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Methylation enrichment pyrosequencing: combining the specificity of MSP with validation by pyrosequencing

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ABSTRACT

It has been suggested that detection of aberrant DNA methylation in clinical specimens such as sputum or saliva may be a valuable tumour biomarker. Any clinically applicable detection technique must combine high sensitivity with high specificity. In this study we describe methylation enrichment pyrosequencing (MEP), which benefits from the high sensitivity and specificity of methylation-specific PCR (MSP) but has a second, confirmatory, pyrosequencing step. The pyrosequencing reaction is rapid, relatively inexpensive and offers significant logistical advantages over previously described validation methods. As proof of principle, we illustrate MEP using assays of p16 and cyclin A1 promoters in a methylated DNA dilution matrix and also in a clinical setting using paired saliva and oral tumour specimens. Our results confirm that mis-priming of MSP, with subsequent false positive results, can occur frequently (perhaps 10%) in assays combining high numbers of PCR cycles and low concentrations of starting DNA. In our clinical example, MEP of saliva-derived DNA was more sensitive than standard non-methylation-specific pyrosequencing as illustrated using p16 and cyclin A1 promoter methylation assays.

INTRODUCTION

There has been remarkable progress in the knowledge of the role of promoter methylation in human cancer. In parallel, there have been many attempts to develop the assessment of DNA methylation as a biomarker (1). Efforts have been made to characterize the epigenetic signature of DNA in circulating body fluids such as plasma (2), urine and saliva (3). The technical challenge is to establish a sensitive technique that will allow the study of the minute amounts of DNA present in these body fluids but yet to avoid false positives.

Methylation-specific PCR (MSP) (4,5) is highly sensitive and has been used widely in this context. However, as there is no inbuilt measure of adequacy of bisulphite treatment, the possibility of false positives due to inadequate conversion of non-methylated cytosine to uracil exists. Another potential source of false positives is mis-priming, and this may be a greater problem when high numbers of PCR cycles or nested primers are used. Previously described methods of controlling for mis-priming include re-analysis by methylation-sensitive restriction enzymes (6) or subsequent bisulphite DNA sequencing (7).

In an attempt to overcome problems with false positives and gain quantitative methylation data, the pyrosequencing methylation assay (PMA) has been described elsewhere (8,9) in which 4–25 CpG dinucleotides are individually assayed together with a non-CpG cytosine (conversion control). This technique shows low variance in replicates over a range of levels of methylation (8). We have developed and successfully applied PMAs to gain quantitative data for a series of oral squamous cell carcinoma (10). PMA, however, does not have as high sensitivity and specificity as MSP because the primers are specifically designed to avoid CpG dinucleotides in their sequence in order to allow the detection of the amount of methylation in a sample quantitatively. Thus it is not the ideal method to sensitively detect methylated 'tumour' DNA in the presence of great quantities of unmethylated 'normal' DNA. In its original form, we feel that PMA may have little value in detecting methylation in clinical samples such as saliva, sputum or plasma.

We describe a novel combination of techniques conferring the sensitivity and specificity of MSP while also benefiting from subsequent validation using pyrosequencing: methylation enrichment pyrosequencing (MEP). Pyrosequencing

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offers significant advantages over conventional sequencing in this context in that it is rapid, relatively inexpensive and allows many samples to be analysed in parallel. We illustrate the technique in parallel with MSP using a dilution matrix of known concentrations of mixed methylated and unmethylated DNA. As a clinical illustration of MEP we then analyse saliva samples from head and neck squamous cell cancer patients in comparison with previous data from paired tumour tissue (10).

MATERIALS AND METHODS

Construction of a DNA dilution matrix

We constructed a dilution matrix to simulate the low concentrations of methylated DNA and possible contamination with unmethylated DNA found in clinical body fluid samples (Table 1). DNA was obtained from human head and neck squamous cell carcinoma tumour tissue. Standard PMA had been used previously to analyse these tumour samples, and two specimens with methylation levels of 50 and 0%, respectively, in the p16 gene promoter were selected. Starting concentrations of 20 ng/µl of these known methylated and unmethylated DNA were used to construct the matrix samples. A similar matrix using specimens with 50 and 0% cyclin A1 promoter methylation was also constructed.

MEP

The positions of MSP, MEP, PMA and pyrosequencing primers for p16 are shown in Figure 1. Details of cyclin A1 primers are available on request. Bisulphite treatment of each matrix sample was undertaken using the EZ DNA Methylation Kit™ (Zymo Research) and elution was carried out using the same volume (50 µl) as the starting volume. Three microliters (60 ng total DNA) of the resulting bisulphite-treated matrix samples were used in 25 µl PCRs.

