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Segregation of partly melted molecules and its application to the isolation of methylated CpG islands in human cancer cells

Review Article

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Summary

Segregation of partly melted molecules is a convenient and efficient method for isolation of DNA fragments associated with CpG islands. DNA fragments digested with restriction endonucleases are subjected to denaturing gradient gel electrophoresis. DNA fragments derived from CpG islands are preferentially retained in the gel after prolonged field exposure because of their lower rate of strand dissociation. An independent technique, methylated-DNA-binding column chromatography, permits separation of DNA fragments on the basis of the number of methyl-CpG sequences in the fragment, and it enables separation of methylated CpG islands from those that are not methylated. Segregation of partly melted molecules and methylated-DNA-binding column chromatography were successfully combined to isolate CpG islands methylated in human adenocarcinomas of the lung. The methylated CpG island library will be valuable in order to elucidate epigenetic process in carcinogenesis.

I. Introduction

In the human genome, G+C content of which is 40%, the appearance of CpG dinucleotides would be expected one every 25 base pair (bp), if all four bases are evenly distributed throughout the genome, but the actual frequency is about one every 150 bp. This indicates that distribution of four bases is not uniform and that a CpG dinucleotide appeared less frequently than the simple assumption of random distribution predicts. Furthermore, the 5-position of cytosine of most CpG's is methylated in the genome, but methylation is rare in certain regions. Mosaic methylation is a characteristic feature of vertebrate genomes.

There are regions in normal somatic cell DNA where non-methylated CpGs are significantly clustered, called CpG islands (Bird, 1986; Cross and Bird, 1995). A CpG island is about 1–2 kilobase (kb) in size, and frequently associated with 5' region of many genes including promoter elements. Although methylation often results in gene silencing, CpG islands are not methylated in all somatic tissues, even where associated genes are not expressed.

A small fraction of CpG islands are methylated in normal somatic cell DNA. So far as is known, CpG islands on inactive X chromosome and those associated with imprinted genes are methylated in normal DNA, genes associated with these CpG islands are transcriptionally silenced. Some tumor suppressor genes are known to be inactivated by CpG island methylation (for a recent review, see Baylin et al, 1998).

Denaturing gradient gel electrophoresis (DGGE) takes advantage of the change in electrophoretic mobility of DNA fragments accompanying partial melting, and permits sequence-determined separation of DNA fragments (Fischer and Lerman, 1979). The electrophoretic mobility of partly melted DNA fragments, consisting of both

helical and dissociated portions, in polyacrylamide gel is much lower than that of fully helical or fully dissociated molecules, and the transition in each fragment to low residual mobility results in stable band pattern. The position corresponding to this transition is called the retardation level. In some cases, two DNA fragments which differ by only one base pair have different melting temperature, and retard in a gradient gel at different levels. DGGE is useful for detection of point mutations (Fischer and Lerman, 1983).

DNA methylation affects *T*m, and correspondingly the retardation level, when it occurs in the domain having the lowest *T*m. Difference in methylation at one base, that is, non-methylation, hemimethylation, or symmetrical methylation, can be resolved by DGGE (Collins and Myers, 1987). We have found that DGGE can be applied to the study of DNA methylation associated with CpG islands (Shiraishi et al, 1995), although DGGE itself does not here depend on methylation. In this article, we describe how DNA fragments associated with CpG islands are isolated on the basis of reduced rate of strand dissociation and its application to the isolation of methylated CpG islands, with emphasis on methodology.

II. Segregation of partly melted molecules

A. Background

DGGE is a technique to separate DNA fragments on the basis of local variation in base composition within the DNA fragments (Lerman et al, 1984). It is effective only when the molecule is comprised of domains having different melting temperatures. The *T*m of the lowest melting domain of sufficient length determines retardation level at which the mobility decreases abruptly in the gel, while the residual regions determine the stability of the partly melted structure. The separation depends on the markedly reduced electrophoretic mobility, which occurs when a part of DNA fragment melts, resulting in a structure that is partly helical and partly random chain. On prolonged field exposure, the retarded fragments will fade away through strand dissociation. If the stability of the helical part is appreciably higher than that of the melted part, the dissociation rate will be low and the retarded partly melted molecule remains in the gel for some hours. It would be reasonable to speculate that DNA fragments derived from the edges of CpG islands consist of at least two different melting domains, because the G+C-rich nature of CpG island sequence results in high *T*m, while flanking non-island sequences are not G+C-rich and would be lower melting. Thus, preferential retention of DNA fragments derived from the edges of CpG islands after prolonged field exposure is strongly expected (**Figure 1**).

