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Hypomethylation distinguishes genes of some human cancers from their normal counterparts

Andrew P. Feinberg & Bert Vogelstein

Cell Structure and Function Laboratory, The Oncology Center, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA

It has been suggested that cancer represents an alteration in DNA, heritable by progeny cells, that leads to abnormally regulated expression of normal cellular genes; DNA alterations such as mutations[1,2], rearrangements[3–5] and changes in methylation[6–8] have been proposed to have such a role. Because of increasing evidence that DNA methylation is important in gene expression (for review see refs 7, 9–11), several investigators have studied DNA methylation in animal tumours, transformed cells and leukaemia cells in culture[8,12–30]. The results of these studies have varied; depending on the techniques and systems used, an increase[12–19], decrease[20–24], or no change[25–29] in the degree of methylation has been reported. To our knowledge, however, primary human tumour tissues have not been used in such studies. We have now examined DNA methylation in human cancer with three considerations in mind: (1) the methylation pattern of specific genes, rather than total levels of methylation, was determined; (2) human cancers and adjacent analogous normal tissues, unconditioned by culture media, were analysed; and (3) the cancers were taken from patients who had received neither radiation nor chemotherapy. In four of five patients studied, representing two histological types of cancer, substantial hypomethylation was found in genes of cancer cells compared with their normal counterparts. This hypomethylation was progressive in a metastasis from one of the patients.

The technique we used is based on the fact that certain restriction endonucleases, which cleave at sites containing the sequence 5′-CG-3′, do not function if the internal cytosine residue is methylated[31–33]. Cytosine is the only significantly modified base in mammalian DNA[34], and the two-base sequence 5′-CG-3′ can be heritably methylated[35–38]. Thus, enzymes that discriminate between methylated and unmethylated CG sequences (for example *Hpa*II and *Hha*I) are sensitive indicators of methylation[32,33]. Indeed, these enzymes have been used in many studies to show an inverse correlation between gene expression and methylation (reviewed in refs 7, 9–11), and in other studies to detect methylation differences in animal tumours and transformed cells[8,18,22–24,29,30]. In the present study, DNA was purified from primary human cancers and from the adjacent normal tissues of the same patients. After cleavage with *Hpa*II or *Hha*I, the DNA samples were transferred to nitrocellulose filters by the method of Southern[39,40]. The level of methylation of specific genes was assessed by hybridization of these filters to ^{32}P-labelled DNA probes. The probes were made from the cDNA clones of human growth hormone[41], γ-globin[42] and α-globin[42], which were chosen on the following bases: (1) the genes to be detected should not be expressed in the normal tissues from which the cancers were derived; (2) the genes should be from widely scattered sites in the genome; and (3) *Hpa*II and *Hha*I cleavage sites should be present in the regions of the genes.

The first cancer studied was a grade D (ref. 43), moderately well differentiated adenocarcinoma of the colon from a 67-yr-old male. Tissue was obtained from the cancer itself and also from colonic mucosa stripped from the colon at a site just outside the histologically proven tumour margin. Figure 1 shows the pattern of methylation of the studied genes. Before digestion with restriction enzymes, all DNA samples used in the study had a size >25,000 base pairs (bp). After *Hpa*II cleavage, hybridization with a probe made from a cDNA clone of human growth hormone (HGH) showed that significantly more of the DNA was digested to low-molecular weight fragments in DNA from the cancer (labelled C in Fig. 1) than in DNA from the normal colonic mucosa (labelled N). In the hybridization conditions used, the HGH probe detected the human growth hormone genes as well as the related chorionic somatotropin

Table 1 Quantitation of methylation of specific genes in human cancers and adjacent analogous normal tissues

