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Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation

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ABSTRACT

In this article, we show that high resolution melting analysis (HRM) is a sensitive and specific method for the detection of methylation. Methylated DNA and unmethylated DNA acquire different sequences after bisulphite treatment resulting in PCR products with markedly different melting profiles. We used PCR to amplify both methylated and unmethylated sequences and assessed HRM for the determination of the methylation status of the *MGMT* promoter region. Reconstruction experiments showed that *MGMT* methylation could be detected at levels as low as 0.1%. Moreover, MS-HRM allows for estimation of the methylation level by comparing the melting profiles of unknown PCR products to the melting profiles of PCR products derived from standards with a known unmethylated to methylated template ratio. We used MS-HRM for the analysis of eight cell lines of known methylation status and a panel of colorectal cancer specimens. The simplicity and high reproducibility of the MS-HRM protocol makes MS-HRM the method of choice for methylation assessment in many diagnostic and research applications.

INTRODUCTION

Methylation of cytosines allows the encoding of epigenetic information directly onto the DNA. In the human genome, methylated cytosines are found in CpG dinucleotides whose palindromic nature allows for the maintenance of methylation patterns by DNA methyltransferases following semi-conservative replication of DNA. Regions of DNA with a relatively high CpG dinucleotide content are referred to as CpG islands (1). CpG islands are distributed in a non-random manner across the human genome and often span the promoter region and the first exon of protein coding genes. Methylation of individual promoter region CpG islands usually acts to turn off (silence) transcription by recruiting histone deacetylases thereby inducing the formation of inactive chromatin (2).

Promoter region methylation of genes, particularly those genes with pivotal functions in relation to tumour suppression, apoptosis and DNA repair is one of the hallmarks of cancer (2). Alterations of the pattern of DNA methylation are an early event in cancer and continue on through the evolution of the cancer. Furthermore, distinct tumour types often have characteristic signatures of methylated genes (3,4) and these can be used as markers for early detection and/or monitoring the progression of carcinogenesis. More importantly, the methylation of certain genes, in particular DNA repair genes, can cause sensitivity to specific chemotherapeutics and methylation of those genes can thereby act as a predictive marker if those chemotherapeutic agents are used (5).

The methylation status of the *MGMT* gene has been shown to be a predictive marker in various cancers treated with alkylating agents (6–8). The MGMT protein removes methyl/alkyl adducts from the O^6-position of guanine and therefore protects the cell from undergoing transition mutations. The tumour-specific methylation of the *MGMT* promoter and subsequent abolition of MGMT protein activity will render tumour cells susceptible to alkylating agents used in cancer chemotherapy. Consistent with this, the survival of patients whose tumour was methylated at the *MGMT* promoter was significantly longer than that of patients with tumours that did not show methylation of *MGMT* when those patients were treated with alkylating agents (6–8).

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Currently, no methylation detection method has been established for reliable, fast and cost-effective locus specific methylation testing that is readily applicable for both research and diagnostic settings. The research-based methods have various limitations and pitfalls and contradictory results can be obtained using different protocols, therefore none of them have found ready applicability in diagnostics (9).

A new more reliable method for promoter methylation analyses in clinical samples is needed. The new approach reported here is based on high resolution melting (HRM) which was originally developed for SNP genotyping (10). HRM relies upon on the precise monitoring of the change of fluorescence as a DNA duplex melts. Like many real-time PCR techniques, HRM utilizes the ability of certain dyes to fluoresce when intercalated with double-stranded DNA. Two advances have made high resolution melting possible. The first is the introduction of intercalating dyes that do not inhibit PCR reactions at the concentrations necessary for them to fully saturate the target DNA duplexes (10). The second is the development of instrumentation that is able to monitor the changes of fluorescence with high accuracy.

We have applied HRM technology to the detection of methylation. By comparing the melting profiles of unknown samples with the profiles of fully methylated and unmethylated references amplified after bisulphite modification, we were able to detect methylation with high sensitivity and moreover estimate the extent of methylation of the screened samples.

