
The 5-methylcytosine content of DNA from human tumors

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ABSTRACT

The overall 5-methylcytosine (m^5C) content of DNA from normal tissues varies considerably in a tissue-specific manner. By high-performance liquid chromatography, we have examined the m^5C contents of enzymatic digests of DNA from 103 human tumors including benign, primary malignant and secondary malignant neoplasms. The diversity and large number of these tumor samples allowed us to compare the range of DNA methylation levels from neoplastic tissues to that of normal tissues from humans. Most of the metastatic neoplasms had significantly lower genomic m^5C contents than did most of the benign neoplasms or normal tissues. The percentage of primary malignancies with hypomethylated DNA was intermediate between those of metastases and benign neoplasms. These findings might reflect an involvement of extensive demethylation of DNA in tumor progression. Such demethylation could be a source of the continually generated cellular diversity associated with cancer.

INTRODUCTION

Tissue-specific differences in the levels of 5-methylcytosine (m^5C) in mammals have been demonstrated in total genomic DNA (1-4) as well as at certain sites in the vicinity of various genes (5-13). There is much evidence that demethylation of specific gene regions of vertebrate or animal viral DNA is sometimes associated with or necessary for transcriptional activation or maintenance of transcriptional activity (5-13). Nonetheless, much of the tissue-specificity in DNA methylation levels cannot be associated with the control of transcription because of the magnitude of these differences; as much as 3×10^7 more m^5C residues in the DNA of the nucleus of one cell as that in another of the same mammal (3,4). Furthermore, the types of DNA sequences and tissues exhibiting these differences suggests that although tissue-specificity in DNA methylation is related to differentiation, it is not always correlated with the control of transcription (3,4).

Since differences in the extent of DNA methylation among normal human tissues are considerable (3), we have compared the genomic m^5C contents of a

wide variety of malignant human neoplasms to those of various benign neoplasms and normal tissues of human origin. Although both sets of samples show much diversity in m^5C content, a much higher percentage of malignant tumors, especially metastases, had DNA with unusually low m^5C levels than did either benign tumors or normal tissues. In agreement with these results, hypomethylation of five genes has been associated recently with human neoplasms (14,15).

MATERIALS AND METHODS

Purification of DNA. DNA was isolated from fresh surgical or autopsy samples obtained from humans and stored at $-40^{\circ}C$ within 4 h of surgery or 16 h post mortem. The neoplastic samples were chosen so as to avoid grossly necrotic tissue and so as to minimize contamination with normal tissue. Purification of the DNA was as previously described (3) except in the case of fibrous tissues, which were treated with 100 units/ml of collagenase (Type IV, Sigma) for 3 h at $37^{\circ}C$ after homogenization of the minced tissue in 10 mM Tris-HCl, 50 mM EDTA, 0.2 M NaCl, pH 8.0, and rinsing and resuspension in 10 mM Tris-HCl, 10 mM $CaCl_2$, pH 7.6. Subsequently these samples were defatted and treated with proteinase K in the presence of 50 mM EDTA, pH 8.0, and detergent as previously described (3). The A_{260}/A_{280} ratio of all samples was >1.85 .

For restriction analysis, a high-molecular-weight DNA preparation (≥ 50 kb) was obtained from purified, human brain and placental DNA samples by the following modification of Hirt's procedure (16). Sodium dodecyl sulfate and NaCl were added at final concentrations of 0.5% and 1 M, respectively, to 2 mg of DNA. After standing at $0^{\circ}C$ for 10 min, the precipitate was collected by centrifugation at $10,000\times g$ for 10 min. The pellet was resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8, gently extracted once with phenol and then with chloroform/isoamyl alcohol (24:1, v/v) and precipitated at room temperature with 0.1 volume of 3 M sodium acetate, pH 5.2, plus 2 volumes of ethanol. The pellet was rinsed with 70% ethanol, evaporated briefly just to remove the ethanol, and finally resuspended in the above Tris, EDTA buffer. This method yielded DNA solutions devoid of detectable inhibitors of endonucleases.