That DGGE can be used for the isolation of DNA fragments associated with CpG islands was implicit when Fischer and Lerman described detection of point mutations by DGGE showing that it depends on partial melting (Fischer and Lerman, 1983). Later on, Myers et al proposed and showed that addition of 300-bp G+C-rich sequence (GC-clamp) to DNA fragments lacking a high *T*m domain would ensure partial melting (Myers et al, 1985a, b). Sheffield et al also showed that addition of short (40–45 bp) GC-clamp was sufficient to protect the DNA fragment from strand dissociation and permit retention in a gel (Sheffield et al, 1989). It can be expected that CpG island sequences would serve as “natural” GC-clamps, as demonstrated by attachment of one to the mouse major globin promoter (Myers et al, 1985a, b).

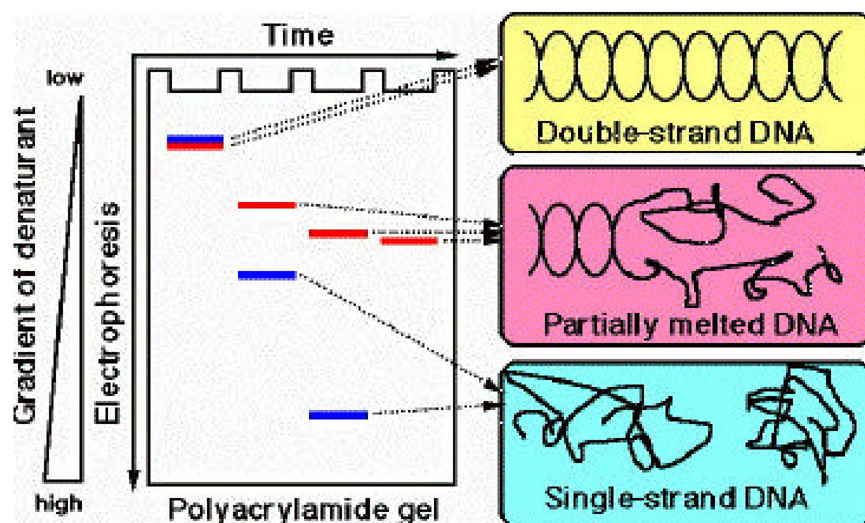

Figure 1. Schematic model of separation. The polyacrylamide gel contains a linear gradient of chemical denaturant (urea and formamide), low at the top and high at the bottom. Red and blue bars indicate DNA fragments having a G+C-rich region and those without a G+C-rich region, respectively. Once part of a DNA molecule melts, pronounced drop in electrophoretic mobility occurs, and low residual mobility restricts migration into more strongly denaturing regions. If the helical portion is stable enough, that fragment persists in the gel, while all others become dissociated and run out of the gel.

B. Preferential isolation of DNA fragments associated with CpG islands

Long cloned DNA fragments have to be fragmented appropriately in order for island fragments to be enriched by DGGE. It was shown that digestion of DNA fragments with four restriction endonucleases, *Tsp*509 I (AATT), *Mse* I (TTAA), *Nla* III (CATG), and *Bfa* I (CTAG), yields DNA fragments of appropriate size for DGGE (Shiraishi et al, 1995). Since occurrence of these sites are rare in CpG islands but abundant in the remaining bulk genomic DNA, this treatment keeps the integrity of CpG island relatively intact, while the rest generally undergoes severe fragmentation.

When cloned DNA molecules containing entire region of some known genes with CpG island were digested with four restriction endonucleases and subjected to DGGE, DNA fragments associated with CpG islands formed stable bands which persisted through continued application of the field, but others did not (Shiraishi et al, 1995). DNA fragments that persisted in the gel were recovered from all CpG islands that were analyzed.

