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

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Introduction

DNA methylation is the addition of a methyl group to a piece of DNA, specifically to cytosines located in CG dinucleotide repeat regions. Regions of the genome containing a high frequency of these dinucleotides are referred to as CpG islands[1] and are often located in the promoter region of genes. Virtually all vertebrate animals have methylation sites in their genome. In humans, approximately 1% of DNA bases undergo DNA methylation.

Detection of methylated sites in genomic DNA is important for two reasons. First, if the promoter region of a gene is methylated, the gene will not be transcribed and its associated protein will not be produced. Methylation of the promoter region for tumor suppressor genes is of great interest for cancer diagnostics[2]. When the tumor suppressor gene is switched off because of methylation in the promoter region, the cell's growth will be unchecked and cancer may form. Likewise, if methylation occurs in mismatch repair gene promoter regions, genetic mutations within a cell will proliferate, thereby increasing the risk of tumorigenesis.

Second, DNA methylation is important in genomic imprinting[3]. At conception, two copies of each chromosome are inherited from an individual's parents, one copy from the father and one copy from the mother. Normally, it cannot be determined from which parent each chromosome originated; however, in some cases, differential expression of a gene occurs depending upon which parental chromosome the gene resides. Differential expression of these genes has been linked to methylation patterns and/or histone modifications. Detecting patterns of methylation may be useful in determining parental chromosome origin.

The classic examples of genomic imprinting are Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS)[4]. PWS is a genetic disorder in which several genes on chromosome 15 are missing or unexpressed on the paternal chromosome. Deletion of the same region on the maternal chromosome causes Angelman syndrome. Normal (wildtype) individuals have one copy of the unmethylated paternal allele and one copy of the methylated maternal allele. Because the methylation pattern of each parental chromosome is known, the methylation pattern of the undeleted or functional chromosome 15 region can be used to determine the chromosome's origin. Other genetic diseases related to genomic imprinting through DNA methylation include Beckwith-Wiedemann Syndrome (BWS) and Russell-Silver Syndrome (RSS).
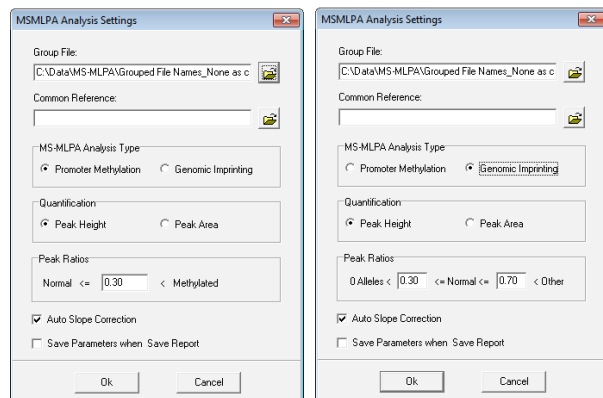
GeneMarker DNA analysis software has been successfully paired with the Multiplex Ligation-dependent Probe Amplification (MLPA®) technique for detecting genetic deletions and duplications in various diseases including cancer. Recently, the MLPA technique has been improved to detect methylation sites within promoter regions and for genomic imprinting applications[5]. Promoter Methylation kits from MRC-Holland include ME001B Tumor Suppressor, ME002 Tumor Suppressor, and ME011 for MisMatch Repair genes. Genomic Imprinting kits from MRC-Holland include ME028 PWS-AS and ME030 BWS-RSS. GeneMarker's new Methylation Specific – MLPA (MS-MLPA) module quickly and accurately detects methylation sites for researchers studying promoter methylation and genomic imprinting diseases. GeneMarker's ease of use and professional reporting options are an excellent choice for MS-MLPA applications.

Procedure

1. Launch GeneMarker software
2. Import raw capillary electrophoresis data
3. Apply size standard and peak filtering options with Run Wizard
4. Select a panel in Panel Editor and align Markers and Bins accordingly
5. Run panel with size called samples
6. Select **Applications → MS-MLPA**
7. The MS-MLPA Analysis Settings box will appear
8. Import grouping information created in the Filename Group Tool into the Group File field
9. Select an MS-MLPA Analysis Type

Genomic Imprinting

9. Select Genomic Imprinting Analysis Type
10. Choose to analyze by Peak Height OR Peak Area
11. Enter the Peak Ratio values between which a peak will be considered normal
12. Click OK
13. Analyze data for non-control peaks above the Other Peak Ratio set in the Analysis Settings box
14. Click the Print icon to create an MS-MLPA Clinical Report

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Promoter Methylation

10. Select Promoter Methylation Analysis Type
11. Choose to analyze by Peak Height OR Peak Area
12. Enter the Peak Ratio value below which a peak will not be considered methylated
13. Click OK
14. Analyze data for non-control peaks above the Methylated Peak Ratio set in the Analysis Settings box
15. Click the Print icon to create an MS-MLPA Clinical Report

Results

The MRC-Holland MS-MLPA kits take advantage of the methylation sensitive endonuclease HhaI which will cleave unmethylated DNA fragments. Methylated fragments are not digested by the endonuclease and will therefore produce a peak signal in the electropherogram. In addition to novel methylation detection probes, MRC-Holland includes multiple control probe genes in the MS-MLPA kit for monitoring the efficiency of the endonuclease activity.