Analysis of the extent of methylation. The m^5C content was determined from the total deoxynucleoside composition of enzymatic digests subjected to HPLC by a modification (Gehrke et al., in preparation) of our previous method (17,18). Two to four HPLC analyses were performed on each DNA

sample. For the z test of proportions each data point for tumor DNA was the mean m^5C content from replicate HPLC determinations on a single tumor and for normal tissue or cell DNAs each data point was the mean m^5C content from replicate tissue samples from different individuals.

An aliquot of one tumor DNA sample was fractionated by reassociation kinetics (3) before digestion and HPLC. Another aliquot of this DNA, two other tumor DNAs, and normal brain and placental DNAs were subjected to restriction analysis with HpaII or MspI (5) in both 0.8% and 0.4% agarose gels. The gel electropherograms were photographed under ultraviolet light after staining with ethidium bromide and the negatives obtained in their range of linear response were subjected to microdensitometry. The number-average molecular weight (M_n) was determined by dividing the densitometry tracings into 20 equal-sized fractions and using the equation:

$$M_n = \frac{1}{\sum 0.01 w_i / M_i}$$

where w_i is the percent of DNA in each fraction of molecular weight M_i .

RESULTS

We analyzed the genomic m^5C content of 21 benign human tumors and 82 malignancies by HPLC of digests of high-molecular-weight DNA samples. The average relative standard deviation in the m^5C levels from replicate determinations was 2-3%. DNA from various benign tumors had 0.77-1.03 mol% m^5C (Table 1). This range was almost the same as that previously observed in normal human tissues (3). Primary and secondary malignancies showed an even broader spectrum of m^5C levels (Tables 2 and 3). One testicular seminoma had a remarkably low value of 0.35 mol% m^5C and the rest of the tumor DNAs were in the range of 0.60-1.03 mol% m^5C (Tables 2 and 3). The distribution

Table 1. 5-Methylcytosine content of DNA from benign human tumors

Tumor classification	Mean mol% m^5C^a	Tumor classification	Mean mol% m^5C^a
Uterine leiomyoma	0.77, 0.83, 0.84, 0.88, 0.89, 0.90, 0.92, 0.97, 1.03	Breast cytosarcoma phylloides	0.83
Ovarian cystadenoma	0.89, 1.02	Thyroid adenoma	0.93, 0.96
Breast fibroadenoma	0.79, 0.85, 0.88, 0.88 0.89, 0.91	Brain ganglioneuroma	0.92

^aEach value for the mol% m^5C (percentage of total bases that is m^5C) in the DNA represents an average from two to four digests of DNA from an individual tumor.

Table 2. 5-Methylcytosine content of DNA from metastatic neoplasms

Site of metastasis	Type of neoplasm	Mean mol% ^5C ^a	Site of metastasis	Type of neoplasm	Mean mol% ^5C ^a
Lymph node	melanoma	0.67 ^b , 0.76 ^b	Peritoneum	uterine carcinoma	0.78
	Wilm's tumor	0.90 ^c		unclassified carcinoma	0.66 ^b
	pancreatic adenocarcinoma	0.72	Intestine	ovarian adenocarcinoma	0.89 ^b
	uterine carcinoma	0.74	Colon	rectal carcinoma	0.79
	breast adenocarcinoma	0.74 ^d , 0.89 ^b	Liver	melanoma	0.70 ^b
	rectal carcinoma	0.79	Liver	endometrial carcinoma	0.87
	pheochromocytoma	0.89 ^b	Omentum	prostate carcinoma	0.76 ^{c,d}
Skin	epidermoid carcinoma	0.92 ^b	Brain	melanoma	0.95 ^b
	hemangiopericytoma	1.03	Lung	breast carcinoma	0.79

^aEach value is derived from a different neoplasm and represents the mean mol% ^5C from two to four replicate digests of DNA from that tumor.

^bThe patient received neither chemotherapy nor radiation treatment of the tumor before its removal.

^cThe patient received chemotherapy before removal of the tumor.