Patient	Carcinoma	Probe	Enzyme	% Hypomethylated fragments N	C	M
1	Colon	HGH	*Hpa*II	<10	35	—
			*Hha*I	<10	39	—
		γ-Globin	*Hpa*II	<10	52	—
			*Hha*I	<10	39	—
		α-Globin	*Hpa*II	<10	<10	—
			*Hha*I	<10	<10	—
2	Colon	HGH	*Hpa*II	<10	76	—
			*Hha*I	<10	85	—
		γ-Globin	*Hpa*II	<10	58	—
			*Hha*I	<10	23	—
		α-Globin	*Hpa*II	<10	<10	—
			*Hha*I	<10	<10	—
3	Colon	HGH	*Hpa*II	<10	41	—
			*Hha*I	<10	38	—
		γ-Globin	*Hpa*II	<10	50	—
			*Hha*I	<10	22	—
		α-Globin	*Hpa*II	<10	<10	—
			*Hha*I	<10	<10	—
4	Colon	HGH	*Hpa*II	<10	<10	—
			*Hha*I	<10	<10	—
		γ-Globin	*Hpa*II	<10	<10	—
			*Hha*I	<10	<10	—
		α-Globin	*Hpa*II	<10	<10	—
			*Hha*I	<10	<10	—
5	Lung	HGH	*Hpa*II	<10	44	86
			*Hha*I	<10	46	83
		γ-Globin	*Hpa*II	<10	82	>90
			*Hha*I	<10	<10	<10
		α-Globin	*Hpa*II	24	78	>90
			*Hha*I	<10	13	23

Autoradiographs such as those shown in Figs 1–3 were scanned with a Clifford Densicomp Model 445 densitometer and the scans digitized using a Hewlett-Packard 9874A digitizer. For each enzyme and probe, the detectable fragments were divided into two groups. Group I consisted of the high-molecular weight fragments that were the major fragments detected in the digests of the normal tissues. Group II consisted of more fully digested fragments of lower molecular weight. The proportion II/I+II was determined for each digest and is recorded as % hypomethylated fragments. As controls for the quantitation, varying amounts of undigested DNA or *Msp*I digested DNA were pooled, co-electrophoresed, blotted to nitrocellulose and hybridized with each of the probes. There was a nearly linear relationship ($r=0.82$) between the proportion of *Msp*I-digested DNA in the sample and the ratio II/I+II over a 10-fold range. Because the film response was linear only over this range, however, ratios between 0% and 10% are listed as <10%, and ratios between 90% and 100% are listed as >90%. N, normal tissue; C, primary cancer; M, metastasis.

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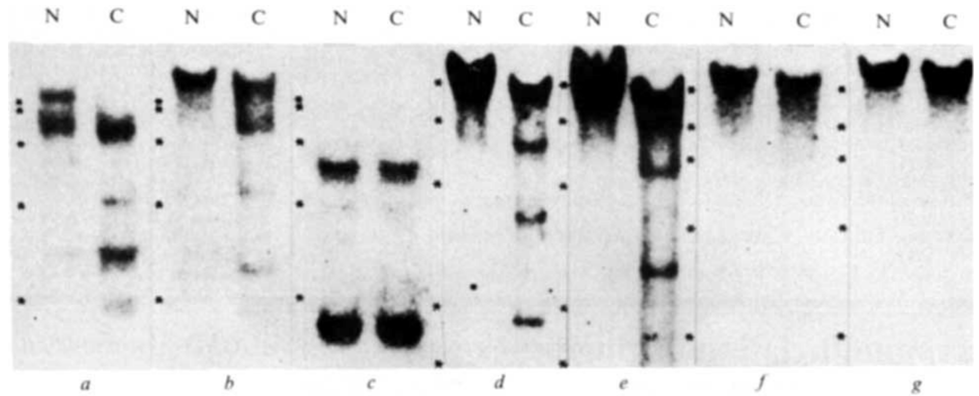

Fig. 1 Methylation pattern of adenocarcinoma of the colon and normal colonic mucosa from patient 1. Lanes C and N were prepared from DNA digests of the colon carcinoma and normal colonic mucosa, respectively. *a HpaII* digest, HGH probe; *b, HhaI* digest, HGH probe; *c, MspI* digest, HGH probe; *d, HpaII* digest, γ-globin probe; *e, HhaI* digest, γ-globin probe; *f, HpaII* digest, α-globin probe; *g, HhaI* digest, α-globin probe. The asterisks on the left of *a–g* indicate molecular weight markers (*Hin*dIII-digested bacteriophage λ DNA, of sizes 9,500, 6,700, 4,400, 2,000 and 570 bp from top to bottom, respectively).

