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CGT Laboratory

MS-MLPA

Methylation-Specific Multiplex Ligation-dipendent Probe Amplification

Introduction

Cogentech's Cancer Genetic Test (CGT Lab) laboratory has developed molecular diagnostic tests to detect and evaluate pathogenic variants that predispose patients and their family members to a higher risk of developing cancer, allowing the oncologist to make timely and accurate diagnoses for better targeting of treatment the patient. Diagnostic tests for different types of hereditary cancers and tests for variants predictive of response to certain therapies, particularly Parp inhibitors, are performed in our laboratories.

There is evidence that some tumor types are prone to methylation defects: in particular, in recent years, the use of a universal test on colorectal and endometrial carcinomas for the identification of LYNCH syndrome has been recommended, which involves, among other things, analyzing the methylation status of the MLH1 gene promoter to correctly identify subjects for genetic testing of MMR genes, and then, in the presence of pathogenic susceptibility variants, possibly being able to cascade predictive testing on their family members.

MS-MLPA is a semi-quantitative, nonautomated technique capable of assessing both the copy number and methylation status of about sixty sequences by multiplex PCR.

MS-MLPA (Methylation-Specific Multiplex Ligation-dependent Probe Amplification)

The principle of MS-MLPA is based on the amplification of up to 60 probes per kit, each of which is specific for a given DNA sequence. The reaction results in a series of amplicons of specific length between 64 and 500 nt, separated by capillary electrophoresis.

After an initial denaturation of the sample DNA, probes are added that consist of two oligonucleotides that must hybridize with directly adjacent target sequences in order to be ligated into a single probe.

During the subsequent PCR reaction, all bound probes are amplified simultaneously using the same primer pair, resulting in a series of unique amplicons. One of the primers is labeled with a fluorochrome, allowing visualization of the amplification products after separation of the fragments by capillary electrophoresis. Separation of the fragments produces a sample-specific electropherogram.

Some of the probes are specific for a region that contains a restriction site for the methylation-sensitive HhaI endonuclease. After the hybridization step, the reaction is split and one part will continue the standard MLPA protocol (to give the copy number information) while the other half will first be digested with the restriction enzyme before amplification (to get the methylation status information)

If the DNA is unmethylated, methylation-specific probes will be ligated and

simultaneously digested by HhaI and thus will no longer be amplified. In contrast, when the target sequence of the MS-MLPA probe is methylated, the methyl group will prevent HhaI digestion. An undigested and bound probe can be amplified during PCR, resulting in a normal peak signal.

MS-MLPA is a relative technique: only relative differences can be detected by comparing the peaks of the sample with those of reference samples. The inclusion of reference samples within each experiment is therefore essential.

The amplification conditions are semiquantitative so that the relative height of each individual peak, compared with the relative heights of probe peaks in various reference DNA samples, reflects the copy number of the corresponding target sequence in a sample. The inclusion of reference samples in the same analysis is therefore essential. Elimination of one or more target sequences is visible as a relative decrease in peak height, while an increase in relative peak height reflects an increase in copy number.

The software used for analysis are Gene Marker v.3.0.1 (SoftGenetics) and Coffalyser.Net v. 220513.1739 (MRC-Holland).

The analytical protocol used has 99% sensitivity and 97% specificity in identifying extensive deletions/duplications (Vorstman et al., Hum Mutat;27(8), 2006); and high sensitivity and specificity in identifying alterations in promoter methylation levels (Nygren et al., Nucleic Acids Res. 33:e128, 2005).

SALSA MS-MLPA kits provided by MRC-Holland are commercially available (most marked CE-IVD) with specific probes for genes of interest, In particular ME011 for Mismatch repair genes. The test can be performed on genomic DNA (gDNA) obtained from the patient's blood collection or extracted from paraffin-embedded tumor tissue (FFPE).

Limits of the analysis

MS-MLPA cannot detect any deletion or duplication or methylation that lies outside the probes' target sequence and cannot detect balanced inversions or translocations.

In addition, variants under the probes or in close proximity can give false positives so this should always be checked if only one probe is lost.

In the case of poor denaturation of sample DNA, they can give false positives especially extremely GC-rich chromosome regions are not denatured at 98°C when more than 40 mM NaCl or KCl is present.

Most MS-MLPA probes detect methylation of the first cytosine nucleotide at a single HhaI site found within the sequence detected by the probe (GmCGC). If methylation is absent for this particular CpG site, it does not necessarily mean that the entire CpG island is not methylated

Methylation status may be different in different tissues; for example, methylated sequences in blood-derived DNA might be unmethylated in DNA derived from amniotic fluid.

MRC Holland provides a sheet for each kit that describes the design of the probes and the purpose of the panel to be referred to for analysis.

Conclusions

MS-MLPA is a reliable, reproducible and fast method, thanks in part to the stability and efficiency of the commercial kits used and the accuracy and precision of the analysis software our laboratory has.