MATERIALS AND METHODS

DNA samples and controls

Colorectal cancer samples were provided by the Peter MacCallum Cancer Centre Tissue Bank. DNA was extracted from those samples by using the DNeasy Tissue Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA from cell lines was purified by using the salting out method (11). As a positive/methylated control in our experiments, we used CpGenome^{TM} Universal Methylated DNA (Chemicon, Millipore, Billerica, MA). DNA from peripheral blood mononuclear cells was used as a negative/unmethylated reference. To create the range of methylated and unmethylated allele dilutions, the above two controls were mixed in 0.1, 1, 10 and 50% methylated to unmethylated template ratios. Each of our experimental runs included the range of methylated/unmethylated standards.

Bisulphite modification

We used the MethylEasy^{TM} Kit (Human Genetic Signatures, Sydney, Australia) for bisulphite modification of the DNA. The starting amount of DNA for all the bisulphite modifications was 1 μg and all the modification reactions were performed according to the manufacturer's protocol with the exclusion of the second 70% ethanol wash.

High resolution melting analysis (HRM)

PCR amplification and high resolution melting analysis were carried out sequentially on a Rotor-Gene^{TM} 6000 (Corbett Research, Mortlake, Australia). PCR was carried out in a 20 μl total volume containing: 1× Buffer, 4 mM Mg^{+2}, 200 μM of each of the four dNTPs, 250 nM of each primer, 5 μM Syto9 dye (Invitrogen, Carlsbad, CA), 1U HotStarTaq polymerase (Qiagen) and 1 μl of bisulphite modified template (theoretical concentration 20 ng/μl). The amplification consisted of 15 min at 95°C, followed by 50 cycles of 5 s 95°C, 5 s at the primer annealing temperature (T_a) and 10 s at 72°C. High resolution melting analyses were performed at the temperature ramping and florescence acquisition setting recommended by the manufacturer i.e. temperature ramping from 70–95°C, rising by 0.1°C/2 s. All the reactions were performed in triplicate. The melting curves were normalized by calculation of the 'line of best fit' in between two normalization regions before and after the major fluorescence decrease representing the melting of the PCR product using the software provided with the Rotor-Gene^{TM} 6000. This algorithm allows the direct comparison of the samples that have different starting fluorescence levels.

MGMT MethylLight assay

The *MGMT* MethylLight assay used for validation of our experiments was as previously reported (12). The reaction consisted of: 1× Buffer, 4 mM Mg^{+2}, 200 μM of each of the four dNTPs, 500 nM of each primer, 200 nM of probe, 1U HotStarTaq polymerase and 1 μl of bisulphite-modified template (theoretical concentration 20 ng/μl). The real-time amplifications were carried over and analysed on a Rotor-Gene^{TM} 3000 machine (Corbett Research). After 15 min at 95°C, 50 cycles of 95°C for 15 s and 60°C for 30 s were performed. The assay was optimized on the same range of methylated/unmethylated template mixes as in MS-HRM analyses. All the PCR amplifications were performed in triplicate.

MGMT MS-HRM and *BNIP3* MS-HRM assays

The primer sets for all MS-HRM assays were designed according to the principles recently set out to compensate for PCR bias (13). The primers were designed to amplify both methylated and unmethylated template. The primers used and the amplified sequences are shown in Table 1.

RESULTS

The sensitivity of the MS-HRM assay

The sensitivity of the *MGMT* MS-HRM assay was tested by using dilutions of fully methylated control DNA into peripheral blood DNA. The inclusion of CpGs in the primer sequence gave us the possibility to direct the PCR bias towards the methylated templates by manipulating the annealing temperature of PCR amplification and therefore making our assays more sensitive for methylation detection. All assays showed annealing temperature-dependent sensitivity (Figure 1). The first assay that we designed (*MGMT* MS-HRM1) targeted a 175-bp long

Table 1. Primers, regions amplified and amplicon's information for the MS-HRM assays