Methods: Tissues were frozen in liquid nitrogen, then pulverized and the DNA extracted essentially as described elsewhere[51]. The DNA was cleaved with 50-fold excess of restriction endonuclease, as assessed by the amount of restriction endonuclease required to digest pBR322 DNA included in a matched duplicate digest of the human DNA. DNA digests (5 μg per lane as assessed by a fluorimetric assay[52]) were electrophoresed on 0.8–1.5% agarose gels at 70 V for 4 h, then transferred to nitrocellulose by the modification of Southern's[39] procedure described by Wahl *et al.*[40]. pBR322 plasmids containing cDNA inserts of human growth hormone (chGH800/pBR322; ref. 41), human γ-globin (JW101; ref. 42) and human α-globin (JW101; ref. 42), were grown in L-broth, and plasmid DNA was isolated by standard techniques[53,54]. The inserts from these plasmids were purified and the DNA labelled with ^{32}P-dCTP to a specific activity of 10^9 d.p.m. μg^{-1} using a technique described elsewhere[55]. The blots obtained by this method were identical to those obtained when probes were labelled by nick-translation, but exposure times were significantly reduced. The probes were hybridized to the filters for 36–60 h and then washed according to a protocol supplied by K. Peden[56]. The autoradiographs shown were exposed for 2–4 days using pre-exposed Kodak XAR-5 film with DuPont Lightning Plus intensifying screens[57].

Fig. 2 Methylation patterns of colonic adenocarcinoma and normal colonic mucosa from patients 2 and 3. Autoradiographs were prepared from blots made as described in Fig. 1 legend. Lanes labelled C and N were prepared from DNA digests of the colon carcinoma and normal colonic mucosa, respectively. *a, HpaII*, HGH probe, patient 2; *b, HhaI*, HGH probe, patient 2; *c, HpaII*, γ-globin probe, patient 2; *d, HhaI*, γ-globin probe, patient 2; *e, HhaI*, HGH probe, patient 3; *f, HpaII*, γ-globin probe, patient 3; *g, HhaI*, γ-globin probe, patient 3. Asterisks (molecular weight markers) are as in Fig. 1.

Fig. 3 Methylation pattern of small cell carcinoma of the lung, normal lung, and liver metastasis from patient 5. Autoradiographs were prepared as described in Fig. 1 legend. Lanes labelled C, M and N were prepared from DNA digests of the lung carcinoma, liver metastasis and normal lung, respectively. *a, HpaII*, HGH probe; *b, MspI*, HGH probe; *c, HpaII*, γ-globin probe; *d, HpaII*, α-globin probe; *e, HhaI*, α-globin probe. Asterisks (molecular weight markers) are as in Fig. 1.

genes[44]. Figure 1*a* shows that the internal cytosine in the *HpaII* recognition sequence 5′-CCGG-3′ of the cancer cells was substantially less methylated than that of their normal counterparts in the region of one or more of these genes. Cleavage with *HhaI* showed that the internal cytosine in the sequence 5′-GCGC-3′ was also significantly hypomethylated in the cancer cells, as assessed with the HGH probe, compared with the normal counterparts (Fig. 1*b*). In contrast, the HGH probe revealed no difference between the normal and cancer cells in methylation of the external cytosine residues in the sequence 5′-CCGG-3′. This was shown by treating the DNA samples with *MspI*, an enzyme that cleaves the recognition sequence 5′-CCGG-3′ regardless of the state of methylation of the internal cytosine[45], but does not cleave if the external cytosine is methylated[46,47]. In all the cases examined here (Figs 1*c*, 3*b* and unpublished data), no differences in the *MspI* patterns between the DNA from the normal and cancerous tissues were detected with either the HGH probe or the globin probes. This result is of interest because it has been shown, at least in an experimental system, that the sequence 5′-CmCGG-3′ is stably inherited, while the sequence 5′-mCCGG-3′ is not[48]. In addition, for all the patients, no differences between the normal and cancerous tissues were detected with the enzymes *Hin*dIII or *Eco*RI, which are not sensitive to methylcytosine residues[33].