Hot-start PCR was carried out with HotStarTaq® Master Mix Kit (Qiagen Ltd) and the PCR conditions for both p16 and cyclin A1 MEP were 95°C for 14 min 30 s; 50 cycles of 94°C for 30 s, 63°C for 45 s, 72°C for 25 s; 72°C for 10 min. Pyrosequencing was performed using the PSQ96MA System (Biotage) according to manufacturer's protocol, including single-strand binding protein.

MSP

Primers and PCR conditions used for MSP were as described previously by Herman *et al*. (5) using 35 cycles.

Patients and samples

Ten consecutive patients with biopsy proven oral squamous cell carcinoma were selected and saliva samples were obtained before surgery using Oragene™ collection vials (DNA Genotec, Ottowa, Canada). At the time of surgery, tumour biopsies were taken and snap frozen in liquid nitrogen. UICC pTNM stages are recorded in Table 2. DNA was extracted from 2 mm^3 tissue and from saliva samples using a DNeasy™ tissue kit (Qiagen Ltd). DNA concentration was measured by spectrophotometry and subsequently adjusted to 40 ng/µl. Bisulphite treatment was undertaken as before. Each sample was then subjected to analysis using

Table 1. Dilution matrix

	M:U 1:1	M:U 1:8.5	M:U 1:18	M:U 1:100	M:U 1:200	M:U 1:2000	M:U 1:20 000	M:U 1:20 0000
No dilution	M: 10 ng/µl U: 10 ng/µl	M: 2 ng/µl U: 17 ng/µl	M: 1 ng/µl U: 18 ng/µl	M: 200 pg/µl U: 20 ng/µl	M: 100 pg/µl U: 20 ng/µl	M: 10 pg/µl U: 20 ng/µl	M: 1 pg/µl U: 20 ng/µl	M: 100 fg/µl U: 20 ng/µl
1:10 dilution	M: 1 ng/µl U: 1 ng/µl	M: 200 pg/µl U: 1.7 ng/µl	M: 100 pg/µl U: 1.8 ng/µl	M: 20 pg/µl U: 2 ng/µl	M: 10 pg/µl U: 2 ng/µl	M: 1 pg/µl U: 2 ng/µl	M: 100 fg/µl U: 2 ng/µl	M: 10 fg/µl U: 2 ng/µl
1:100 dilution	M: 100 pg/µl U: 100 pg/µl	M: 20 pg/µl U: 170 pg/µl	M: 10 pg/µl U: 180 pg/µl	M: 2 pg/µl U: 200 pg/µl	M: 1 pg/µl U: 200 pg/µl	M: 100 fg/µl U: 200 pg/µl	M: 10 fg/µl U: 200 pg/µl	M: 1 fg/µl U: 200 pg/µl
1:1000 dilution	M: 10 pg/µl U: 10 pg/µl	M: 2 pg/µl U: 17 pg/µl	M: 1 pg/µl U: 18 pg/µl	M: 200 fg/µl U: 20 pg/µl	M: 100 fg/µl U: 20 pg/µl	M: 10 fg/µl U: 20 pg/µl	M: 1 fg/µl U: 20 pg/µl	M: 100 ag/µl U: 20 pg/µl

M:U, ratio of methylated to unmethylated DNA.
The first column indicates the dilution factor applied to the given starting mixture of 20 ng/µl total DNA. The body of the table gives the concentrations of each DNA present.

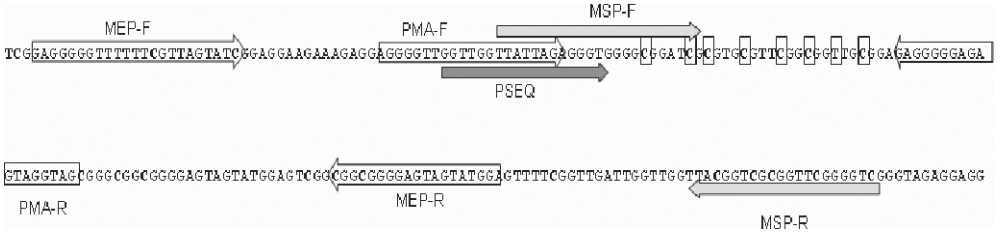

Figure 1. Diagrammatic representation of primer alignment on sodium bisulphite-treated p16 promoter sequence. Cytosines in squares indicate the nucleotides interrogated by pyrosequencing in PMA and MEP assays (i.e. CpG dinucleotides). The T nucleotide in a square indicates the bisulphite control of these assays (site of non-CpG cytosine). MEP, methylation enrichment PCR primers; PMA, pyrosequencing methylation assay primers; PSEQ, pyrosequencing primer; MSP, methylation-specific PCR primers (primers specific for unmethylated target DNA contain T in squares; primers specific for methylated DNA target contain C in squares).