Assay name	Primer sequences	Position (UCSC Genome Browser, March 2006)	Screened CpGs/amplicon length
MGMT MS-HRM1	F- GCGTTTCGGATATGTTGGGATAGT R- CCTACAAAACCACTCGAAACTACCA	chr10:131,155,459-131,155,631	18/173 bp
MGMT MS-HRM2	F- GCGTTTCGGATATGTTGGGATAGT R- AACGACCCAAACACTCACCAAA	chr10:131,155,459-131,155,568	12/109 bp
MGMT MS-HRM3	F- CGTTTGCGATTTGGTGAGTGTT R- CCTACAAAACCACTCGAAACTACCA	chr10:131,155,538-131,155,631	5/94 bp
BNIP3 MS-HRM	F- GGTTGCGGGATGTGTTTTAGTTG R- ACCCCGCCCTACCCTATAAATTC	chr10:133,645,340-133,645,467	13/128 bp

fragment of the *MGMT* promoter (Table 1). This assay did not give reproducible methylation signals at the 0.1% methylation measurement point. We addressed this by redesigning the primers to amplify shorter fragments of the template. Two new assays *MGMT* MS-HRM2 and *MGMT* MS-HRM3 amplified fragments of 109 and 94 bp, respectively (Table 1). Both *MGMT* MS-HRM2 and *MGMT* MS-HRM3, when run at the annealing temperature that significantly favoured amplification of methylated template, were able to reproducibly detect methylation in the samples containing 0.1% methylated template shown in Figure 2B and C.

Profiling of methylation content of the samples by MS-HRM

We tested the consistency of normalized melting profiles derived from samples with different ratios of methylated and unmethylated template. The normalized melting profiles of the PCR product amplified from the same mix of methylated and unmethylated template were consistent between replicates and between different runs (data not shown). Furthermore, the shapes of normalized melting profiles were amplification independent as samples with different starting amount of template displayed very similar profiles.

The consistency of HRM profiles allows the design of MS-HRM for estimation of the methylation content of unknown samples on the basis of similarities of normalized HRM profiles. Nevertheless, when designing MS-HRM, the length and the number of differences between methylated and unmethylated PCR products needs to be taken into account. A short product will give high sensitivity but limited resolution between different levels of methylation because of the smaller differences in melting profiles between methylated and unmethylated products (Figure 2 and unpublished data). On the other hand, a longer product will give readily distinguishable HRM profiles for PCR products derived from samples with different ratios of methylated and unmethylated template which will allow for the estimation of the methylated proportion of an unknown sample on the basis of similarities of HRM profiles of standards and unknown (Figures 1, 3A and 4). However, the annealing temperature at which the PCR amplification is in equilibrium between methylated and unmethylated product has to be empirically determined prior to analyses.

Validation of MS-HRM results against the MethylLight assay

We validated the performance of the *MGMT* MS-HRM1 assay against a previously described MethylLight assay for the *MGMT* promoter region (12). In our hands, the performance of the *MGMT* MS-HRM1 assay for the detection of *MGMT* promoter region methylation was equivalent to the MethylLight assay as both of the assays gave reproducible results until the 0.1% methylation dilution (data not shown). However, the sensitivity of the *MGMT* MS-HRM2 and MS-HRM3 assays were superior to that observed for the MethylLight *MGMT* assay (12). *MGMT* MS-HRM2 as well as *MGMT* MS-HRM3 gave fully reproducible methylation signals from the standard sample containing 0.1% methylated template in the background of unmethylated DNA (data not shown).

Application of the *MGMT* MS-HRM assay to cell lines

We also tested DNA from eight cell lines (MDA-MB-468, HS578T, SW480, MDA-MB-435, MDA-MB-231, PC3, T47D and SW48) for which the methylation status of the *MGMT* promoter had been previously reported (14) by using the *MGMT* MS-HRM1, *MGMT* MS-HRM3 and MethylLight assays. Four of the eight cell lines studied (SW480, MDA-MB-435, MDA-MB-231 and SW48) showed complete (100%) methylation (Figure 3).