Differences in methylated sites were also found in the region of the γ-globin gene in patient 1, using the 5′-mCG-3′-sensitive enzymes (Fig. 1*d, e*). No substantial differences in methylation were found in the region of the α-globin gene (Fig. 1*f, g*).

The second patient studied was a 63-yr-old male with a grade D, poorly differentiated colonic adenocarcinoma. This patient

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also showed clear-cut decreases in the degree of methylation of the growth hormone-related genes and the γ-globin gene (Fig. 2*a–d*). However, the pattern of the bands was in some cases different from that of the first patient (compare Figs 1 and 2). There was a heterogeneous pattern of methylation from cell to cell within each tumour, as indicated by the presence of several low-molecular weight bands of varying intensity on Southern hybridization. In contrast, these sites were almost uniformly methylated in normal colonic mucosa. These results are not consistent with a clonal (unique) methylation pattern in the tumours, but suggest that a varying proportion of the cells in each cancer have failed to perform the normal methylation at individual 5'-CG-3' sites. The resultant heterogeneous pattern of methylation has obvious parallels to the heterogeneity in biological properties often noted in cancer[49].

Patient 3 was a 75-yr-old female with a grade D, moderately well differentiated colonic adenocarcinoma. Hybridizaton of both HGH and γ-globin probes to *Hpa*II and *Hha*I DNA digests also revealed substantial hypomethylation in the cancer in the region of these genes, compared with normal tissue (Fig. 2*e–g*). Patient 4, a 69-yr-old female with a grade D, poorly differentiated colonic adenocarcinoma, showed no differences in methylation between the cancerous and normal tissues for the three genes studied (Table 1). This analysis obviously does not preclude DNA hypomethylation in other areas of the genome in this patient.

A second type of cancer, small cell carcinoma of the lung, was removed from patient 5, a 53-yr-old male. DNA was extracted from the primary cancer, from normal lung tissue, and from a liver metastasis. Each of the three probes showed substantial hypomethylation at the *Hpa*II and *Hha*I sites in the cancer tissue compared with normal lung tissue (Fig. 3). It is significant that the liver metastasis, which was undoubtedly derived from the same cell type as the primary tumour, showed even greater hypomethylation than the primary cancer. Thus, hypomethylation was progressive from primary cancer to metastasis in this patient. This pattern of increasing degree of hypomethylation, from normal to primary cancer to metastasis, was evident with all the probes used (Fig. 3*a*, *c–e*).

The relative degree of hypomethylation of the three genes studied was assessed by densitometry. Four of the five cancers showed 3–9-fold differences in the degree of methylation in at least two of the gene regions studied (compared with the normal tissue of the same patient), as assessed by the method described in Table 1. Methods for detecting general hypomethylation might not have shown differences between the DNA samples from the cancerous and normal tissues studied here. For example, Fig. 4 shows an ethidium bromide-stained gel containing *Hpa*II- and *Msp*I-digested DNA samples from patient 5. This gel was used to make the blots shown in Fig. 3*a*, *b*. No gross differences in methylation were detected among the normal, cancerous and metastatic tissues (Fig. 4), even though three different probes all showed significant hypomethylation on Southern analysis (Fig. 3). Therefore, our data suggest that specific sites in genes of cancer cells may be hypomethylated even in the absence of a general hypomethylation detectable with less sensitive techniques.