^dThe tumor was irradiated at some time prior to its removal.

No superscript is used if data on chemotherapy or irradiation were unavailable.

Table 3. 5-Methylcytosine content of DNA from primary malignant neoplasms

Tumor classification	Mean mol% 5C^a	Tumor classification	Mean mol% 5C^a
Rectal carcinoma	0.79 ^b , 0.87	Pheochromocytoma	0.74 ^b
Nodular lymphocytic lymphoma	0.80 ^c	Kidney carcinoma	0.91 ^b , 0.95 ^b
Histiocytic lymphoma	0.78 ^d , 0.88 ^b , 0.95 ^c	Lung adenosquamous carcinoma	0.79, 0.84
Leiomyosarcoma	0.97 ^{c,d}	Lung squamous carcinoma	0.79
Ovarian carcinoma	0.69, 0.71 ^b , 0.76 ^c , 0.80, 0.81, 0.85, 0.87, 0.95	Lung small cell carcinoma	0.76 ^d
Mixed müllerian carcinoma	0.60	Parotid epidermoid carcinoma	0.81 ^c
Breast adenocarcinoma	0.76, 0.81 ^c , 0.83, 0.83 ^b , 0.83, 0.86 ^b , 0.87, 0.89 ^b , 0.90 ^b , 0.90 ^b , 0.96 ^b , 0.97, 0.99 ^b	Parapharyngeal epidermoid carcinoma	0.89 ^b
Colon carcinoma	0.78, 0.80, 0.80 ^b , 0.81, 0.81, 0.81 ^b , 0.84 ^b , 0.86, 0.88, 0.90 ^b , 0.91 ^c	Testicular seminoma	0.35 ^b , 0.95
Gastric carcinoma	0.74 ^b , 0.91 ^b	Brain glioblastoma multiforme	0.66 ^b , 0.76 ^b , 0.92 ^b , 0.97 ^b
Pancreatic carcinoma	0.72 ^b	Brain astrocytoma	0.84 ^b
Prostate carcinoma	0.87 ^b	Brain oligodendroglioma	0.96 ^b
Granular cell tumor	0.92 ^b		

^aEach value is derived from a different tumor and represents the mean mol% 5C from two to four replicate digests of DNA from that tumor.

^bThe patient received neither chemotherapy nor radiation treatment of the tumor before its removal.

^cThe patient received chemotherapy before removal of the tumor.

^dThe tumor was irradiated at some time prior to its removal.

No superscript is used if data on chemotherapy or irradiation were unavailable.

of m^5C levels in DNAs from various malignancies and that in DNA from normal tissues was very different as will be described below. In contrast, the total cytosine (cytosine plus m^5C) content of the examined DNAs showed no significant variation and for malignancies was 20.5 mol% (standard deviation, 0.4 mol%), which is not significantly different from the mean value previously found for normal human tissues (20.9 mol%; 3).

Much higher percentages of malignant DNAs than of normal DNAs were hypomethylated as can be seen in Table 4 where the DNA samples were grouped according to whether they had methylation levels in the range of most normal human tissues (>0.84 mol% m^5C), somewhat lower methylation levels (0.80–0.84 mol% m^5C) or whether they were considerably hypomethylated (<0.80 mol% m^5C). Like the DNA from most normal adult tissues, the DNA from most of the benign neoplasms had more than 0.84 mol% m^5C , whereas that from most of the secondary malignancies had less than 0.80 mol% m^5C ; these differences between benign neoplasms or normal tissues and metastases were statistically significant ($P < 0.001$ by the z test of proportions). Of the normal tissues, only placenta had less than 0.80 mol% m^5C in its DNA. Placenta is an atypical organ, whose cells have some of the properties associated with malignant cells (19) and whose globin genes are unusually hypomethylated (20). The distribution of DNA methylation levels for primary cancers was intermediate between those of benign neoplasms and secondary neoplasms. For example, no benign neoplasms or normal tissues had DNA with less than 0.75 mol% m^5C , whereas 13 and 30%, respectively of the primary and secondary malignancies were in this category, (Tables 1–4).