Cosmid clones randomly selected from a human genomic library were analyzed similarly (**Figure 2**). Digestion yielded hundreds of fragments. When the digests are subjected to DGGE, only a few selected fragments, three per cosmid clone on the average, were retained in the gel after 11 hours run. Nucleotide sequence analysis revealed that about half of the retained fragments were considered to be derived from CpG islands (Shiraishi et al, 1995). Thus, the method, named segregation of partly melted molecules (SPM), provides a useful means to isolate DNA fragments associated with CpG islands (Shiraishi et al, 1995). A representative result is shown in **Figure 3**. A retained fragment, R16-2, contained the sequence identical to the 5'-end of the prostacyclin synthase cDNA and a putative promoter region (Shiraishi and Sekiya, 1996). It is well known that the isolation of 5' upstream region of a gene is notoriously difficult by the conventional cDNA synthesis approach. Therefore, SPM is also useful for isolation of promoter regions.

The melting map of the fragment, R16-2, is shown in **Figure 3**. A region of high *T*m is identified in about position 400 to the end. It is expected that this G+C-rich region is thermally stable and serves to tether the partly melted molecule. This region is also inferred to be the 5' edge of the island, since there is a clear boundary of CpG distribution at about position 350.

C. Application to gene hunting

Since 56% of human genes are reported to be associated with CpG islands (Larsen et al, 1992), detection of CpG islands in unsequenced DNA fragments can provide markers for unidentified genes. When P1 artificial chromosome clones covering a 400-kb region of human chromosomal region 11q13, a well known region enriched with CpG islands (Craig and Bickmore, 1994), were subjected to SPM analysis (**Figure 4**), the expected numbers of CpG islands were isolated (Shiraishi et al, 1998). This result suggested that SPM is an efficient method for isolation of bits of gene sequences from long unsequenced DNA fragments.

The isolation of DNA fragments associated with CpG islands by means of DGGE stands on the different basis from that of current practice that makes use of

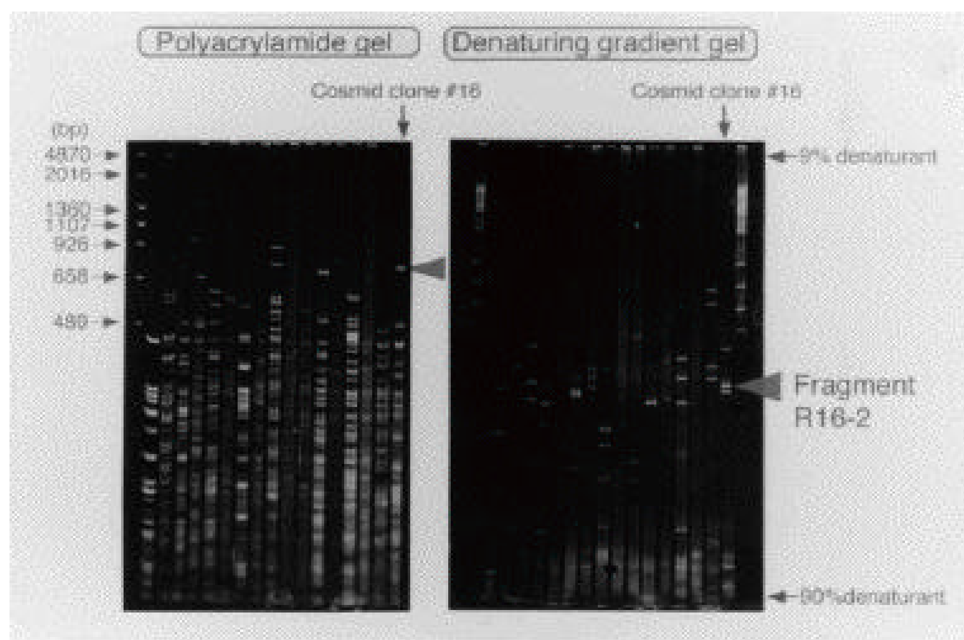

Figure 2. SPM analysis of cosmid clones. Cosmid clones were serially digested with four restriction endonucleases, *Tsp*509 I, *Mse* I, *Nla* III, and *Bfa* I. Conventional polyacrylamide gel electrophoresis shows that numerous fragments were yielded after digestion. After DGGE, only limited number of fragments was retained in the gel (Shiraishi *et al*, 1995). R16-2 is a fragment whose profiles are shown in Figure 3 (Shiraishi and Sekiya, 1996).