Thus, we observed substantial hypomethylation in several specific genomic regions for four of five human cancers, compared with adjacent normal cells from the same patients. A metastasis from one patient showed an even greater degree of hypomethylation than the primary tumour. Further studies are required, using other tumours and different probes, to determine the prevalence of this form of genomic alteration in neoplasia. However, this study clearly shows that such DNA alterations exist in at least some human cancers. These alterations may be widespread, as the genes studied are localized on three different chromosomes[50]. Whether such alterations underlie the abnormal pattern of gene expression found in cancer remains a subject for future investigation.

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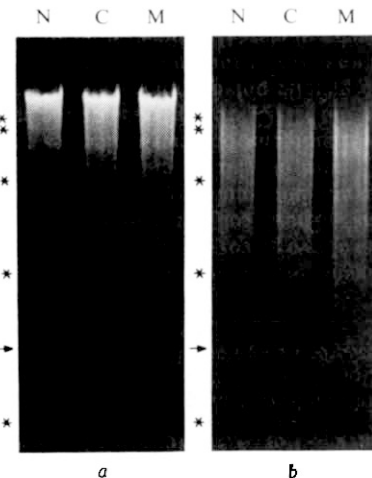

Fig. 4 Ethidium bromide-stained gel of DNA samples digested with *Hpa*II and *Msp*I. DNAs were prepared from lung carcinoma (C), liver metastasis (M) and normal lung (N) of patient 5, and 5 μg of each were digested with *Hpa*II (a) or *Msp*I (b) as described in Fig. 1 legend. The DNA samples were electrophoresed on a 1% agarose gel, stained with ethidium bromide (2 $\mu g\ ml^{-1}$) and photographed under UV light. The DNA was then transferred to nitrocellulose for the experiments shown in Fig. 3*a*, *b*. Asterisks (molecular weight markers) are as in Fig. 1. The arrow indicates the bromophenol blue dye marker.

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Chloroplast DNA exists in two orientations

Jeffrey D. Palmer

Carnegie Institution of Washington, Department of Plant Biology, Stanford, California 94305, USA

An almost universal feature of the circular chloroplast genome is a large inverted repeat sequence, some 10–25 kilobase pairs (kb) in size, which separates the remainder of the molecule into single copy regions of ~80 kb and 20 kb[1–3]. A number of physical properties—formation of head-to-head dimers[1], copy-correction between the inverted repeat segments[1,3,4], resistance to intramolecular recombinational loss[1–3], and maintenance of a highly stable chloroplast genome resistant to rearrangement[2]—have been attributed to the presence of this large inverted repeat. However, one property which an inverted repeat might be expected to confer—reversal of polarity of the single copy sequences located between the repeats[1]—has not yet been demonstrated for the chloroplast genome. I now show that chloroplast DNA prepared from a single plant of common bean (*Phaseolus vulgaris*) consists of two equimolar populations of molecules differing only in the relative orientation of their single copy sequences. A model is presented to explain these results, and comparisons are made to similar cases of inversion heterogeneity in 2-micrometre plasmid DNA from yeast[5,6] and in herpes simplex virus DNA[7,8].

The polarity of the unique sequences between the inverted repeats found in chloroplast DNA molecules can be determined by restriction fragment analysis, but it requires the use of an enzyme that does not cleave within the inverted repeat[9] but does cleave the unique regions asymmetrically with respect to the inverted repeat. Recently, restriction enzyme maps have been constructed for the chloroplast genome of the common bean (*P. vulgaris*), and it has been found that *Sal*I does not cleave the inverted repeat, but does cut in an asymmetric fashion four times in the large and once in the small single copy regions (Fig. 1).

Digestion of common bean chloroplast DNA with *Sal*I yields seven fragments, the sizes of which add up to 249 kb (Fig. 2, track 2). The four largest *Sal*I bands appear with approximately half the intensity of the three smallest bands. This same fragment pattern was seen in multiple digests of the same DNA using an excess of *Sal*I, and also in complete *Sal*I digests of chloroplast DNA prepared from a different cultivar (Hawkesbury Wonder) from that shown in Fig. 1 (Kentucky Wonder Bean). However, analysis with a number of other restriction enzymes, including *Pst*I (Fig. 1) and *Xho*I[10,11], gives a genome size of 150 kb, ~100 kb lower than that obtained with *Sal*I. The simplest explanation for this discrepancy is that molecular heterogeneity is revealed by *Sal*I, which does not cleave the inverted repeat (Fig. 1), but not by enzymes such as *Pst*I (Fig. 1) and *Xho*I[10,11], which do.

