Screening for DNA Hypermethylation Using the LightCycler Instrument

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Introduction

Alterations of DNA methylation patterns are frequent in human cancers and are now regarded as an important factor in carcinogenesis [1]. In particular, CG-rich regions surrounding the transcriptional start site of many genes (CpG islands) are largely exempt from methylation in normal cells, but become hypermethylated in the genome of cancer cells. CpG-island hypermethylation is typically associated with transcriptional silencing of the respective gene or promoter. Therefore, DNA hypermethylation is a mechanism that needs to be investigated if down-regulation of a gene is observed in cancers, but also in other diseases. Several methods are available for this purpose (see [2] for an overview). Nowadays, most researchers rely on bisulfite treatment of DNA, which converts cytosines to uracils and leaves methylcytosine unaffected. Therefore, unmethylated cytosines appear as thymines in subsequent polymerase chain reactions (PCRs), whereas methylcytosines are maintained as cytosines. The gold standard is bisulfite sequencing, which yields information on every methylatable CpG site within investigated sequences. This method is, however, rather elaborate and expensive.

A faster and more convenient method is methylation-specific PCR, which can be used for genes well-characterized by bisulfite sequencing. There is, however, a need for a rapid and cheap method to screen candidate genes for hypermethylation prior to extensive bisulfite sequencing. For this purpose, we have found a denaturing high-performance liquid chromatography (dHPLC) technique very useful [3]: Heteroduplex DNAs formed from unmethylated and methylated sequences treated with bisulfite can be sensitively detected. This method is rapid and convenient, but some experience with the dHPLC technique and, of course, with the equipment is a prerequisite. To establish a simpler method, we have explored melting temperature analysis using the LightCycler Instrument.

This method is straightforward. Following bisulfite treatment and PCR amplification using the LightCycler Instrument, the products from methylated and unmethylated DNA differ in GC content. This results in a detectable difference in melting points that is sufficient to distinguish fully methylated DNA from fully unmethylated DNA [4]. However, hypermethylation is often partial, and DNA from tissues and cell lines often contains mixtures of unmethylated and methylated DNA. Therefore, the usefulness of the method depends on its ability to identify methylation changes at some sites and in a fraction of DNA.

Materials and Methods

High-molecular-weight genomic DNA from whole blood samples and cancer cell lines was isolated using a blood and cell culture DNA kit. Extensive digestion with proteinase K was used to avoid artifacts from incomplete bisulfite conversion.

Specifically methylated DNA samples were generated from genomic DNA isolated from blood leukocytes by specific *in vitro* methylation using *Hpa*II methylase, *Hha*I

gcttgtgatc ttttcagttt ctccagctgc tggctttttg gacacccact cccccgccag 1921

gaggcagttg caagCGCGga ggctgCGaga aataactgcc tcttgaaact tgcagggCGa 1981

agagcaggCG gCGagCGctg ggcCGgggag ggaccaccCG agctgCGaCG ggctctgggg 2041

ctgCGgggca gggctggCGc cCGgagcctg agctgcagga ggtgCGctCG ctttcctcaa 2101

caggtggCGg CGgggCGCGC GcCGggagac ccccctaat gCGggaaaag caCGtgtcCG 2161

catttagag aaggcaaggc cggtgtgttt atctgcaag

Figure 1: Unmodified sequence from the promoter region of the estrogen receptor β gene *(ESR2)* (*capital letters*: CpG sites *red*: *Hha*I sites *green*: *Hpa*II sites *underlined*: unmodified forward and reverse primer sequence)

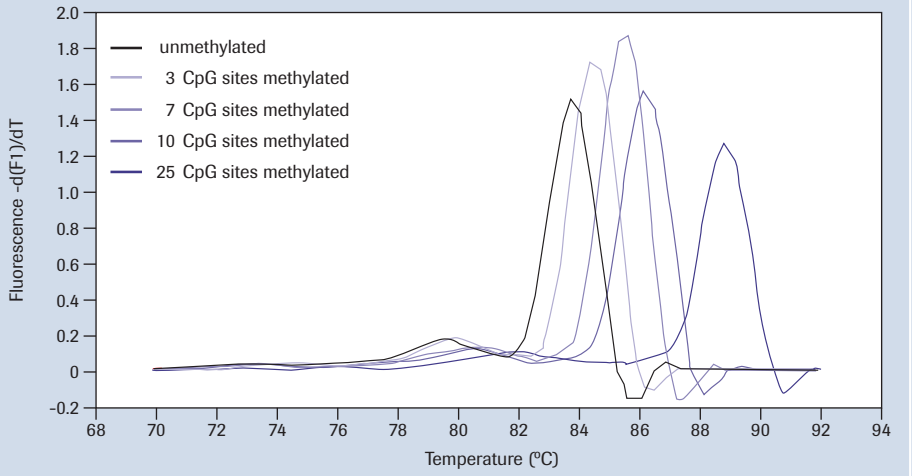

Figure 2: Melting-curve analysis for *ESR2*. Melting peaks of PCR products from leukocyte DNA und DNA treated with *Hpa*II, *Hha*I or *Sss*I methylases, resulting in methylation of 3, 7, 10, or 25 CpG sites.

