Methylated CpG Island Amplification of Representational Difference Analysis for Oral Cancer

<Review>

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ABSTRACT

CpG islands are G+C-rich regions approximately 1 kb long that are free of methylation and contain the promoters of many mammalian genes. It has been demonstrated that aberrant methylation of CpG islands is associated with gene silencing of tumor suppressor genes. To examine DNA methylation changes in oral squamous cell carcinoma (OSCC), we have applied a novel genome screening technique, methylated CpG island amplification coupled with representational difference analysis. Using DNA from an OSCC cell line as tester and DNA from normal tongue tissue as driver, DNA sequences aberrantly methylated in OSCC can be obtained. We therefore propose that MCA is a useful technique to study methylation and to isolate CpG islands differentially methylated in cancer.

Key words : Methylation, CpG island, Oral squamous cell carcinoma (OSCC)

1. Introduction

The precise molecular mechanisms involved in the development and progression of oral squamous cell carcinoma (OSCC) remains unclear. With regard to epigenetic alterations, it has been reported that DNA methylation of 5' CpG has been shown to be a major cause of inactivation of tumor suppressor genes[1,2]. CpG islands are clusters of CpG dinucleotides that can be found in the 5' region of about half of human genes[3]. Methylation of cytosine within the 5' CpG islands is associated with transcriptional inactivation of the involved gene. Therefore aberrant methylation of CpG islands is an important mechanism of gene inactivation in cancer and other states such as aging and inflammation[4]. Such methylation often involves numerous CpG islands and emerging data suggests that methylation profiles may be of some utility in disease detection, prognosis, and risk assessment. In order to investigate methylation profile, we have examined DNA methylation in the development and progression of head and neck cancers, including OSCCs, and reported on the association between DNA methylation and the clinocopathological features of oral cancer[5]. However, the measurement of DNA methylation abnormalities at multiple gene loci is currently cumber-

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some, time-consuming, and not easily amenable to automation. Furthermore, the identification of noble gene sequences hypermethylated in cancer is relatively difficult and inefficient using current technology. Methylated CpG island amplification (MCA) was developed to overcome these problems[6]. The principle underlying MCA involves amplification of closely spaced methylated SmaI sites to enrich methylated CpG islands. The MCA technique is outlined in Fig.1. About 70-80% of CpG islands contain at least two closely spaced (1kb) SmaI sites (CCCGGG). Only those SmaI sites within these short distances can be amplified using MCA, ensuring representation of the most CpG rich sequences. Briefly, DNA is digested by SmaI, which cuts only unmethylated sites, leaving blunt ends between the C and G sites. DNA is then digested with the SmaI isoschizomer XmaI, which does cut methylated CCCGGG sites, and which leaves a 4-base overhang. Adaptors are ligated to this overhang, and PCR is performed using primers complementary to these adaptors. To identify CpG islands differentially hypermethylated in cancer (or any appropriate condition); MCA amplicons can be used as templates for subtraction techniques such as representational difference analysis (RDA, ref. 7), using DNA from cancer as tester and DNA from normal tongue tissue as driver. Because this combination is positive selection, contamination of tumor samples with normal cells has little unfavorable effect on the subtraction. In addition to disease-related genes, this technique could potentially be used to identify novel imprinted genes, as well as genes on the X-chromosome.

Compared to other techniques used to detect methylation and/or differentially methylated genes. MCA has several advantages: 1) A large number of samples can be analyzed rapidly at multiple loci, 2) many steps of MCA can be automated, 3) MCA allows for an unbiased representation of CpG islands without requiring prior knowledge of their DNA sequence, and 4)