Differences in DNA methylation levels of neoplastic cells compared to the levels in normal cells are underestimated from the results since some of the tumor samples used for DNA extraction were probably considerably contaminated with normal cells in spite of our attempts to obtain tissues which contained predominantly tumor cells. The variety of methylation levels seen in the gastric carcinomas (Table 3) correlates with the microscopically determined extent of contamination of these samples with normal cells. Similarly, the diversity in the m^5C content of DNA from leiomyomas can be explained by their pleiomorphism and that of the colorectal samples by varying numbers of included lymph nodes (Table 3). Nonetheless, some of the tumor samples, either on the basis of pathological analysis or comparison of their DNA methylation levels to those of adjacent normal tissues appear to be composed predominantly of neoplastic cells. For example, two of the primary lymphomas and six of the lymph node metastases (Tables 2 and 3) had

Table 4. Distribution of genomic 5-methylcytosine contents in normal and neoplastic tissues from humans

Type of sample	Total number of tissue samples	Percentage distribution of tissue samples according to the m^5C content of the DNA ^a		
		<0.80 mol% m^5C	0.80-0.84 mol% m^5C	>0.84 mol% m^5C
Benign tumors	21	10	14	76
Primary malignancies	62	27	26	47
Secondary malignancies	20	60	0	40
Normal tissues	43	7	13	80

^aThe data in this table are derived from analyses of DNA from tumors cited in Tables 1-3 and nine previously described types of normal tissues or *in vivo*-derived cell populations (3) and six more types of normal tissues. The latter had the following mean mol% m^5C from replicate samples or HPLC determinations: colon, 0.89; rectum, 0.87; pancreas, 0.85; adrenal cortex, 0.86; stomach, 0.86; esophagus, 0.83. For normal tissues the percentage distribution refers to the distribution of the 15 different types of samples with the genomic m^5C contents averaged for the multiple samples of each type.

DNA methylation levels far below those of normal, circulating or splenic lymphocytes and those of whole, normal spleen and thymus (0.96, 0.93, and 1.00 mol% m^5C , respectively; 3).

The intragenomic distribution of m^5C was examined in the case of the primary gastric adenocarcinoma DNA with 0.74 mol% m^5C . The examined Cot fractions and their mol% m^5C were as follows: Cot $<10^{-3}$, 0.93; Cot 10^{-3} -0.05, 1.26; Cot 0.05-50, 0.83; Cot >50 , 0.52. The percentages of the genome in each of these fractions was 2, 17, 9, and 71, respectively. The ratios of mol% m^5C in each of these Cot fractions to that of the unfractionated tumor DNA was similar to the analogous ratios from normal human tissues. This indicates that if demethylation accompanied tumorigenesis it was not confined only to the highly repeated sequences which are normally m^5C -rich (3-5 and 17).

We also digested this gastric adenocarcinoma DNA, a primary pancreatic adenocarcinoma DNA (0.72 mol% m^5C) and a primary rectal adenocarcinoma DNA (0.79 mol% m^5C) with HpaII, which is inhibited by methylation at CG sequences, the primary site of vertebrate DNA methylation (5). Upon electrophoresing these digests and analogous digests of brain DNA (0.98 mol% m^5C ; 3), a greater extent of cleavage and, therefore, hypomethylation of the tumor DNAs relative to the brain DNA was observed (Fig. 1). In contrast, MspI, an isoschizomer of HpaII, which is generally insensitive to methylation at the CG site (5), digested the tumor DNAs and the brain DNA equally well (Fig. 1). Placental DNA, which is the most hypomethylated of all tested normal in vivo-derived human DNAs (0.76 mol% m^5C and 22% less m^5C residues than brain DNA; 3) appeared to be methylated at HpaII sites to a similar extent as these tumor DNAs (Fig. 1) and had ~20% more unmethylated HpaII sites than did brain DNA. However, precautions for and limitations of this common type of quantitative restriction analysis of methylation in unfractionated genomes should be noted. In order to quantitate the extent of digestion, high-molecular-weight preparations of DNA should be used and electrophoresis carried out in $<0.5\%$ agarose gels to analyze large fragments and also in $>0.5\%$ agarose for the medium-sized and small fragments. A random distribution of fragment sizes is usually assumed in order to apply the relation that the number-average molecular weight is half of the weight average molecular weight; however, this assumption is incorrect for a HpaII digest of vertebrate DNA (21). Lastly, a small fraction of the methylated HpaII sites are inaccessible to this analysis (22,23).