Promoter Methylation

In Figure 1, the undigested patient DNA sample appears as the red background trace in the electropherogram and is used as the reference. The same patient's digested DNA sample appears as the blue trace in the electropherogram. Based on the Quantification method chosen in the MSMLPA Analysis Settings box (Peak Height or Peak Area); the digested sample trace is compared to the undigested reference trace. The ratio value of the comparison is plotted by basepair size in the Ratio Plot below the electropherogram.

In the Ratio Plot, the blue plot points represent the control probe peak ratios. As expected, the control probes of the sample trace are in a 1:1 ratio with the control probe peaks of the reference trace. A green threshold line appears at Ratio = 0.3 and represents the Methylated Peak Ratio value set in the MS-MLPA Analysis Settings box. Green plot points below this threshold do not contain a significant peak in the sample trace compared to the reference trace because the fragment was digested by the endonuclease. Since the fragment was digested by the endonuclease, it is assumed the gene is unmethylated. Red plot points that appear above the green threshold line represent an amplified undigested fragment. Undigested fragments, which are not control probes, are considered sites of methylation. In Figure 1, one copy of gene ESR1 is methylated because its representative red plot point appears above the Methylated Peak Ratio threshold line at around Ratio = 0.5
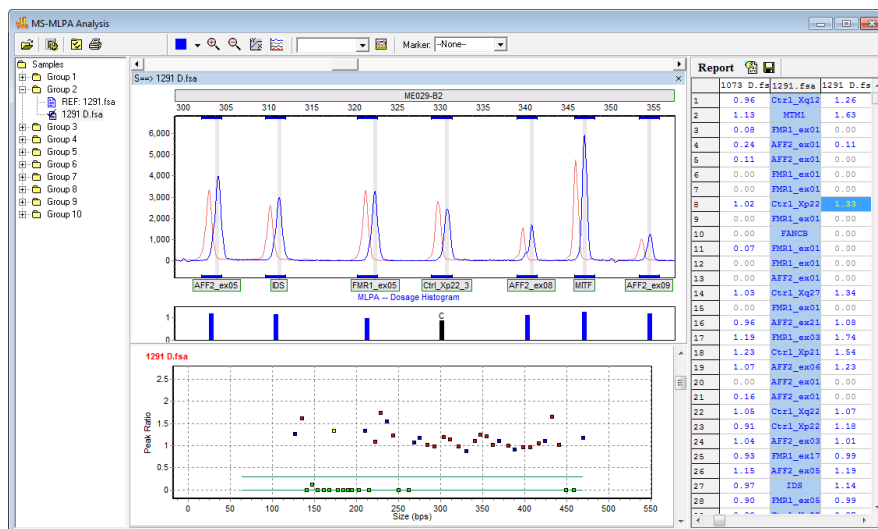

Figure 1: Methylation of the promoter region of tumor suppressor gene ESR1 is indicated by the red plot point that appears above the methylation threshold line.

Genomic Imprinting

Prior to detecting genomic imprinting with MS-MLPA, a researcher must determine if the genes of interested have been deleted with the traditional MLPA technique. Once the copy number has been determined, digestion with methylation sensitive endonucleases can be performed to detect imprinting[6].

In Figure 2, a normal patient's sample appears on the left and a sample from a patient with Prader-Willi Syndrome appears on the right. In the normal patient's sample, five green plot points appear between the green methylation threshold lines. These plot points represent five genes of interest located in the PWS/AS region on chromosome 15. As discussed previously, unmethylated fragments are completely digested by the methylation sensitive endonuclease and methylated fragments remain undigested. The five plot points that appear around Ratio = 0.5 represent genes that contain one methylated allele and one unmethylated allele. A normal patient, without a deletion at the PWS/AS site, will receive one copy of the methylated region from the mother and one copy of the unmethylated region from the father.