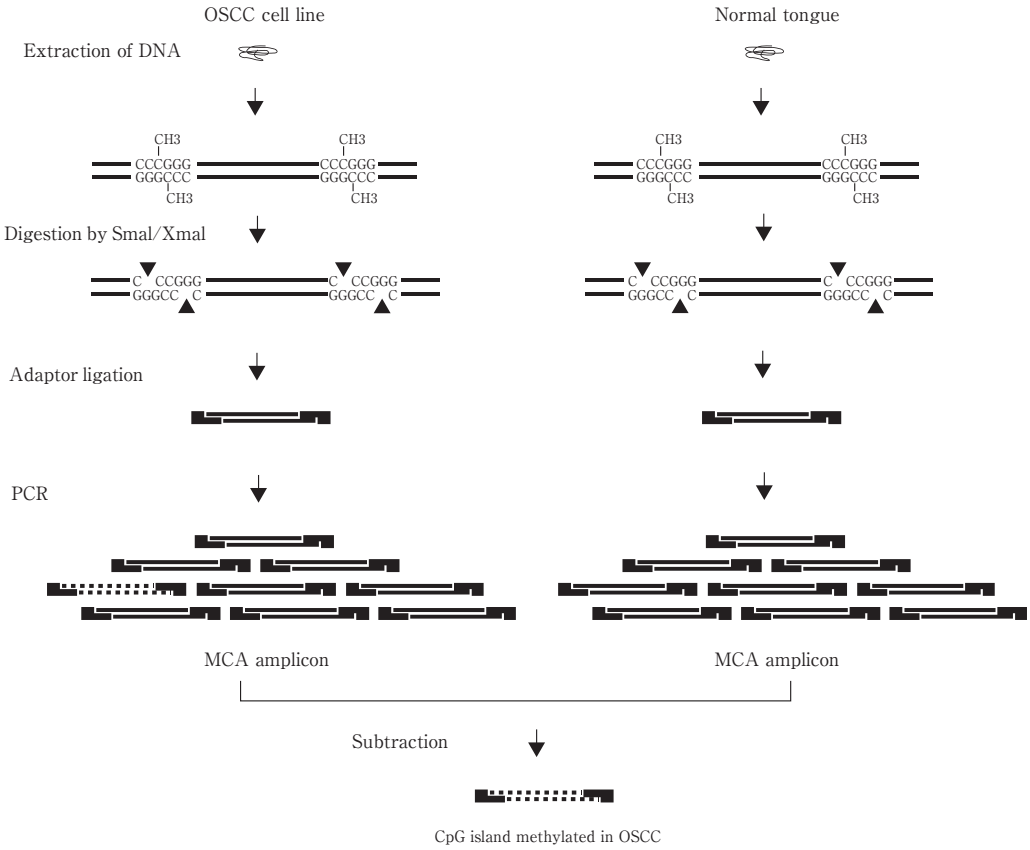

Fig. 1 Strategy to identify differentially methylated CpG islands in OSCC.

novel differentially methylated CpG islands can be amplified and cloned relatively simply, without the need for acrylamide gel or two-dimensional (2-D) gel electrophoresis. However, MCA also has a few potential disadvantages in that 1) it requires relatively good-quality DNA, precluding the use of paraffin-embedded samples, 2) it examines only a limited number of CpG sites within CpG islands, 3) CpG islands that do not contain two closely spaced (1kb) SmaI sites cannot be analyzed, 4) false-positive results can occur due to incomplete digestion using the methylation-sensitive restriction-enzyme SmaI.

2. Material

2.1 MCA

1. Restriction enzymes SmaI, XmaI
2. T4 DNA ligase
3. Taq DNA polymerase
4. 10X polymerase chain reaction (PCR) reaction buffer; 670mM Tris-HCl (pH8.8),40mM $MgCl_2$, 160mM $NH_4(SO_4)_2$, 100mM β-Mercaptoethanol, 1mg/ml bovine serum albumin(BSA)
5. Tris-EDTA (TE), pH8.0
6. DNA precipitation reagents; Phenol/Chloroform, pH 8.0-9.0, 3M NaOAc (for general precipitation), 5M NH_4OAc(for precipitation and quantitation when dNTPs are present),100% ETOH
7. Agarose gel electrophoresis reagents
8. Sequencing instruments
9. Restriction enzymes for combination bisulfite restriction analysis (COBRA) methods