This was confirmed by hybridizing cloned mung bean *Pst*I fragments of 18.8 kb and 9.7 kb, which are defined by the same

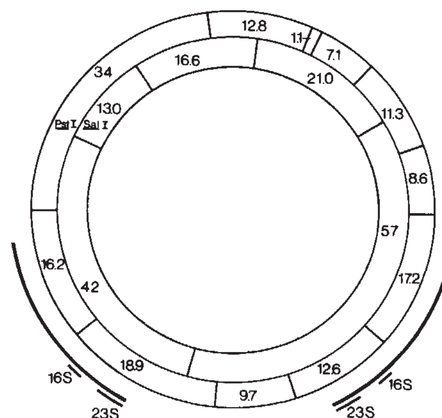

Fig. 1 Restriction map of common bean chloroplast DNA[10] showing the position of the inverted repeat (long heavy lines) and a single orientation of the unique sequence regions. Fragment sizes are in kb.

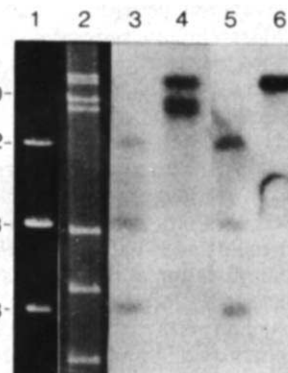

Fig. 2 Physical heterogeneity of common bean chloroplast DNA. Chloroplast DNA was purified[23] from a single common bean plant (*Phaseolus vulgaris* cv. Kentucky Wonder Bean). *Eco*RI and *Sal*I restriction fragments of phage λ DNA, together with intact λ DNA (track 1), and *Sal*I fragments of common bean chloroplast DNA (track 2) were separated by electrophoresis in a 0.4% agarose gel[23]. Duplicate nitrocellulose filter blots of the gel were prepared according to a modification[23] of the bidirectional transfer method of Smith and Summers[24]. Filters were hybridized[23] using as probes ^{32}P-nick-translated[25] cloned[26] mung bean chloroplast DNA *Pst*I fragments of 18.8 kb (tracks 3 and 4) and 9.7 kb (tracks 5 and 6). Size of λ fragments are given in kb. Hybridization of the pBR322–chloroplast DNA recombinant plasmids to λ phage restriction fragments in tracks 3 and 5 results from a small amount of cross-homology between pBR322 and λ DNAs[27].

*Pst*I sites as the common bean fragments of 18.9 kb and 9.7 kb[10] (Fig. 1), to nitrocellulose filter blots of the gel lanes shown in tracks 1 and 2 of Fig. 2. The 18.8-kb fragment hybridizes to the 42-kb and 46-kb *Sal*I fragments, and also to an unresolved band which probably represents the two largest *Sal*I fragments of 57 and 53 kb (Fig. 2, track 4). The 9.7-kb probe hybridizes only to the broad 57–53-kb band (Fig. 2, track 6). The most reasonable explanation for these data is that common bean chloroplast DNA consists of two distinct molecular species, present in approximately equal proportions, which differ only with respect to the relative orientation of their single-copy DNA regions (Fig. 3, bottom).

The top portion of Fig. 3 presents a schematic representation of the hypothetical intermediates in the interconversion of the two circular chloroplast DNA isomers. It is envisioned that reciprocal recombination between the paired inverted repeat segments leads to direct interconversion of the dumbbell-shaped intermediates, which may then relax to give the circular conformation typically seen in the electron microscope[1,12,13]. That no dumbbell-shaped molecules have been observed in the electron microscope[1,12,13] suggests either that such structures are fragile and difficult to isolate or that the vast majority of chloroplast DNA molecules are in the unpaired, circular confor-

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