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The isolation of DNA fragments associated with CpG islands by means of DGGE stands on the different basis from that of current practice that makes use of clustering or presence of restriction sites characteristic for CpG islands, some of which are *Bss*H II (GCGCGC), *Eag* I (CGGCCG), and *Sac* II (CCGCGG) (Lindsay and Bird, 1987; Bickmore and Bird, 1992; Valdes et al, 1994). The SPM method would be advantageous when unbiased isolation of DNA fragments associated with CpG islands is attempted since these restriction sites are not always present in all CpG islands.

D. Rationalization

The system depends on both extensive digestion of DNA fragments with restriction endonucleases and relative rate of dissociation of partly melted molecules, which are locally G+C-rich during DGGE. However, theory for dissociation rates at temperature lower than that sufficient for full dissociation is not established. Instead, a heuristic approach was adopted to explain the molecular basis for retention and examine the generality of the principle. RHST is an index, which attempts to relate the rate of dissociation to the length and sequence of the unmelted portion of the molecule, where the index is the reciprocal of the estimated relative rate (Shiraishi et al, 1995, 1998). RHST would be large for retained fragments and small for disappearing fragments.

$$\mathrm{RHST} = \left\{\exp\left(\left[T\mathrm{m(i)} - T\mathrm{ret}\right]/\,\mathrm{q}\right) - 1\right\}$$

where *T*m is the calculated temperature for each pair at which the unimolecular probability of helicity of each pair in the stable segment falls to a chosen threshold. *T*ret represents the equivalent temperature at the gradient level of the retarded

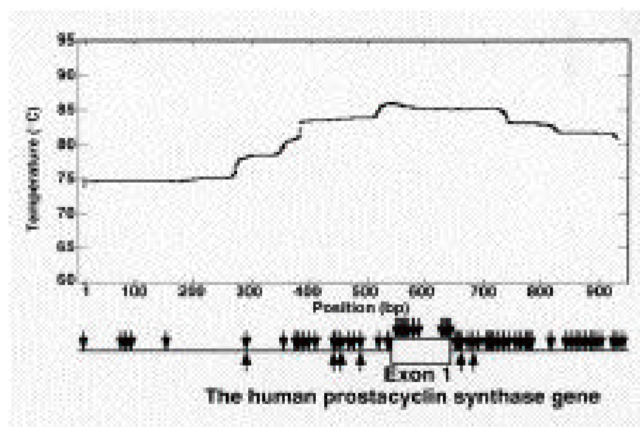

Figure 3. The melting map of fragment R16-2 and corresponding gene structure. The contour shows the midpoint of the melting equilibrium at each base pair (Lerman and Silverstein, 1987). Arrows down and up indicate CpG dinucleotides and GC-box sequences (GGGCGG or CCGCCC), respectively.

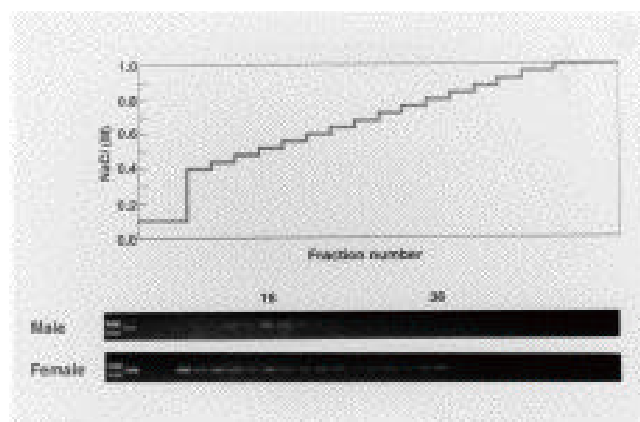

Figure 5. Separation of methylated and non-methylated CpG islands in genomic DNA. DNAs from male and female tissues were digested with Tsp509 I, subjected to an MBD column, and eluted with a stepwise gradient of salt concentration as described (Shiraishi *et al*, 1999a). DNA from each fraction was subjected to PCR-based detection of fragments containing the CpG island of the human *HPRT* gene.

fragment; q is an arbitrary constant that is uniform for all fragments. The summation extends only over pairs calculated by MELT (Lerman and Silverstein, 1987) to be helical at the retardation level. It was shown that RHST values could discriminate retention and non-retention when the followings were assumed; *T*m taken to be equivalent (bath temperature + denaturant) gel temperature at which melted domain is approximately 75% helical, the mobility in the gel is reduced to about 28% of initial velocity, equivalent to 95-bp melting, and q value of 1.546 (Shiraishi et al, 1998). All retained fragments showed RHST values not less than 3.56×10^3, while non-retained ones showed RHST values not greater than 3.19×10^3.