Table 2. Tumour characteristics and p16/cyclin A1 methylation results

Patient no.	pT stage	pN stage	p16 (% methylation)					Cyclin A1 (% methylation)				
			Tumour DNA PMA	Saliva DNA PMA	MEP1	MEP2	MEP3	Tumour DNA PMA	Saliva DNA PMA	MEP1	MEP2	MEP3
1	4	0	0	0	–	–	–	0	0	–	–	+ (91)
2	2	0	0	0	–	–	+ (100)	4	0	–	+ (70)	+ (100)
3	1	0	0	0[1]	–[a]	–[a]	–[a]	25	F[a]	–[a]	–[a]	+[a] (100)
4	3	0	1	0	+ (98)	–	+ (98)	0	0	+ (100)	–	–
5	1	0	0	0	–	–	–	0	0	–	–	–
6	2	0	3	0	+ (99)	+ (100)	+ (99)	6	0	+ (85)	–	+ (100)
7	4	0	40	22	+ (97)	+ (100)	+ (97)	12	13	+ (97)	+ (100)	+ (100)
8	3	1	0	0	–	–	–	8	0	–	+ (100)	+ (100)
9	4	2b	0	0	–	–	–	22	4	+ (98)	+ (100)	+ (100)
10	2	2b	18	3	+ (99)	+ (100)	+ (100)	30	0	+ (99)	+ (100)	+ (100)

PMA: quantitative pyrosequencing methylation analysis. Numbers represent methylation index (MtI, average % ^{m}C for all CpGs interrogated); F, pyrosequencing fail; MEP, qualitative methylation enrichment pyrosequencing; +, pyrosequencing-confirmed MEP positive; numbers in parenthesis indicate MtI of PCR product; –, no PCR product or pyrosequencing fail.
[a]Saliva DNA concentration <10 ng/μl (all others diluted to 40 ng/μl for analysis).

both MEP (as above) and PMA of the p16 and cyclin A1 gene promoters as described previously (8).

Analysis of pyrosequencing results

The methylation index (MtI) for each sample was calculated at each gene promoter as the mean value of % ^{m}C for all examined CpGs interrogated.

RESULTS

DNA dilution matrix

Matrix DNA samples were subjected to p16 MSP using primers specific for methylated or unmethylated DNA and in two independent MEP assays for both p16 and cyclin A1. Confirmation of PCR product quality and freedom from contamination was established on 2% agarose gels with ethidium bromide staining (Figure 2). These initial gel results confirm the sensitivity and reproducibility of MEP both between replicates and in the presence of different amounts of unmethylated DNA (Figure 2b, row 1 lane 6 and row 2 lane 4; Figure 2c, row 1 lane 8 and row 2 lane 6).

Pyrosequencing to confirm the identity of the PCR products was carried out on all 30 p16 and 40 cyclin A1 MEP PCR products that produced bands on agarose gels, including all outliers; 27/30 p16 and 36/40 cyclin A1 products had MtI (average % methylation) >95% for both replicates. A typical pyrogram for p16 is shown in Figure 3. However, for p16 MEP, 3/30 of the bands did not correlate with the target sequence of the promoter region under investigation and thus 'failed' during pyrosequencing. Two out of these could be identified on the agarose gels by size difference, but one appeared to be a PCR product of the correct size. For cyclin A1 MEP, 3/40 of the bands did not yield the expected product on pyrosequencing and a further single case was successfully sequenced but was entirely unmethylated. In total, 7/70 (10%) of the positive bands appeared to result from mis-priming.

Patient samples

PMA of tumour-derived DNA has revealed previously that 4 of the 10 cases displayed some degree of p16 promoter methylation (MtI range 1–40%) and 7 of 10 showed cyclin A1 promoter methylation (MtI range 4–30%) (Table 2). MtI values were reproducible, even when they lay between 1 and 5%. PMA of the saliva DNA was positive from only 2/4 p16 and 2/7 cyclin A1 positive tumours (Table 2).