HS578T displayed a less characteristic melting profile which we interpreted as being due to heterogeneous methylation. Whereas there was no evidence for heteroduplex formation between PCR products arising from unmethylated and fully methylated templates, heteroduplexes could form if the individual PCR products differed at only a few bases. Therefore, the curve was differently shaped as a consequence of complex melting pattern of multiple heteroduplexes (Figure 3). The results from *MGMT* MS-HRM were consistent with MethylLight data for the cell lines (Figure 3B).

Application of the *MGMT*-MS-HRM assay to clinical specimens

The diagnostic applicability of MS-HRM assay was tested on a panel of 19 colorectal cancer samples. The *MGMT* MS-HRM3 assay was used in those experiments and we

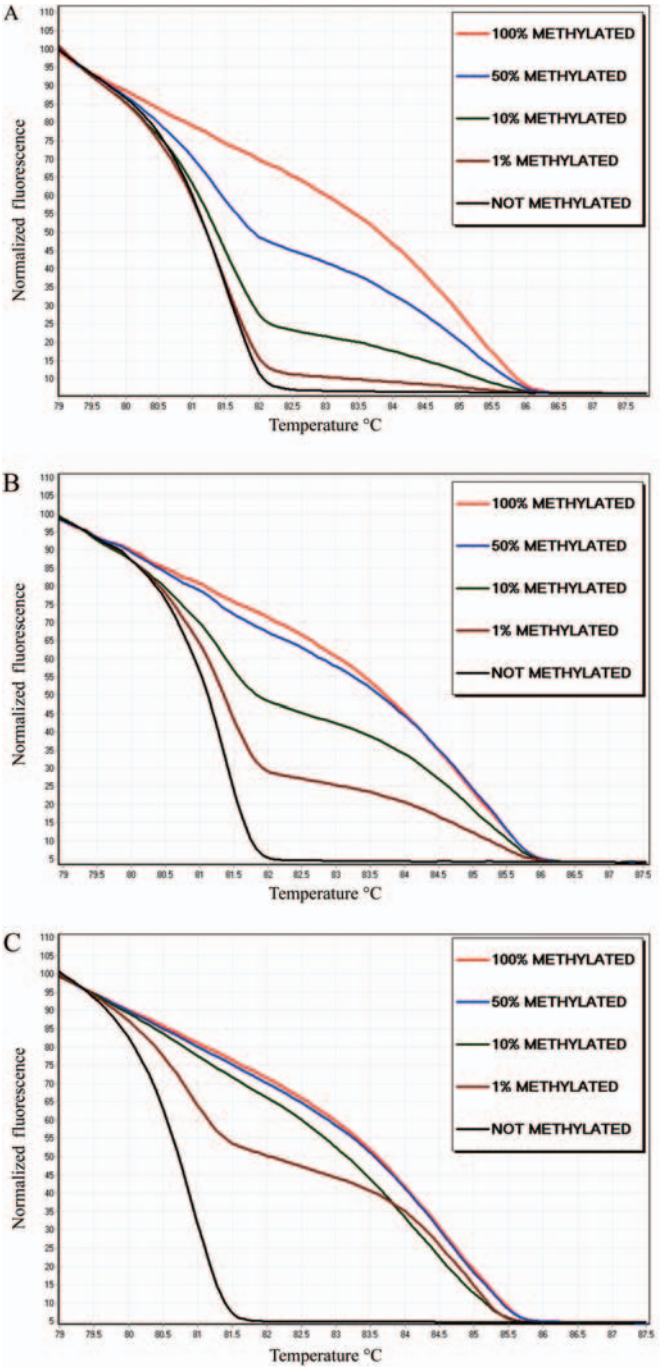

Figure 1. The effect of annealing temperature on the sensitivity of the MS-HRM assay. The *MGMT* MS-HRM1 assay was run at the following annealing temperatures. (**A**) 60°C, (**B**) 62°C and (**C**) 63°C.