2.2 RDA and Cloning PCR Products

1. 3XEE buffer; 30mM EPPS(Sigma), pH8.0, 3 mM ethylenediaminetetraacetic acid(EDTA), pH8.0
2. 5M NaCl
3. cDNA spin column (Amersham)
4. Mung bean nuclease (NEB)
5. pBluescript (Stratagene)

2.3 Oligonucleotides

RMCA primers

RMCA24 ; 5'-CCACCGCCATCCGAGCCTTTCTGC-3'
RMCA12 ; 5'-CCGGGCAGAAAG-3'
JMCA24 ; 5'-GTGAGGGTCGGATCTGGCTGGCTC-3'
JMCA12 ; 5'-CCGGGAGCCAGC-3'
NMCA24 ; 5'-GTTAGCGGACACAGGGCGGGTCAC-3'
NMCA12 ; 5'-CCGGGTGACCCG-3'

3. Methods

3.1 Preparation of MCA Amplicons

3.1.1 Digestion of Genomic DNA

1. Digest 5μg of DNA (tester and driver) using 120units of SmaI over night.
2. Add 100units of Xmal and incubate at 37℃ for 6hr.
3. Add 400μl of PC,vortex,centrifuge 5min at 15,000rpm and extract the supernatant, two times.
4. Precipitate the DNA ; Add 1/10th volume 3M NaOAc and 2 volumes 100% ETOH. Store at -70℃ for 1hr and centrifuge 30min at 15,000rpm. Pour 70% ETOH out, air-dry the pellets.
5. Resuspend in 15μl TE and determine DNA concentration using a spectrophotometer.

3.1.2 Ligation of RMCA Adaptor

1. Prepare an adaptor mixture by diluting the primers to 100μM and combining 50μl of RMCA24 (100μM) and RMCA12 (100μM).
2. Incubate at 65℃ for 2min and cool to room temperature for 1hr.
3. Mix the following ; 500ng of digested DNA, 10μl of adaptor mixture, 1μl of T4 DNA ligase, 3μl of 10X ligase buffer and water to a total volume of 30μl.
4. Incubate at 16℃ for 16hr.

3.1.3 PCR Amplification

1. Prepare tubes containing 20μl of 10X PCR buffer, 4μl of RMCA 24primer (50μM), 15 units of Ex Taq DNA polymerase, 2.4μl dNTP mix (25mM), 10μl of DMSO, H_2O to a total volume of 197μl.

2. Add 3μl of ligation mixture, vortex, spin down and cover with 2 drops of mineral oil.
3. To fill the 3'-recessed ends of the ligated fragments, incubate at 72℃ for 5min.
4. Perform 25cycles of PCR (95℃ for 1min and 72℃ [for RMCA] for 3min).
5. After the reaction, electrophorese 10μl of PCR products in a 1.1% agarose gel to check the quality of the amplification.

3.2 First Round RDA

3.2.1 Elimination of RMCA adaptor

1. Transfer PCR products ; tester (200μl) to 1.5ml tube, driver (2ml) to 1.5ml tube X 10.
2. Add PC, vortex, centrifuge for 5min at 15,000rpm and extract the supernatant, twice.
3. Precipitate the DNA ; Add 2/3 volume 5M NH_4OAc and 2 volumes 100% ETOH. Store at -70℃ for 1hr and centrifuge for 30min at 15,000rpm. Pour 70% ETOH out, air-dry the pellets.
4. Dissolve tester amplicon in 30μl TE, driver amplicon in 200μl TE.
5. Check the concentration of the tester and the driver amplicon with spectrophotometer.

3.3 Separation of JMCA adaptor

3.3.1 Mix the following;

tester ; 5μl of tester amplicon, 10μl of 10XH buffer, 40units of XmaI, H_2O to a total volume of 95μl. Incubate at 37℃ overnight.

driver ; 100μl of driver amplicon, 12μl of 10XNE buffer 4, 80units of SmaI, H_2O to a total volume of 110μl. Incubate at 25℃ overnight.