MEP was performed in triplicate on all the saliva-derived DNA samples using p16 and cyclin A1 specific primers. Of the 60 MEP reactions, 30 of the 31 positive bands on agarose gels gave positive results on subsequent pyrosequencing (Table 2). In 9/10 cases where tumour DNA demonstrated methylation by PMA, MEP replicates from saliva samples produced either 2/3 or 3/3 positive results (i.e. contained methylated promoter DNA). The exception was patient 3, where, for unknown reasons, the saliva-derived DNA concentration was <10 ng/μl. PMA failed in this sample and 1/3 positives were obtained by triplicate MEP analysis only for cyclin A1. Patients 1, 2 and 4 each generated a single positive result from their saliva samples despite not having detectable methylation by PMA of that gene promoter in their corresponding tumour.

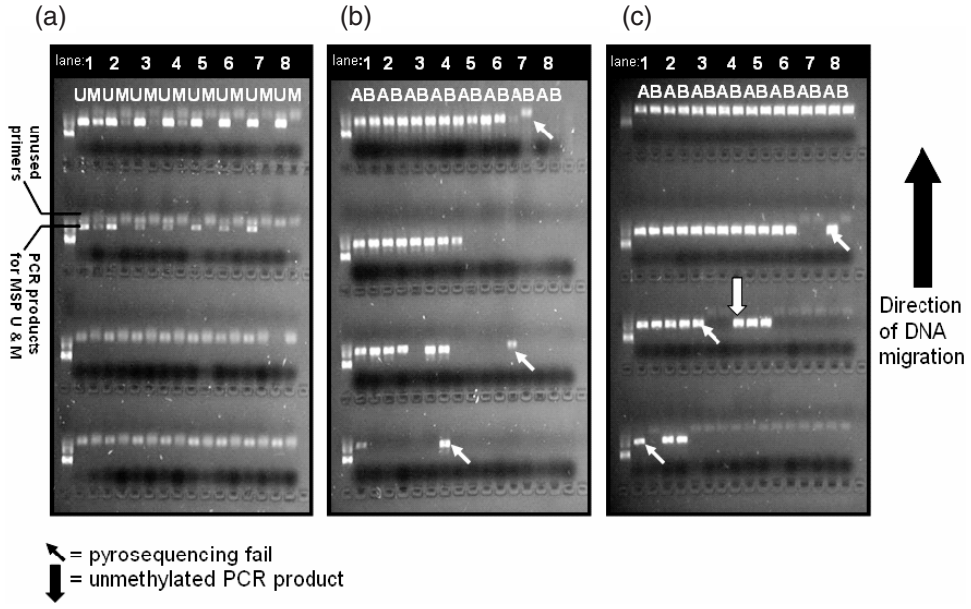

Figure 2. Agarose gels of PCR products following (**a**) p16 MSP, (**b**) p16 MEP, and (**c**) cyclin A1 MEP. U, PCR using MSP primers specific for unmethylated DNA; M, PCR using methylation-specific MSP primers; A and B, two independent MEP assays. Rows 1–4 (top to bottom) correlate with rows 1–4 in dilution matrix Table 1; i.e. row 1, no dilution of starting M:U concentrations; row 2, 1/10 dilutions, etc. Lanes 1–8 correlate with columns 1–8 in dilution matrix Table 2; i.e. lane 1, M:U ratio of 1:1; lane 2, M:U ratio of 1:5, etc.