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In the sample from the patient with PWS, it is observed that the peak ratios for the five methylationspecific fragments are in a 1:1 ratio with the undigested fragments. It was determined previously using copy-number-change MLPA analysis that this patient's PWS/AS region contained a deletion of one chromosome. In the PWS case, since the remaining gene fragments were not digested, it is deduced that the remaining chromosome is methylated and therefore comes from the mother. The father's PWS/AS genes have been deleted.
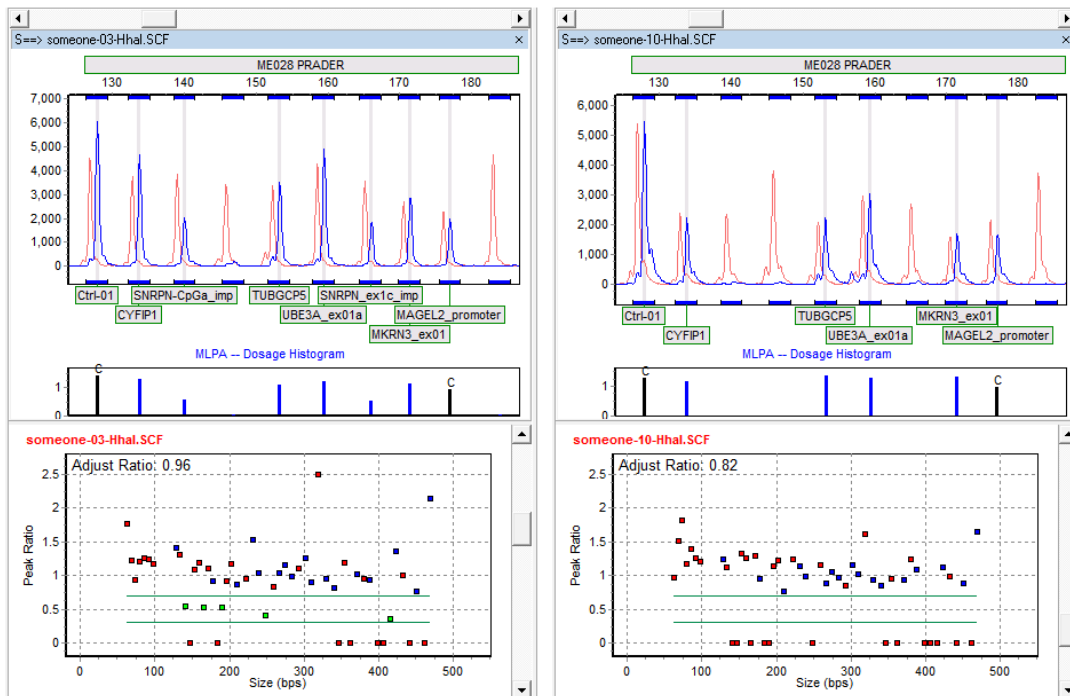
Figure 2: The image on the left is a normal patient sample. Five genes of interest (methylationspecific) from the PWS/AS region of chromosome 15 appear in the "Normal" region between the methylation detection threshold lines. The image on the right is a sample from a PWS patient. The five genes of interest are in a 1:1 ratio with the reference trace indicating methylation at these five sites (one copy of 15q11.2 and one copy of 15q12 have been deleted).

Discussion

Promoter Methylation

It is important to consider, when working with cancer samples, the possibility of "contamination" with normal cells. When screening for methylation sites in the promoter region of tumor suppressor or mismatch repair genes, the methylation signal may be reduced due to the presence of unmethylated, normal cells in the sample. In Figure 1, a green plot point appears just below the methylation threshold line which was set at Ratio = 0.3. To determine whether a plot point that appears above zero but below the methylation threshold should be considered a methylation site, it is necessary to evaluate first the resolution and quality of the trace. If resolution and quality are good, then it is possible that the peak signal intensity was reduced due to the presence of unmethylated, normal cell DNA in the sample. Careful laboratory procedures and researcher experience are important factors in making accurate methylation determinations.

Genomic Imprinting

As mentioned previously, it is important to first determine copy number change in the region of interest with the classic MLPA technique prior to analyzing for methylation sites with MS-MLPA. If classic MLPA indicates gene deletion in the area of interest, then MSMLPA may be informative for genomic imprinting determination. However, if classic MLPA does not indicate a deletion in the region of interest, it is still possible the phenotypic symptoms will appear. In this case, it is important to consider uniparental disomy or UPD. UPD is the inheritance of two copies of a chromosome or gene region from the same parent. UPD can occur as a random event during the formation of egg or sperm cells or may happen in early fetal development. Uniparental inheritance of an imprinted gene can result in the loss of gene function which can lead to conditions such as Prader-Willi and Angelman Syndromes. Detection of genomic imprinting with MS-MLPA technique will remain the same in the case of UPD. Two methylated alleles from a maternal disomy will appear in the case of PWS or two unmethylated alleles from a paternal disomy will indicate AS[7].

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Mutation Surveyor® for Sequence Methylation Analysis:

Mutation Surveyor is a powerful software using a unique physical trace comparison technology to detect nucleotide changes between two sequence traces. Mutation Surveyor has a new function to simplify the analysis of DNA methylation using bisulfite conversion of cytosine to uracil followed by nucleotide sequencing.

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