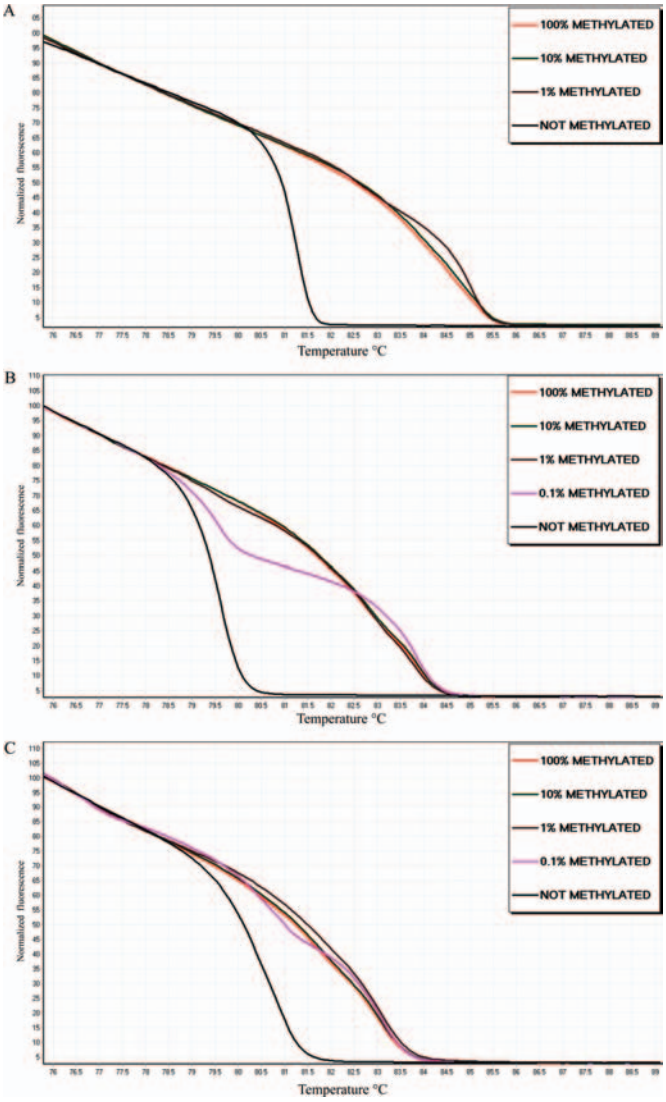

Figure 2. The sensitivity of different MS-HRM assays for *MGMT* methylation. (**A**) *MGMT* MS-HRM1, (**B**) *MGMT* MS-HRM2 and (**C**) *MGMT* MS-HRM3. All the assays were run at the annealing temperature of 64°C which enables the highest sensitivity of methylation detection. The results from the 0.1% methylation dilution for *MGMT* MS-HRM1 were not reproducible between replicates and this dilution was excluded from the figure.

also validated the MS-HRM results against the MethylLight assay (12). MS-HRM assays detected *MGMT* methylation in 8 of the 19 samples (42%). However, two of the above samples showed a very low methylation level (less than the 0.1% standard). Methylation of one of the above samples was only detectable by the *MGMT*-MS-HRM3 assay. We repeated all the runs to test the reproducibility of our results in between two different experiments and obtained identical results.

Verification of accuracy of MS-HRM approach: *BNIP3* MS-HRM assay

To verify that the MS-HRM approach is widely applicable in methylation studies, we developed a MS-HRM assay for the promoter region of *BNIP3* (Table 1). *BNIP3* has been reported to undergo aberrant methylation in various cancer types (15–17). As with the *MGMT* MS-HRM assay, we used the range of methylated/unmethylated mixes at different PCR annealing temperatures to determine the best conditions. In this model system, the *BNIP3* MS-HRM assay was able to unambiguously detect methylation at the 0.1% level (Figure 4). During evaluation of this assay, we performed the *BNIP3* MS-HRM