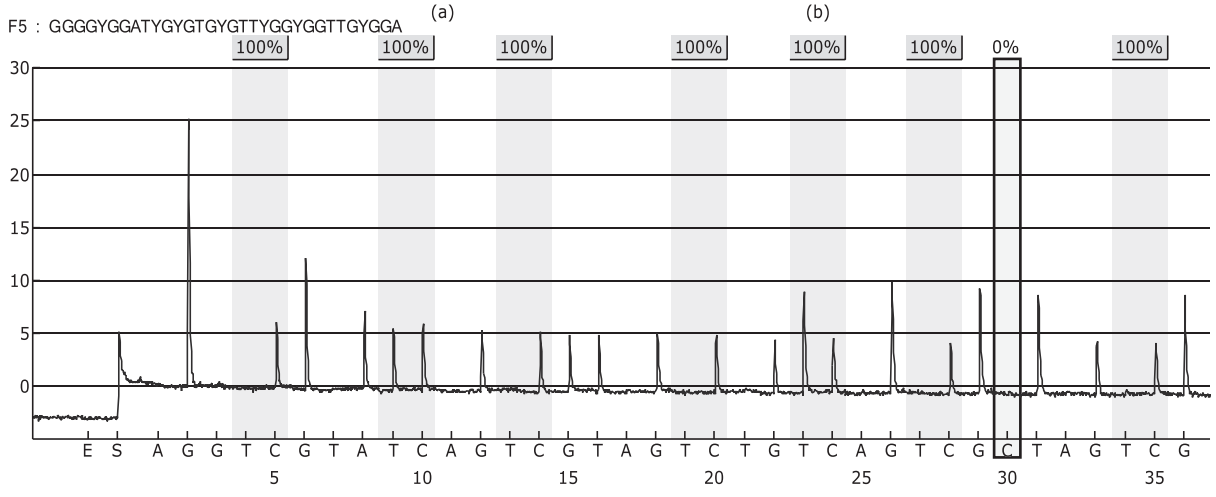

Figure 3. Pyrogram for a typical p16 MEP showing 100% methylation. Shaded bars encompassing T/C pairs, seven interrogated CpG (Figure 1) (T, conversion of unmethylated C to U; C, non-conversion of methylated C); box, control, non-CpG cytosine (0% cytosine incorporated). a: Sequence context ATYG. Equal height of T and C peaks at 5 units indicates 100% methylation of C. b: Sequence context GTTYGG. Height of T peak (10 units) is twice the height of C peak (5 units) indicating 100% methylation.

DISCUSSION

We describe a combination of techniques conferring both the sensitivity and specificity of MSP and benefiting from subsequent validation using pyrosequencing: MEP. As in MSP (but unlike the standard PMA) PCR primers are specific for methylated CpG sites within the promoter under investigation. The resultant methylation-enriched amplicon is then subjected to pyrosequencing which determines the methylation status of a number of CpG sites as well as the completeness of bisulphite treatment. This validation step allows the number of PCR cycles to be increased to 50, enhancing the sensitivity of MEP over MSP, with the re-assurance that any false positives can be subsequently identified. In the context of this limited clinical example, and using the criteria of 2/3 MEP positives, the test had 91% sensitivity and 100% specificity. The one false negative (patient 3 saliva) might arguably be excluded due to poor DNA yield, thus giving 100% sensitivity also. If a single MEP reaction is used, this technique appears less accurate, with a 6/33 (19%) false negative and 3/27 (11%) false positive rate. This may not prove sufficiently accurate for clinical usage and even after

taking the extra confirmatory sequencing steps implicit in MEP, duplicate or triplicate testing may be necessary.

The presence of a single positive result in MEP analysis from the saliva of three patients in this study whose tumours did not show methylation may reflect isolated false positives or possibly very low levels of methylation (<0.5%) in their respective tumours, which were only tested by PMA and not MEP. Alternatively aberrant methylation from small numbers of cells derived from other oral or other upper aerodigestive tract epithelium may be a possibility.

As with MSP, and contrary to standard PMA, quantification of the amount of methylated DNA present in the original sample cannot be determined by this method. It is also notable that some MEP products had lower methylation percentages than might be expected, i.e. 70, 85 and 91%. These resulted from corresponding tumours with low levels (4, 6 and 0%, respectively) of methylation at the cyclin A1 promoter and might underline the stochastic effects of these methylation 'specific' PCRs in the context of large amounts of contaminant DNA present in such clinical samples.

The finding that PCR products of the expected size can totally fail in subsequent pyrosequencing confirms our suspicions that methylation-specific primers mis-prime in ~10% of cases, resulting in false positives when the number of PCR cycles is increased for use with low concentrations of methylated DNA. These false positives are identified in MEP by the pyrosequencing stage of the reaction.

We have illustrated the clinical utility of MEP with a small series of paired oral cancer tumour and saliva specimens. Presumably the tumour DNA in the saliva was somewhat diluted by DNA shed from normal oral epithelium. Under these conditions, standard quantitative PMA was insufficiently sensitive to detect promoter methylation in several cases, but MEP was much more valuable (when positive in at least two of three tests performed). This example, using a non-invasive technique of sample collection, shows the potential for MEP to be used to detect aberrant DNA in a clinical sample that will also contain abundant normal DNA. We suggest this technique is suited for use with clinical samples with very low concentrations of methylated DNA and/or the presence of contaminating unmethylated DNA, and consequently may have a role in research exploring DNA methylation as a clinical biomarker, e.g. in dysplastic lesions or early malignancy.

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Conflict of interest statement. None declared.

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