Journal of Molecular Diagnostics, Vol. 11, No. 2, March 2009

DOI: 10.2353/jmoldx.2009.080109

Technical Advance

High Quality Assessment of DNA Methylation in Archival Tissues from Colorectal Cancer Patients Using Quantitative High-Resolution Melting Analysis

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High-resolution melting (HRM) analysis is a novel tool for analysis of promoter methylation. The aim of the present study was to establish and validate HRM analysis for detection of promoter methylation on archival formalin-fixed paraffin-embedded tissues from colorectal cancer patients. We first evaluated HRM assays for O^6-methylguanine-DNA methyltransferase (*MGMT*) and adenomatous polyposis coli (*APC*) promoter methylation on a methylated DNA dilution matrix and DNA extracted from eight fresh or formalin-fixed paraffin-embedded human cancer cell lines. Then we used these assays for the analysis of *MGMT* and *APC* promoter methylation in a subset of archival formalin-fixed paraffin-embedded colorectal tumor specimens. All samples with promoter methylation of *MGMT* or *APC* and randomly selected samples without promoter methylation were analyzed twice. All results generated by HRM were validated with *MGMT* and *APC* MethyLight assays. *APC* and *MGMT* promoter methylation data were consistent and reproducible throughout the dilutions and all three replicates in the methylated DNA dilution matrix and between two experiments in clinical samples. There was high concordance between HRM and MethyLight results. HRM for *APC* promoter methylation revealed consistent results between fresh and formalin-fixed paraffin-embedded human cancer cell line DNA. The methylation status in archival tumor specimens from patients with colorectal cancer can therefore be determined with high quality by HRM. The ability to analyze archival tissues greatly facilitates further research and its clinical implementation. ***(J Mol Diagn 2009, 11:102–108; DOI: 10.2353/jmoldx.2009.080109)***

Promoter hypermethylation is one of the hallmarks of carcinogenesis associated with transcriptional silencing and loss of expression of genes encoding for diverse cellular pathways.[1] Most of the evidence exists for tumor suppressor genes.[2,3] DNA-methylation-based assays are promising tools for detection of biomarkers for early cancer diagnosis, risk assessment, and response to therapy.[1,4] As a result, a variety of methods to detect aberrant DNA methylation in cancer patients have been developed.[5]

The most popular approaches rely on treatment of genomic DNA with sodium bisulfite,[6] which converts cytosine into uracil while 5-methyl cytosine remains unmodified, thus allowing identification of cytosine methylation status following PCR amplification.[5] Among the PCR-based methods, methylation-specific PCR is the most widely used technique for detection of methylation. This assay uses primers specific for methylated, bisulfite-modified DNA.[7] One major drawback of methylation-specific PCR is that it provides only qualitative information regarding the methylation status of the analyzed sequence and, therefore, cannot distinguish low versus high levels of methylation. Quantitative measurement of methylation is important because low levels of methylation (below the threshold of transcriptional silencing) may not be biologically important.[8,9] Also, quantification of promoter methylation may enable early detection of cancer and early metastatic spread.[10]

To enhance specificity and sensitivity of DNA-methylation based assays, alternative techniques have been developed and widely used in basic research. These

Supported by the Austrian National Bank Fund (grant 12680 to N.D.).

Accepted for publication December 12, 2008.

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include fluorescence-based real-time PCR analysis (MethyLight), as a quantitative and high throughput technology, and bisulfite sequencing of multiple clones. However, they are neither cost-effective nor fast enough to be implemented for routine clinical diagnosis.[11]