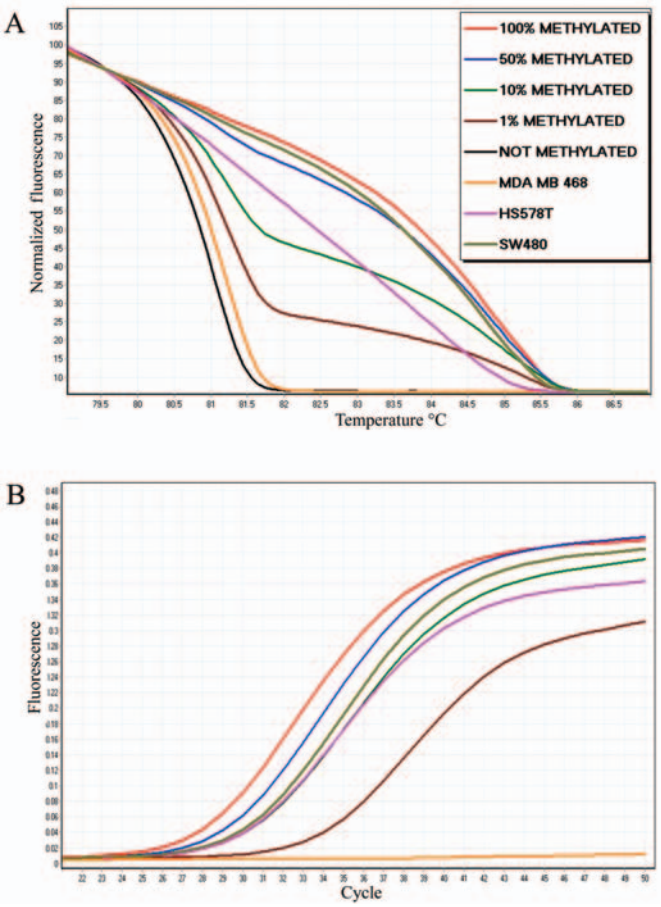

Figure 3. Validation of the *MGMT* MS-HRM1 assay by the *MGMT* MethylLight assay. The samples are the series of dilution standards and three of the cell lines (MDA MB 468, SW480 and HS578T). Panel A shows the *MGMT* MS-HRM1 assay and panel B shows the MethylLight assay. The MS-HRM assay was run at an annealing temperature of 61°C.