3.3.2 Preparation for Spin Column

1. Add PC of the same amount of each mixture, vortex,centrifuge 5min at 15,000rpm and extract the supernatant and place the extract in a new tube.

3.3.3 Elimination of adaptor DNA

1. Resuspend the resin in the column by vortex.
2. Loose the cap one-fourth twin and snap off the bottom closure.
3. Place the column in a 1.5ml screw-cap microcentrifuge tube support.
4. Pre-spin at 3,000rpm(Eppendorf model 5415 C) for 1min.
5. Discard the cap, spin at 3,000rpm for 1min.
6. Place the column in a new 1.5ml tube, remove and discard the cap and slowly apply, being careful not to disturb.
7. Spin at 3,000rpm for 2min, discard the column and transfer the solution to a new tube.

3.3.4 Elimination of adaptor by electrophoresis

10μl of the purificated solution were checked by electrophoresis (3.0% Nusieve) to see whether the adaptor had been eliminated.

3.3.5 Precipitation of DNA

1. Add 1/10 volume of 3M NaOAc and 2 vol of 100% ETOH, and store at -70℃ for 1hr.
2. Centrifuge at 15,000rpm for 25min, and wash the pellet with 170μl of 70% ET-OH.
3. Dry the pellet,dissolve it in 20μl of TE (tester), 100μl of TE(driver).
4. Check the concentration of purified DNA with photometer.

3.4 Ligation of JMCA Adaptor

3.4.1 Preparation of JMCA adaptor

1. Prepare an adaptor mixture by diluting the primers to 100μM and combining 50μl of JMCA24 (100μM) and JMCA12 (100μM).
2. Incubate at 65℃ for 2min and cool to room temperature for 1hr.

3.4.2 Ligation of JMCA adaptor

1. Mix the following ; 500ng of digested DNA, 10μl of Jadaptor mixture, 1μl of T4 DNA ligase, 3μl of 10X ligase buffer and water to a total volume of 30ul.
2. Incubate at 16℃ for 16hr.

3.5 Competition

1. Add 70μl of TE to 30μl of ligation mix, PC extract.
2. Change the vol of ligation mix.

3. Add 40μg of driver amplicon to tester amplicon.
4. Add 1/10 volume of 3M NaOAc and 2 vol of 100% ETOH, and store at -70℃ for 1hr.
5. Centrifuge at 15,000rpm for 25min, and wash the pellet with 170μl of 70% ETOH.
6. Dry the pellet, and dissolve it in 4μl of 3XEE. (add 2μl of 3XEE, vortex vigorously, and more addition of 2μl of 3XEE), cover it with 1 drop of mineral oil.
7. Incubate at 96℃ for 10min, and add 1μl of preheated 5M NaCl and 50μl of preheated mineral oil, vortex and spin down (competition solution ; 5μl).
8. In this step, do not cool the solution.
9. Incubate at 67℃ for 22hr.

3.6 Amplification of the target DNA fragment

1. 100μl of 1M NaCl, 8μl of JMCA24 primer.
2. Incubate 100μl of 1M NaCl at 67℃ in a 0.5 ml tube.
3. In a 0.5ml tube, mix the following PCR mixture ; 20μl of 10X PCRIII buffer, 2.4μl of 2.5 mM dNTP mixture, 15units of Ex Taq polymerase, 10μl of DMSO, H_2O to a total volume of 180μl.
4. Add 2 drops of mineral oil, incubate at 72℃.
5. Add 45μl of incubated 1M NaCl (67℃) to the competition solution (72℃),rapidly vortex and replace it to the thermal cycler (competition solution ; 50μl).
6. Transfer 5μl of competition mixture solution to PCR mixture, vortex and spin down.
7. Incubate 85℃ for 3min(rapidly after transfer of 5μl of the competition solution).
8. Raise the temperature to 95℃, and add 4μl of JMCA24 primer.
9. PCR is as follows ; 72℃ for a few min (preincubation), 85℃ for 3min, 72℃ for 5min, 95 ℃ for 1min and 70℃ for 3min/10 cycles,4℃ soak/cool on ice.