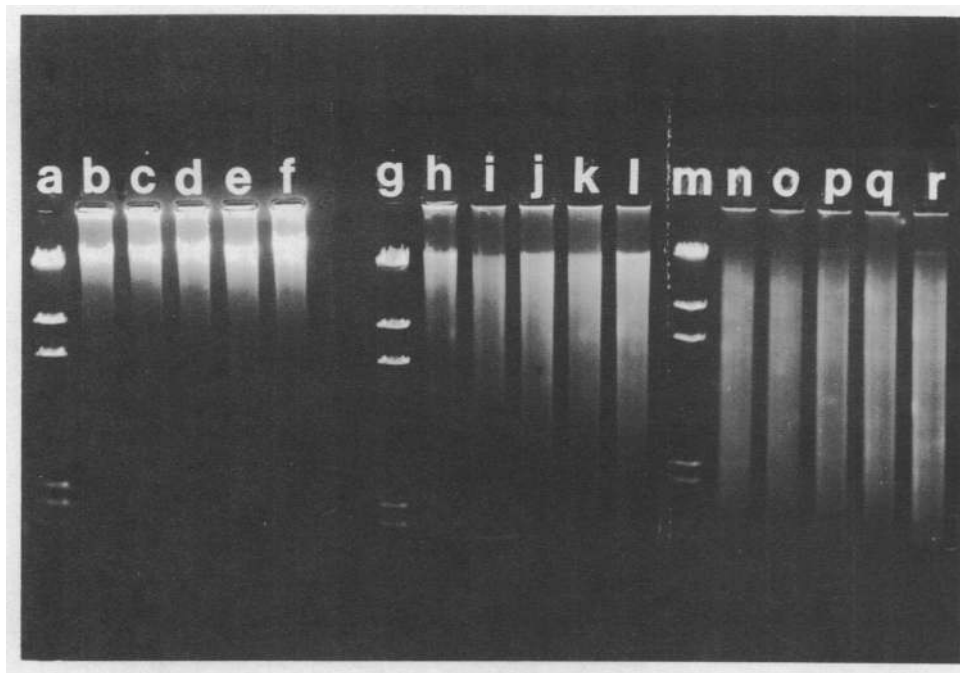


Fig. 1 Restriction analysis of the extent of methylation of DNA from normal human tissues and from three malignancies. Ten micrograms of brain, placental, rectal carcinoma, pancreatic carcinoma, and gastric carcinoma were electrophoresed in a 0.8% agarose gel (27) without prior digestion (b-f, respectively) or after digestion with 100 units of HpaII (h-l, respectively) or MspI (n-r, respectively) for 4 h under standard conditions (28) and visualized by ethidium bromide-induced fluorescence. Lanes a, g, and m contain molecular weight marker bands from *Hind*III digest of λ DNA with 23.7, 9.5, 6.6, 2.3, and 2.1 kb fragments, respectively. That no contaminating inhibitors of the enzymatic reaction were present was demonstrated in duplicate digestions for each sample of human DNA in the presence of [32 P] λ DNA.

