since it cannot test multiple chromosomal loci⁽³³⁾.

Numerous FISH protocols have been published, varying fundamentally in the choice of probe, target retrieval and detection steps⁽²⁹⁾. The basic standard protocol includes the following steps: deparaffinization, pretreatment/target retrieval, denaturation of probe and target DNA, hybridization, post-hybridization washes, detection and image analysis in fluorescence microscopy (**Figure 4**). Initially, slides are deparaffinized in xylene or other substitutes, followed by rehydration in a series of graded alcohols. During fixation, formalin causes protein-protein and protein-nucleic acid cross-links that preserve tissue morphology. However, this procedure results in DNA denaturation and masking, reducing the probe recognition and binding to target DNA. In order to obtain successful FISH results, target retrieval is a critical step to demask DNA target. In general, this procedure varies according to individual preferences, fixation time and tissue type. Some pathology laboratories prefer boiling the slides in an appropriate buffer (concentrated sodium citrate buffer

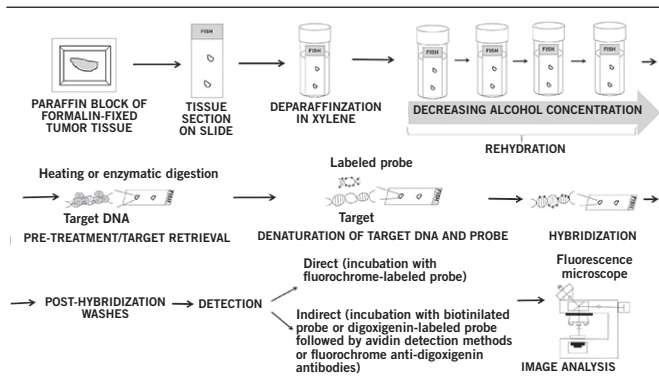


FIGURE 4 – Standard steps of FISH LOH screening

FISH: fluorescence in situ hybridization; LOH: loss of heterozygosity; DNA: deoxyribonucleic acid.

Eight basic steps are included: deparaffinization in xylene, rehydration in graded alcohols, pre-treatment/target retrieval, denaturation of target DNA and probe, hybridization, post-hybridization washes, direct or indirect detection and image analysis in a fluorescence microscope.

or ethylenediaminetetraacetic acid [EDTA]) in a microwave oven, while others use enzymatic digestion to break formalin-induced protein-protein cross-linking. For target retrieval, the commonly used enzymes are serine protease, proteinase K and pepsin. In addition, before enzymatic digestion, a pre-treatment step is usually performed to ease the subsequent protease action. Following target retrieval, the target DNA and labeled probe are denatured by heating. Afterwards, the hybridization step begins at a lower temperature. In summary, a competition between unlabeled and labeled sequences in the probe occurs. Thus, unlabeled sequences in the probe hybridize to complementary sequences in the target DNA to block unstained signals, while the labeled probe sequences hybridize to the target DNA, marking the target sequence. Typically, hybridization requires a minimum time (12 hours), but some laboratories use longer times (72 hours) to help increase signal strength. Both denaturation and hybridization steps occur in the presence of salt and formamide. After hybridization, the slides are washed in stringent conditions using a saline-sodium citrate (SSC) buffer at an optimal temperature to remove the non-specific bound probe. Following the post-hybridization step, the slides are counterstained by 4',6-diamidino-2-phenylindole (DAPI), which binds to DNA and is used to visualize nuclei. Subsequently, the hybridized probe can be visualized by direct or indirect methods. The fluorescent labeled probe can be visualized directly in the fluorescence microscope and results in rapid staining with a low background, but this method is less sensitive than the indirect method. The sensitivity of the fluorescent labeled probe could be increased utilizing probes covalently labeled by reporter molecules, such as biotin or

digoxigenin. Biotinylated-probes can then be detected by avidin, streptavidin methods or anti-biotin antibodies, while digoxigenin-labeled probes are visualized by incubation with anti-digoxigenin antibodies coupled with a fluorochrome. Some laboratories include in their protocols an amplification step using anti-avidin antibodies and fluorescent labeled avidin or fluorescent secondary antibodies.

There are different types of probes for FISH assays. The three most common are: alpha-satellite, telomeric and locus-specific or gene-specific probes⁽³⁴⁾. Alpha-satellite probes hybridize with repeated DNA sequences in the pericentromeric region of chromosomes. Telomeric probes are specific for repeated sequences present in the termini of chromosomes and used to identify chromosomal terminal deletions. Lastly, locus-specific probes are specific to certain chromosomal regions, groups of repeated DNA sequences or single genes. These probes are designed to study structural chromosomal aberrations, such as deletions, translocations and gene amplifications. In addition, locus-specific probes are used to assess 1p/19q co-deletions in gliomas. Apart from the locus-specific probes, a second probe labeled with a different reporter is included as a reference hybridization and enumeration control. FISH probes are commercially available or homemade using vector cloning, including bacterial artificial chromosomes (BAC), P1 artificial chromosomes (PAC), yeast artificial chromosomes (YAC) and cosmids.