3.7 Digestion of the single strand DNA with Mung-Bean nuclease

1. Cool the PCR solution on ice.
2. Add 20μl of 10X Mung-Bean nuclease buffer, 10μl of Mung-Bean nuclease to PCR solution.
3. Incubate PCR solution at 30℃ for exactly 30min in the thermal cycler.
4. Transfer the PCR solution to new 1.5ml tube.
5. PC extract and 2/3 volume of 5M NH_4OAc, 2 volume of 100% ETOH.
6. Store at -70℃ for 1hr and centrifuge for 30 min at 15,000rpm. Pour 70% ETOH out, air-dry the pellet and dissolve in total of 50μl of H_2O.
7. Mix the following ; 20μl of 10X PCR buffer, 2.4μl of 2.5mM dNTP mixture, 3μl of Ex taq polymerases, 50μl of PCR products, 10 μl of DMSO, H_2O to a total volume of 180μl.
8. Incubate at 95℃ for 3min, and add 4μl of JMCA24 primer (50μM).
9. PCR is as follows ; 95℃ for 3min, 95℃ for 1 min and 77℃ for 3min/20cycles, soak at 4℃.
10. PCR products were checked by electrophoresis (1.1% agarose gel).
11. PC extract and 2/3 volume of 5M NH_4 OAc, 2 vol of 100% ETOH.
12. Store at -70℃ for 1hr and centrifuge for 30 min at 15,000rpm. Pour 70% ETOH out, air-dry the pellet and dissolve in total of 30μl of TE.
13. Check the concentration of purified DNA with photometer.

3.8 Second Round RDA

1. Elimination of JMCA adaptor ; same step
2. Preparation of NMCA adaptor ; same step
3. Ligation of NMCA adaptor ; same step
4. Competition ; same step
5. Amplify target DNA fragments.

3.9 pBluescript II KS(+) phagemid

1. 10μl of pBluescript (0.7μg/μl), 20μl of 10X NE buffer, 6μl of XmaI, H_2O to a total volume of 200μl. Incubate at 37℃overnight.
2. Check the ligated mixture (1.0% Agarose gel).
3. PC extract and 1/10 vol of 3M NaOAc, 2 vol of 100% ETOH.
4. Store at -70℃ for 1hr and centrifuge for 30

min at 15,000rpm. Pour 70% ETOH out, air-dry the pellet and dissolve in total of 20μl of TE.
5. 20μl of pBS-XmaI mixture, 10μl of 10X CIP buffer, 3μl of CIP (20 units/μl), H_2O to a total volume of 100μl.
6. Incubate at 37℃/10min, 50℃/10min, 56℃/10min, 65℃/10min.
7. PC extract X2 and 1/10 vol of 3M NaOAc, 2 vol of 100% ETOH.
8. Store at -70℃ for 1hr and centrifuge for 30 min at 15,000rpm. Pour 70% ETOH out, air-dry the pellet and dissolve in total of 30μl of TE.
9. Check the concentration of purified DNA with photometer.

3.10 Elimination of NMCA adaptor

1. Tester; incubate at 37℃ for 4hr.
2. PC extract column; same step
3. Remove NMCA adaptor by spin column procedure.
4. Precipitate with 1/10 vol of 3M NaOAc and 2 vol of 100% ETOH.
5. Store at -70℃ for 1hr and centrifuge for 30 min at 15,000rpm. Pour 70% ETOH out, air-dry the pellet and dissolve in total of 23μl of TE.
6. Check the concentration of purified DNA with photometer.
7. Ligation: 1μl of RDA^{2nd}/XmaI/column(50ng), 1μl of pBluescriptII SK(+)/XmaI/CIP, 5μl of Takara I, H_2O to a total volume of 10μl. Incubate at 16℃ for 22hr and 4℃ soak.
8. After ligation, add 10μl of DNA to 120μl of TE and PC extract and 2/3 vol of 5M NaOAc, 2 vol of 100% ETOH.
9. Store at -70℃ for 1hr and centrifuge for 25 min at 15,000rpm. Pour 70% ETOH out, air -dry the pellet and dissolve in total of 10μl of TE. Perform Electroporation.
10. Add competent cell to 3μl of DNA.
11. Seed 100μl to LB plate.
12. 37℃ overnight culture, and then soak at 4℃.