Fragments more or less uniformly dense in G+C are not retained in the gel since they are already at the edge of dissociation as partial melting begins. Their RHST values

are small. Both experimental results and calculation showed that not the entire island but rather the edge of the island can be generally isolated by the SPM method.

High RHST values are not only found for CpG island fragments; some DNA fragments containing repetitive sequences also have high RHST values. Alu repetitive sequences are CpG-rich and contain poly(A) stretches (for a recent review, see Schmid, 1996), and can have high RHST values. When P1 clones from human chromosomal region 11q13 was analyzed by SPM method, about 40% of retained fragments contained Alu sequences, and RHST values of these fragments were high (Shiraishi et al, 1998). These results reflects the observation that both CpG islands and Alu sequences are enriched in R band regions of chromosomes (Korenberg and Rykaowski, 1988; Craig and Bickmore, 1994), and strongly suggest that RHST is a good indicator of retention.

III. Isolation of methylated CpG islands in human cancer cells

A. Methods for analysis of DNA methylation

It is well known that cancer is caused by accumulation of genetic and epigenetic aberrations. Epigenetic aberrations are those that can not be explained by alteration of nucleotide sequence, and CpG island methylation is representative. Epigenetic process in carcinogenesis is less well understood compared with genetic process, partly due to limitation of available methods. Before describing application of SPM to the isolation of methylated CpG islands, we briefly summarize methods for analysis of DNA methylation in order to know their features.

1. Sensitivity to digestion with some restriction endonucleases

Until the appearance of bisulfite modification method described in the next section, sensitivity to digestion with some restriction endonucleases, first described in 1978 (Bird and Southern, 1978), was practically the only method to analyze methylation status of a complex genome. The most frequently used combination of restriction endonucleases is that of *Msp* I and *Hpa* II. Both endonucleases recognize CCGG sequence and cleave DNA at that site. However, when the internal C is methylated, the modified sequence becomes resistant to cleavage by *Hpa* II, but not by *Msp* I. Subsequent Southern hybridization or PCR permits discrimination of methylation and non-methylation as presence or absence of appropriate fragments. Restriction landmark genomic scanning (RLGS) is a method involving two-dimensional gel electrophoresis that permits genomic scan on the basis of methylation status of *Not* I sites (Hatada et al, 1991).

The results of methods using restriction endonucleases are strongly affected by incomplete digestion, which often results in overestimation of methylation, especially in PCR-based experiments. Moreover, the analysis is restricted to methylation status of specific restriction sites, which are not always a representative of the target region, and that non-methylation is negatively displayed as the absence of a signal.

2. Modification by chemical reagents

Although some chemical reactions permit discrimination of cytosine and methylcytosine (for recent reviews, see Rein et al, 1998; Thomassin et al, 1999; Oakeley 1999), their application to the analysis of genomic methylation status was very limited due to complexity of mammalian genomes.

The pyrimidine ring of cytosine is cleaved by hydrazine treatment, but when 5-position of cytosine is methylated, the ring is not cleaved. These properties were applied to determine methylation status of the mouse IgM heavy chain constant region gene *C*μ in combination with Southern hybridization (Church and Gilbert, 1984). However, in addition to technical difficulties, indication of the presence of methylcytosine as the absence of signal makes interpretation difficult.

A major breakthrough was brought to analysis of DNA methylation when chemical modification of cytosine by sodium bisulfite, conversion of cytosine to uracil (Hayatsu et al, 1970; Shapiro et al, 1970), was combined with PCR to permit positive display of 5-methylcytosine in individual DNA strand (Frommer et al, 1992). Treatment of single-stranded DNA with sodium bisulfite, followed by hydrolysis, converts nonmethylated cytosine into uracil, while methylated cytosine remains intact. Since adenine makes a base pair with uracil, subsequent PCR converts original nonmethylated cytosines to thymine. Consequently a non-methylated C-G pair becomes converted to a T-A pair, while a methylated C-G pair remains as a C-G pair. This reaction can be applied to the analysis of any CpG sequence, and permits positive display of methylcytosine.