methylase, or *Sss*I methylase (NEB) according to the manufacturer's recommendations.

For bisulfite conversion of DNA, the CpGenome DNA Modification Kit (Q-Biogene) was used, following the protocol provided by the manufacturer.

A 273-bp fragment of the estrogen receptor β gene (*ESR2)* promoter region corresponding to residues 1,912-2,185 (GenBank Accession No. AF191544) containing 25 CpGs was amplified in the LightCycler Instrument following bisulfite treatment (Figure 1). The PCR amplification was performed in a 10-µl volume using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit, 20 ng bisulfite-treated DNA, 10 pmol primer Biseq-ESR2-fwd 5'-tt(ct)gttaggaggtagttgtaag-3' and Biseq-ESR2-rev 5'-cacc(ag)accttaccaactctaaaata-3'. The initial denaturing step at 95°C for 10 minutes was followed by 50 cycles of 95°C for 10 seconds, 58°C for 5 seconds and 72°C for 15 seconds. The melting-curve analysis was performed at 68°C according to the protocol supplied with the kit.

Results

We used a sequence from the estrogen receptor β gene (*ESR2*) CpG island to study the sensitivity of melting-curve analysis with the LightCycler Instrument. This *ESR2* sequence is hypermethylated in some prostate and breast cancers [5, 6]. As shown in Figure 1, the 273-base pair fragment amplified contains 25 CpG sites, three of which are located in *Hpa*II sites (CCGG) and seven are located in *Hha*I sites (GCGC). The sequence is completely unmethylated in leukocyte DNA from young healthy individuals.

DNA from healthy individuals was methylated *in vitro* using *Hpa*II methylase, *Hha*I methylase, a combination of these methylases, or *Sss*I methylase. These treatments introduce methylation at 3, 7, 10, or 25 sites. The melting-curve analysis of the PCR products from these DNAs is shown in Figure 2. Complete methylation shifted the melting point by 5.1°C from 83.7°C to 88.8°C. Partial methylation yielded less pronounced differences, but methylation of only three sites resulting in a shift of 0.7°C was well distinguished. Even partial methylation was sensitively detected.

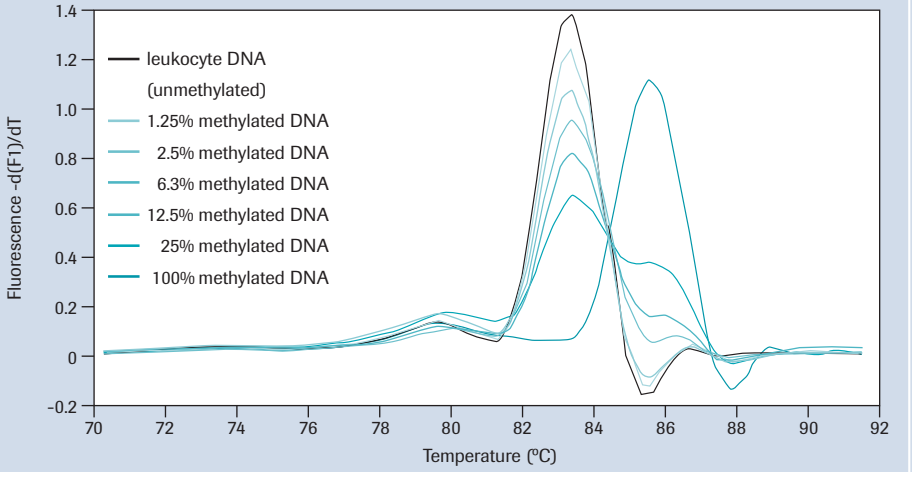

Figure 3: Melting-curve analysis for *ESR2*. Melting peaks of PCR products from leukocyte DNA with different admixtures of DNA treated with *Hpa*II and *Hha*I methylases.