THE EVOLUTION TO HIGH-THROUGHPUT CYTOGENETIC TECHNIQUES

Recently, new techniques have revolutionized cytogenetic testing due to advances in microarrays, allowing an increase on LOH analysis capacity⁽²⁸⁾.

The comparative genomic hybridization array (aCGH) was developed combining the principles of comparative genomic hybridization (CGH) with the use of microarrays. In CGH, two genomes are directly compared for DNA content differences. This methodology uses tumor and normal control DNAs. The two DNAs are differentially labeled with two distinct fluorochromes and compared by examining the ratio of the two fluorochromes on metaphase chromosomes hybridization. If a chromosome deletion occurs on tumor DNA, metaphase chromosomes will be colored only with control fluorochrome, but if amplification occurs, it will be colored preferentially by the tumor fluorochrome, generating a map of DNA copy number changes⁽³⁵⁾. Instead of using metaphase chromosomes, aCGH uses glass slides with small segments of DNA as a target⁽³⁶⁾. The resolution of CGH has been limited to alterations of approximately 5-10 Mb^(37, 38). Because targets fixed on glass

slides are several orders of magnitude smaller than metaphase chromosomes, the theoretical resolution of aCGH is proportionally higher than that of traditional CGH⁽³⁹⁾.

The resolution of microarray technology has been further improved with the introduction of single nucleotide polymorphism (SNP) arrays. In contrast to aCGH, SNP arrays do not rely on competitive binding of reference DNA, but genotype polymorphisms directly in test DNA, and the hybridization signal strength from individual probes allows for the estimation of gene copy number. SNP is a variation at a single position in a DNA sequence among individuals. If more than 1% of a population carry the same nucleotide variation at a specific position in the DNA sequence, then this variation can be classified as a SNP. SNP arrays take advantage of these different alleles; they are differentially fluorescently labeled and hybridized with patient DNA. The total fluorescence and the fluorescence ratio of the two different dyes allow analysis of homozygosity and heterozygosity as well as identification of duplications or deletions. High-density SNP arrays allow testing a large number of polymorphisms in the same study. The applications include whole-genome genetic linkage analysis, identification of genetic abnormalities in cancer for prognostic or diagnostic uses. Most SNP arrays detect between 660 thousand and 2 million SNPs across the length of all chromosomes⁽⁴⁰⁾.

FINAL CONSIDERATIONS

In this review, we described the basic principles, advantages and drawbacks of PCR-based LOH assays and FISH, which are molecular techniques commonly used for 1p/19q testing. Due to therapeutic and prognostic importance of this marker, the understanding of the

fundamental principles of these molecular diagnostic techniques is important for their implementation in routine clinical practice. However, individual expertise, institutional and financial issues should be taken into account before a decision to implement these molecular approaches in a clinical situation can be made. In PCR-based LOH assays, the manual reading of PCR products should be an option in neuro-oncological institutions that have no access to a DNA sequencer for realizing capillary electrophoresis. Of the two molecular techniques described for testing for 1p/19q status, FISH is usually the favored procedure in many pathology laboratories, but less suitable for high-throughput work than microsatellite analysis. Two observations weigh strongly in the choice of molecular diagnostic techniques for evaluation of 1p/19q loss in oligodendroglial tumors. First, 1p/19q loss has an important prognostic value in oligodendrogliomas and second, the use of this marker refines clinicopathologic data. Finally, the information provided by 1p/19q status can help selection and stratification of patients, and also guide treatment decisions at a clinical setting.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

RESUMO

Nos últimos anos, instituições de oncologia têm implementado o uso de abordagens moleculares para avaliar e conduzir pacientes com câncer. O tipo mais comum de alteração encontrada no câncer é a perda de heterozigosidade (LOH). Na clínica, esse marcador molecular pode ter importância para o prognóstico, o diagnóstico e/ou na decisão do tratamento. Por exemplo, a perda de 1p/19q é um marcador molecular clássico para a avaliação do oligodendroglioma. Além disso, esse marcador está associado ao prognóstico favorável e à quimiossensibilidade em tumores oligodendrogliais. A interpretação do significado clínico dos marcadores moleculares exige que os profissionais da área da saúde entendam os fundamentos básicos teóricos das técnicas de diagnóstico molecular. Embora existam diferentes metodologias para avaliar a LOH, inclusive técnicas de alta performance, esta revisão tem o objetivo de descrever o ensaio de LOH com base na reação da cadeia da polimerase (PCR) e a hibridização in situ fluorescente (FISH), que são as técnicas moleculares mais usadas para avaliação do status 1p/19q em laboratórios de patologia.

Unitermos: oligodendroglioma; reação em cadeia da polimerase; hibridização in situ fluorescente; perda de heterozigosidade.

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