Bisulfite modification method has now become popular, and unraveled previously unknown features of sequence. One of the unexpected findings revealed by bisulfite modification method is that methylated CpG islands, such as those on inactive X chromosomes, are not uniformly or densely methylated, contrary to previous thought. Hornstra and Yang reported that the CpG island associated with the human *HPRT* gene on an inactive X chromosome is not uniformly methylated (Hornstra and Yang, 1994). CpG sites in the GC-box region were methylation-free even in the CpG island on inactive X chromosome. Although the interpretation of this finding awaits further experiments, we are now closer to the precise nature of methylation.

3. Methylated DNA binding column chromatography

An approach that stands on a different principle in terms of discrimination of methylation and non-methylation was developed recently. Rat nuclear protein MeCP2 (Lewis et al, 1992) binds DNA at a mCpG site, but not at a CpG site (Meehan et al, 1992) The DNA binding domain of this protein is comprised of 85 amino acids (Nan et al, 1993). A methylated DNA binding column (MBD column) is an affinity matrix that contains

a polypeptide derived from the methyl-CpG binding domain (Cross et al, 1994). Since the stoichiometry of binding is one protein to one mCpG (Nan et al, 1993), it is expected that highly methylated DNA fragments bind to the column tightly, while poorly methylated DNA fragments bind only weakly.

By MBD column chromatography, DNA fragments with the same nucleotide sequence but different methylation status can be separated (Cross et al, 1994; Shiraishi et al, 1999a). A representative result of MBD column chromatography experiment is shown in **Figure 5**. The human *HPRT* gene is an X-liked gene having a CpG island, and one allele is inactivated in female. There is only one non-methylated *HPRT*-CpG island in male DNA, while there are two *HPRT*-CpG islands in female DNA, one is methylated and the other is not methylated. Most part of the island is contained in a 0.9-kb *Tsp*509 I fragment and there are 86 CpG residues in it (data not shown). When *Tsp*509 I digests of male DNA was analyzed by MBD column chromatography, DNA fragments containing the CpG island were detected only in low salt fraction (fractions around number 16). In contrast, corresponding fragment of female DNA were detected both in lower (fractions around number 16) and higher (fractions around 36) salt fractions. These results show that DNA fragments from methylated CpG islands and those from non-methylated CpG islands can be separated by MBD column chromatography.

Not only number of mCpGs but also mCpG density seems to be a factor that affects affinity (Shiraishi et al, 1999b; Brock et al, 1999), although possibility of sequence-specific preferential binding can not be excluded.

Clearly this method is insensitive to discriminate heterogeneity in methylation within the same DNA fragment and small change in total number of mCpGs. The nature of this method, which is not destructive and not influenced by methylation status of specific restriction sites, is advantageous for comprehensive isolation of methylated CpG islands.

B. Isolation of methylated CpG islands

Isolation of CpG methylated islands in cancer cells has been drawing growing interest since it provides valuable information on cancer epigenetics, which is very limited now. Several methods for this purpose have been reported; arbitrary primed PCR method (Gonzalgo et al, 1997; Huang et al, 1997; Gonzalgo and Jones, 1998), subtraction (Ushijima et al, 1997; Huang et al, 1999, Toyota et al, 1999), and RLGS (Costello et al, 2000) are those primarily dependent on the methylation status of specific restriction sites.

In contrast, MBD column chromatography permits separation of methylated DNA fragment independent of methylation status of any internal restriction sites, and seems to be excellent for unbiased, comprehensive isolation of methylated CpG islands. Using MBD column chromatography, highly methylated DNA fragments in human adenocarcinomas of the lung was enriched and then cloned. By SPM analysis of the clones, DNA fragments associated with CpG islands methylated in cancer were isolated (Shiraishi et al, 1999a). Many CpG islands thus obtained from cancer were also methylated in noncancerous portion of the lung, possibly only in one allele. These results suggest that number of CpG islands specifically methylated in cancer is lower than that of normally differentially methylated ones.

IV Perspective

In this article, we introduced approaches to study CpG island and DNA methylation standing on novel principles; these may play an important role in cancer research (Terada, 1999). There are many issues yet to be clarified in the field of DNA methylation, such as molecular mechanism of gene silencing by methylation and comprehensive identification of genes that are inactivated by methylation and involved in carcinogenesis. Development of new experimental techniques and their application will be a key to solve these problems.

Acknowledgments

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