Thus, there is a clear need for development of a more reliable method for DNA methylation assays. A new approach, high-resolution melt curve analysis (HRM), has recently been reported, and is based on the "melting" properties of DNA in solution.[12] Originally it was developed for single-nucleotide polymorphism genotyping.[13] The principle of this method is that bisulfite-treated DNA templates with different contents of methylcytosine can be resolved by melting analysis due to differences in melting temperatures.[14] HRM offers several advantages over the widely used MethyLight assay.[12] The use of probes in MethyLight assays increases the costs of experiments. Also, quantitative MethyLight requires normalization against a reference gene, which needs to be run for each sample. HRM, by contrast, does not require expensive probes, and no reference gene for normalization, making the experiment relatively simple and cost-effective. Another important aspect is that HRM scans all of the CpGs flanked by the primers binding to the target sequence, regardless of the methylation status of CpGs in the primer-binding site, while MethyLight detects methylation of CpG sites covered by the primers and probes. This enables HRM to distinguish heterogeneous from homogeneous methylation by the shape of the melting curve. This factor is of importance because methylation patterns at promoter CpG islands are typically not homogeneous.[15,16]

Archival tissues represent an enormous source for testing of clinically important issues and most previously mentioned methods have been tested for their performance on formalin-fixed paraffin-embedded (FFPE) tissues. The aim of this study was to validate HRM for methylation status detection on archival FFPE tissues from colorectal cancer patients. All results generated by HRM were validated with MethyLight assays. As a proof of principle we demonstrated HRM using assays for *O*6-methylguanine-DNA methyltransferase (*MGMT*) and adenomatous polyposis coli (*APC*) promoters in a methylated DNA dilution matrix and DNA isolated from fresh and FFPE human cancer cell lines. In a second step, HRM assays were validated in a clinical setting using archival FFPE colorectal tumor specimens. To our knowledge this is the first report on the use of HRM for detection of the promoter methylation status on FFPE tissues.

Materials and Methods

Controls and Patient Samples

CpGenome Universal Methylated DNA (Chemicon, Millipore Billerica, MA, USA) was used as 100% methylated control DNA. DNA extracted from peripheral blood mononuclear cells of normal individuals was used as unmethylated control DNA. Methylation standards were constructed by diluting 100% methylated bisulfite-modified control DNA in a pool of normal bisulfite-modified DNA at ratios of 50%, 25%, 10%, 5%, 1%, and 0.1%. These standards were included in each experimental run.

In addition, the following eight human cancer cell lines were used for validation experiments: human breast cancer cell lines MCF-7, MDA-MB-231, SKBR3, T47D, and MDA-MB-453, and human prostate cancer cell lines DU145, LNCAP, and PC3. All cell lines were obtained from the American Tissue and Cell Collection (Manassas, VA, USA) and were cultured according to the supplier's instructions. We used cell lines obtained either directly from cultures or after formalin-fixation and paraffin-embedding, as adapted from the published protocol by Kerstens et al.[17]

Surgically resected tissues were collected from 66 colorectal carcinoma patients (48 male and 18 female) at the Surgical Department of the Danube Hospital, Vienna, Austria. Median age at surgery was 67 years (range, 41 to 87 years). The cancers were classified according to the UICC TNM guidelines.[18] Seventeen patients were UICC I, Dukes A (25.8%), 11 patients were UICC II, Dukes B (16.7%), 20 patients were UICC III, Dukes C (30.3%), 12 patients were UICC IV, Dukes D (18.2%), and 6 patients (9.1%) had local relapses. Additionally, 9 normal tissue samples were analyzed. The study was approved by the local Ethics Committee.

Extraction of Genomic DNA

Healthy volunteers' peripheral blood mononuclear cell DNA and DNA from cultured cancer cell lines was isolated using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the supplier's recommendation.

For patient tumor samples, an appropriate paraffin block containing tumor tissue was selected for analysis after reviewing the H&E-stained slides. An area of tumor on the H&E-stained slide was identified on a corresponding unstained slide and circled with an indelible fine-tipped pen. DNA was isolated from material scraped from the unstained slide as previously described.[19] Genomic DNA from FFPE cancer cell lines was isolated using the same protocol as for patient tumor samples. DNA quantity was assessed spectrophotometrically and quality of genomic DNA was confirmed by agarose gel electrophoresis.

Sodium Bisulfite Modification

One μg of genomic DNA was subjected to bisulfite conversion with the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The eluted DNA (40 μl volume) was used for the HRM and MethyLight analysis.

MethyLight Assay

The MethyLight assays for *MGMT* and *APC* have been described previously.[11,20,21] Briefly, PCR was performed on