3.11 Colony pick up

1. Pick up colonies, and culture in LB medium at 37℃ overnight.
2. Purify plasmid DNA using automated DNA extract apparatus (PI, Kurabo, Japan).
3. Add 1μl of RNase (1mg/ml) and incubate at 37℃ for 30min.
4. Digest plasmid by SmaI overnight. Mix 4μl of DNA, 1.2μl of 10X T buffer, 1μl of SmaI, H_2O to a total volume of 12μl.
5. Check the inserts by electroporesion (2.0% Nusieve gel).

3.12 Reaction for Sequence

1. Add 4μl of sequence ready premixture (Applied Biosystems), 2μl of 5X Sequence buffer (Applied Biosystems), 1μl of primer (T3 or T7), 5μl of plasmid DNA, H_2O to a total volume of 20μl, mix well up and down.
2. Cycle sequencing is performed by PCR as follows ; 96℃ for 1min and 96℃ for 10sec, 50℃ for 5sec, 60℃ for 4min/25cycles, soak at 4℃.
3. After reaction, mix 2μl of 3M NaOAc, 50μl of 100% ETOH and wait for 15min at room temperature. Centrifuge for 20min at 15,000 rpm. Pour 70% ETOH out, air-dry the pellet.
4. Resuspend pellet in 20μl of Hi-dye formamide.
5. Shake for 10min, incubate at 96℃ for 2min at 4℃ on ice for 5min.

3.13 Identification of Differentially Methylated Sequences by Bisulfite-PCR.

Genomic DNA was treated with sodium bisulphite (SIGMA) as described previously[8]. Briefly, 2μg of DNA were denatured for 10min at 37℃ in 2M NaOH, after which 30μl of (10 mM) hydroquinone (Sigma Chemical Co) and 520 μl of 3M sodium bisulphite (pH 5.0) were added, and the resultant mixture was incubated for 16 hr at 50℃. The modified DNA was then purified using a Wizard DNA Purification System (Promaga, Madison, WI), after which it was again treated with NaOH and precipitated. Fi-

nally, the DNA precipitate was resuspended in 20μl of TE buffer and stored at -20℃ until use.

Combined Bisulfite Restriction Analysis (COBRA) was performed as described previously (Xiong and Laird, 1997). PCR reactions were performed in a volume of 50μl containing 1×PCR buffer (Takara, Tokyo, Japan), 0.25 mM of dNTP mixture, 0.5μM of each primer and 2.5 U of Taq polymerase (Takara). Primers were designed based on the nucleotide sequences after conversion by bisulfite treatment. After amplification, 20μl of products were digested with restriction enzyme. After ethanol precipitation, DNA was electrophoresed in 3% agarose gel, and stained with ethidium bromide.

DISCUSSION

CpG islands vary in their CpG density such that different PCR primers and conditions may be required for effective MCA amplification. For this reason, we have developed two different sets of primers (RMCA24 and RMCA12) that differ in GC content and represent slightly different subsets of CpG islands. In our experience, 70-90% of the amplified MCA products represent Alu sequences. After two rounds of substraction, 50-70% of the subtracted fragments also contains Alu sequences. This may be due to the fact that some Alu's are differentially methylated in cancer. When excluding Alu sequences, 70-80% of the fragments were true-positive, i.e., differentially methylated. The rest were sequences methylated in both tester and drivers. Using MCA/RDA, we have not recovered sequences that are unmethylated in both tester and driver.

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