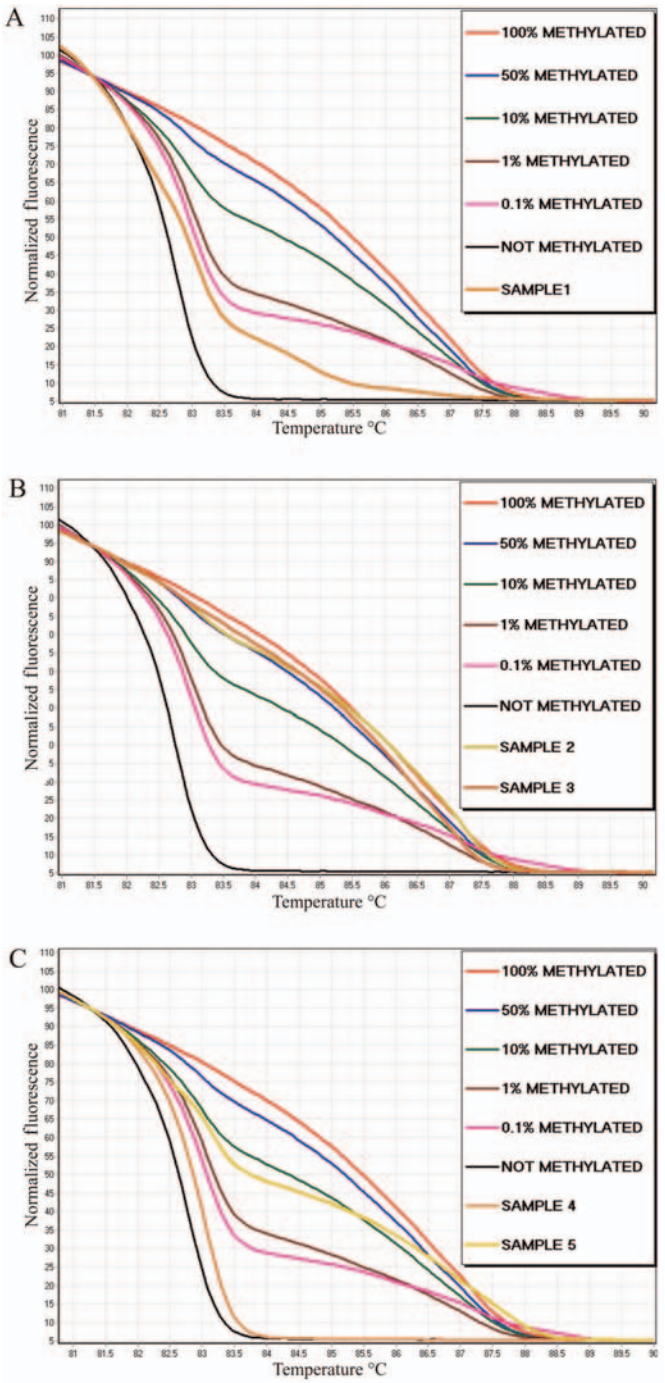

Figure 4. The MS-HRM assay for *BNIP3* methylation. Results of the *BNIP3*-MS-HRM assay for five clinical samples compared to the dilution standards. Samples 1–5 show different methylation levels. The samples have been distributed over three panels to help distinguish the individual samples.

assay for the eight cell lines used in the *MGMT* methylation study. Three of the cell lines SW480, MDA-MB-435 and SW48 were methylated at the *BNIP3* promoter (data not shown). The assay was also used to test for methylation of the panel of 19 colorectal cancer samples. Methylation of the *BNIP3* promoter sequence was detected in 12 out of 19 (63%) of colorectal cancer samples. The methylation levels of 8 of these samples (42%) were less than 10% with 3 of them displaying 0.1% or less methylation (Figure 4). All the results were reproducible between replicates (data not shown).

DISCUSSION

There are many methods for the analysis of methylation at individual loci, each with their characteristic strengths and weaknesses (9). However, only a few protocols have gained widespread use. Genomic sequencing can be considered the gold standard (18,19). It provides the most detailed information but its sensitivity is relatively low (about 20%) and it is generally unsuitable for screening because it is expensive to run, particularly when individual clones are analysed. Pyrosequencing, which has been recently introduced, is more sensitive (about 5%) but is dependent on the availability of the proprietary instrumentation (20).

The most widely used method is methylation-specific PCR (MSP) that uses primers specific for methylated, bisulphite-modified DNA (21). Unmethylated sequences are not normally amplified unless primers specific for unmethylated bisulphite modified DNA are designed. Despite its widespread use, MSP has a number of important limitations (9,22). As with other techniques that rely on PCR primer 3′ mismatching to give specificity, false positives can arise if primers are badly designed or used at too low a temperature. MSP is very sensitive but is not quantitative. This can lead to the classification of a tumour as being methylated for a gene when a small minority of cells only is positive, or more seriously, if the bisulphite conversion of the DNA is incomplete. Consistent with these reservations, in the recent trial of temozolomide in glioblastoma, it was reported that testing for *MGMT* methylation using MSP gave 'highly variable and centre-dependent' results (8).

The majority of applications in methylation studies utilize methylation-independent PCR (MIP) where the primers are designed to amplify the bisulphite-modified sequence regardless of its methylation status. However, the standard algorithms for the design of MIP primers and the protocols used do not always lead to the proportional amplification of methylated and unmethylated sequences (13,20,23). As it is often difficult to avoid CpG dinucleotides in primers designed to amplify CpG islands, some authors have suggested that the Cs in CpG sequences in primers get replaced by a mismatched base (19). Other authors have suggested that since the purpose of these assays is to detect methylation, some bias towards methylated sequences is acceptable and that a limited number of CpGs can be included in the primers, particularly if they are placed away from the 3′ end (24).