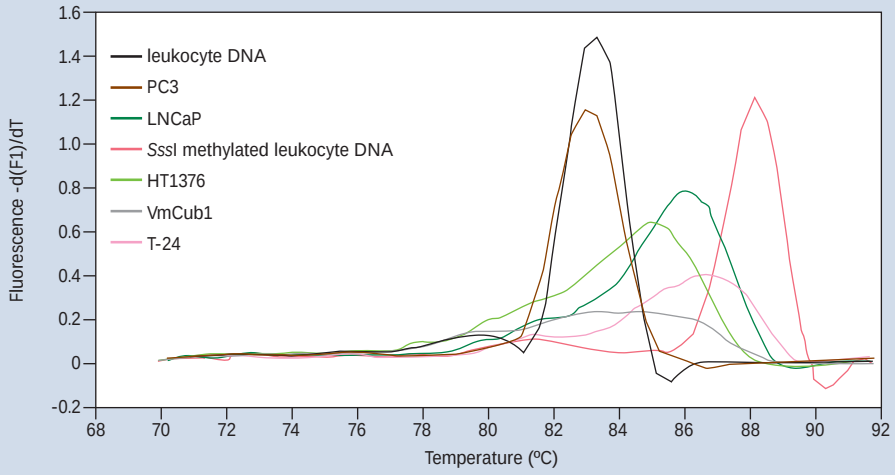

Figure 4: Melting-curve analysis for *ESR2*. Melting peaks of PCR products from different cancer cell lines. As an unmethylated control leukocyte DNA was used. As a methylated control, *Sss*I-treated leukocyte DNA was used.

To ensure that melting-curve analysis with the LightCycler Instrument detected a fraction of methylated DNA against a background of unmethylated DNA, leukocyte DNA was mixed with various amounts of DNA methylated with *Hpa*II and *Hha*I methylases (Figure 3). Admixture of 5–10% of methylated DNA resulted in detectable peak "shoulders" in the melting-curve analysis.

In contrast to DNA methylated by bacterial methylases *in vitro*, DNA from tissue or cell lines represents a mixture of DNAs with varying methylation patterns – if hypermethylation takes place at all. To assess the suitability of melting-curve analysis for such "real-world" samples, DNA from a range of cell lines was employed. These samples often yielded broader curves, as expected for DNA with varying degrees of hypermethylation (Figure 4). Some cell lines and tissues presented with a peak that clearly corresponds to unmethylated DNA (*e.g.*, the prostate cancer cell line PC3). Other peaks, however, extended towards the methylated control. For instance, the prostate cancer cell line LNCaP, known to contain a hypermethylated *ESR2* CpG island from bisulfite sequencing analysis, peaked at >86°C. Interestingly, and somewhat unexpectedly, several bladder cancer cell lines were found to harbor substantial degrees of hypermethylation, as exemplified by HT1376 and T-24 in Figure 4. According to this analysis, VmCub1 appears to contain unmethylated as well as methylated alleles.

Conclusion

The results indicate that melting-point analysis following bisulfite treatment and PCR using the LightCycler is well-suited to screening for DNA hypermethylation of CpG islands in cancer and other diseases. As few as three out of 25 sites and 5–10% methylated DNA were sufficient to detect hypermethylation in the exemplary sequence used. It may be possible to further increase the level of sensitivity by using shorter amplification products with higher CpG content, different salt conditions, or other DNA stains. However, the present level of sensitivity should be quite sufficient to detect physiologically relevant hypermethylation in tumor tissues.

The high sensitivity of the technique is somewhat unexpected in view of theoretical considerations [4]. This is probably due to the fact that hypermethylation of DNA results in an increased GC content after bisulfite treatment. Therefore, PCR products from methylated DNA present as novel peaks at increased temperature compared with peaks obtained under normal circumstances. Any contaminant products, such as primer multimers, however, melt at lower temperatures. Therefore, the background level at higher temperatures is low, and even small shifts and minor shoulders can be relatively easily discerned.

In the present investigation, a simple screen revealed the presence of hypermethylated *ESR2* sequences in bladder cancer cell lines. Estrogen receptors are well-known to play a role in bladder function, but have hardly been studied in the context of bladder cancer. Thus, this screen raises the interesting question of whether such changes also occur in the respective tissues and may be relevant for tumorigenesis. ■

References

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Order INFO

Product	Pack Size	Cat. No.
LightCycler 2.0 Instrument	1 instrument	03 531 414 201
FastStart DNA Master^{PLUS} SYBR Green I Kit	1 kit (96 reactions)	03 515 869 001