DISCUSSION

Despite a wide range of genomic m^5C contents, (0.35–1.03% mol% m^5C), a statistically significant difference was found in the distribution of DNA methylation levels in various malignancies, especially in metastases, as compared to that in benign neoplasms or normal tissues (Tables 1–4). Although in most cases it is not possible to compare the m^5C content of a tumor cell DNA with that of the presumptive, normal cells of the tumor's origin, the percentages of DNA samples with <0.80 mol% m^5C or >0.84 mol% m^5C were strikingly different for malignancies and for normal tissues or benign tumors (Table 4). Out of 15 DNAs from various types of normal tissues or in

vivo-derived cell populations, only 3 had $<0.85 \text{ mol\% } m^5C$ and two of these, namely, placenta and sperm, were not from adult, somatic tissue (3; Table 4). The average m^5C content determined by pooling data for all 15 of these normal samples was 0.89 mol\% . The same average value was obtained from all 21 benign tumor DNAs. In contrast, most of the metastases had $<0.80 \text{ mol\% } m^5C$ in their DNA and the average $\text{mol\% } m^5C$ values for DNA from all of the 20 examined metastases and all of the 62 primary tumors were 0.78 and 0.83 , respectively (Tables 2-4). The differences between these average m^5C levels for malignant and nonmalignant samples were much greater than the average standard deviation for replicate samples (0.02 - 0.03 mol\%). These comparisons suggest that either hypomethylation often accompanied tumorigenesis or that many of these neoplasms were derived from atypical, minor populations of cells with relatively low m^5C contents.

Three primary brain gliomas had DNA that was strikingly hypomethylated compared to that of normal brain ($0.98 \text{ mol\% } m^5C$; 3). The other glioma samples might owe their higher chromosomal m^5C content to a much higher proportion of normal cells. On the other hand, the relatively high DNA methylation levels of the leiomyosarcoma sample (Table 3), which appeared to be composed mostly of cancer cells, may indicate that extensive demethylation need not accompany oncogenesis. However, at least 5×10^6 cytosine residues in the DNA (5% of the total) would have to be demethylated per diploid nucleus in order to conclude by HPLC analysis that genomic demethylation occurred.

It is unlikely that the aneuploidy associated with most cancer cells (24) directly accounts for our results by changing the proportion of chromosomes because the large number of human chromosomes and their DNA sequence complexity should average out their effects on the base composition. Another trivial explanation of the hypomethylation of tumor cell DNA could be that the high cell turnover rate of neoplasms does not allow DNA methylation to keep pace with DNA replication. However, previous studies have shown that there is not a correlation between DNA methylation levels and the average cell turnover times for normal mammalian tissues (3,4). Also, treatment of the cancer patients cannot be correlated with DNA hypomethylation because unusually low genomic m^5C contents were observed in many malignancies from patients who had not been treated with radiation or chemotherapeutic antimetabolites (Tables 2 and 3) and it is improbable that the drugs which are commonly used on patients with solid tumors could cause considerable DNA demethylation. Our observation of the tendency of human

malignancies to exhibit hypomethylation of their DNAs is consistent with the recently observed hypomethylation of the unfractionated DNA of many human tumor cell lines compared to the DNA of diploid cell strains (25). Similarly, DNA from chemically induced hepatocarcinomas in rats was reported to have a lower m^5C content than did normal rat liver DNA (26). Our observation of hypomethylation being more frequent in metastases than in primary tumors is consistent with the finding that the human growth hormone gene was much more hypomethylated in a metastasis than in the primary tumor from which it was derived (14).

The m^5C content of neoplastic samples should reflect the percentage and type of neoplastic cells in the sample, the type of cells which gave rise to the neoplasm, and any changes in DNA methylation which occur during the early stages of oncogenic transformation or during tumor progression. That a much higher percentage of secondary malignancies compared to primary malignancies had hypomethylated DNA might reflect less contamination of the former with normal cells or the selection of hypomethylated cells during the evolution of the neoplasms. We propose that both factors have influenced our results and that tumor progression with its attendant continually generated cellular diversity (27) is often accompanied by extensive replacement of m^5C residues in DNA with cytosine residues. Hypomethylation of DNA during tumor progression could provide epigenetic changes of the type associated with normal differentiation. It could help establish or maintain transcriptional activity or be associated with cancer-related chromosomal rearrangements, gene amplification, and alterations in chromosome conformation. The resulting changes in expression or replication of the genome might be an important component in the diversification of tumor cells, which allows them to successfully exploit their hosts.

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