More recently, it has been shown that some CpGs are necessary in the primer sequence, otherwise PCR bias can lead to a significant underestimate of the degree of methylation (13). We have accordingly adopted the strategy of using primers containing limited numbers of CpGs and manipulating the annealing temperature to control the bias of PCR amplification in the design of MS-HRM assays. At lower annealing temperatures, the primers bind both methylated and unmethylated templates and PCR bias will favour the amplification of unmethylated sequences. At higher annealing temperatures, primer binding will favour methylated sequences, and thus at the optimal annealing temperature, amplification is effectively independent of methylation status. Therefore, MS-HRM can be used to estimate the proportion of methylation of a sample when run with standards. This is especially important when assessing clinical cancer samples for predictive markers such as *MGMT* where discrimination between tumour specimens that are methylated in all cells of the tumour from those that only show methylation in a small subset of their cells may have prognostic value. In the ideal situation, an estimate of the proportion of tumour cells in the sample will be given by pathological examination of the tissue and this can be compared with the estimated proportion of DNA that is methylated. Furthermore, if the tumour samples are of high purity, they can be used to determine whether the tumour is homozygously or heterozygously methylated. The technique would also be applicable to the diagnosis of imprinting disorders that are characterized by the abnormal methylation of imprinted genes.

High resolution melting relies on the use of high sensitivity florescence detection instrumentation, fully saturating intercalating dyes and software allowing the analysis of the melting profiles of PCR products. We developed HRM for discrimination between methylated and unmethylated sequences after bisulphite modification of the target DNA. Sodium bisulphite converts unmethylated cytosines to uracil and leaves methylated cytosines intact. Therefore, the PCR product derived from a methylated template will have a higher melting temperature than that from an unmethylated template and those differences can be resolved by melting analysis.

We have shown that HRM is applicable for the very sensitive detection of methylation in an unmethylated background. With MS-HRM, we were able to unambiguously detect the methylated fraction of DNA in samples containing as little as 0.1–1.0% of methylated DNA, the same range as seen for the MethylLight assay (25).

MS-HRM is an in-tube method meaning that the analysis takes place without the PCR product leaving the tube that it was amplified in. This is of importance for diagnostic laboratories not only because of the rapidity that it affords, but also the elimination of PCR product contamination which has proven to be a major problem both in research and the diagnostic settings.

An in-tube strategy based on the analysis of derivative peaks of melting curves of PCR products to assess methylation has already been reported (26,27). This has not become widely used presumably because of the difficulty of the suggested guidelines for assay design and the technical limitations of reagents, instrumentation and data analysis software which have now been overcome since the development of HRM methodology.

MethylLight, the other in-tube method, is used by a greater number of laboratories. It is a quantitative adaptation of MSP that uses TaqMan probes. Whereas MethylLight assays methylation of CpG sites covered by the primers and probe, MS-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 side. Thus, the results of MS-HRM are not compromised by heterogeneous methylation of a particular CpG dinucleotide or incomplete conversion of some of CpGs within the template, as the latter will fall below the limits of resolution. Heterogeneous methylation can readily be distinguished from homogeneous methylation by the shape of the curves as seen for HS678T. Moreover, the use of probes in MethylLight complicates the design and increases the costs of experiments. Also quantitative MethylLight requires normalization against a reference assay which needs to be run for each sample (24). By contrast, MS-HRM does not require a reference assay for normalization. All of the above make MethylLight experiments relatively complex and expensive.

In summary, MS-HRM is a new approach that can be readily applied to the methylation analysis of *MGMT*. It can also be readily extended to other loci as we have

shown for the the *BNIP3* locus. The sensitivity of MS-HRM allows for detection of even a very small fraction of methylated material which is of importance as tumour samples may contain a low proportion of methylated sequences due to the presence of significant amounts of normal tissue or heterogeneity of the tumour. Furthermore, the high reproducibility and cost effectiveness of HRM makes this method suitable for both research and diagnostic applications.

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