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DNA methylation in cancer: too much, but also too little

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Cancer-associated DNA hypomethylation is as prevalent as cancer-linked hypermethylation, but these two types of epigenetic abnormalities usually seem to affect different DNA sequences. Much more of the genome is generally subject to undermethylation rather than overmethylation. Genomic hypermethylation in cancer has been observed most often in CpG islands in gene regions. In contrast, very frequent hypomethylation is seen in both highly and moderately repeated DNA sequences in cancer, including heterochromatic DNA repeats, dispersed retrotransposons, and endogenous retroviral elements. Also, unique sequences, including transcription control sequences, are often subject to cancer-associated undermethylation. The high frequency of cancer-linked DNA hypomethylation, the nature of the affected sequences, and the absence of associations with DNA hypermethylation are consistent with an independent role for DNA undermethylation in cancer formation or tumor progression. Increased karyotypic instability and activation of tumor-promoting genes by *cis* or *trans* effects, that might include altered heterochromatin-euchromatin interactions, may be important consequences of DNA hypomethylation which favor oncogenesis. The relationship of DNA hypomethylation to tumorigenesis is important to be considered in the light of cancer therapies involving decreasing DNA methylation. Inducing DNA hypomethylation may have short-term anticancer effects, but might also help speed tumor progression from cancer cells surviving the DNA demethylation chemotherapy.
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Introduction

Recently, there has been much emphasis on the critical role of DNA hypermethylation in human carcinogenesis (Baylin and Herman, 2000; Issa, 2000). In comparison, there has been inadequate attention paid to decreased methylation of DNA in cancer. The discovery of extensive cancer-associated DNA hypomethylation in the human genome (Feinberg and Vogelstein, 1983a,b; Gama-Sosa *et al*., 1983b) preceded that of cancer-linked DNA hypermethylation. DNA hypomethylation in cancer often affects more of the genome than does hypermethylation so that net losses of genomic 5-methylcytosine (m^5C) are seen in many human cancers (Gama-Sosa *et al*., 1983b).

The role in carcinogenesis of cancer-linked hypermethylation of transcription control regions is clear because of the consequent transcriptional silencing of genes important for prevention of cancer (see other reviews in this issue). The biological significance of DNA hypomethylation in cancer is less understood. Nonetheless, experiments involving DNA methylation inhibitors *in vivo* and *in vitro* (Carr *et al*., 1984; Denda *et al*., 1985; Thomas and Williams, 1992) or analysis of DNA methyltransferase-deficient mice (unpublished data, L Jackson-Grusby and R Jaenisch cited in Ehrlich, 2000) indicate the importance of induced DNA hypomethylation to oncogenesis. Studies of tumorigenesis in rodents fed methyl-deficient diets (reviewed in Poirier, 1994 and Ehrlich, 2000) are in accord with a causative role for DNA hypomethylation in cancer, although the contribution of carcinogenic effects of these diets other than changes in DNA methylation is unclear (Pogribny *et al*., 1995). As described below, the high frequency of cancer-associated DNA hypomethylation, the nature of the affected sequences, and the absence of associations with DNA hypermethylation are consistent with an independent role for undermethylation of certain DNA sequences in cancer formation or tumor progression.

DNA modification in cancer: hypo- or hypermethylated relative to what?

Hypomethylation and hypermethylation of DNA are relative terms and denote less or more methylation than in some standard DNA. When applied to cancer epigenetics, that standard is normal tissue. However, there are considerable differences in the amounts and distribution of DNA methylation among different vertebrate tissues because DNA methylation is not only species-specific but also tissue-specific (Ehrlich *et al*., 1982; Gama-Sosa *et al*., 1983a). For example, at the extremes of 5-methylcytosine (m^5C) contents of DNA from normal human postnatal somatic tissues, cerebellum DNA has 17% more m^5C than heart DNA. The respective percentages of genomic C methylated in

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these tissues are 4.03±0.14 and 3.43±0.12 (from 12 determinations each) as determined by high performance liquid chromatography (HPLC) of DNA digested to deoxynucleosides (Ehrlich *et al*., 1982; Ehrlich *et al*., submitted). Moreover, these analyses are for organs containing mixtures of cell types. Pure populations of uncultured cells may differ even more in their genomic m^5C content. Therefore, unless the studied tumor DNA is being compared to a relatively pure population of cells which is known to be the cell of origin of the tumor, and that is usually not the case, it is best to use DNA from a variety of normal tissues as the control. Another consideration in identifying cancer-specific differences in genome methylation is that the studied cells should be uncultured cell populations whenever possible because of the frequent changes in DNA methylation that occur upon cell culture (Smiraglia *et al*., 2001). In this review, only cancer-specific DNA methylation determined from uncultured cell populations will be discussed. Most of these changes, like most of the normal tissue-specific differences in DNA methylation patterns, involve CpG sites, the predominant, but not only, location of m^5C in the vertebrate genome (Ramsahoye *et al*., 2000).

How do cancer-associated hypomethylation and hypermethylation differ in extent, frequency, and types of cancers affected?

Extent and frequency of human cancer-associated hypomethylation of DNA

As we first demonstrated by HPLC in a collaboration with Charles Gehrke (Gama-Sosa *et al*., 1983b), various cancers often have a low overall m^5C content compared to many different kinds of postnatal tissues. Although both malignant and normal samples display considerable diversity in their DNA's m^5C content, a high percentage of malignant tumors, especially metastases, have DNA with unusually low m^5C contents relative to the normal tissues (Table 1). Differences in DNA methylation levels between tumors and normal tissues were probably underestimated in this study and many other cancer studies because of contamination with normal cells. In collaboration with Andrew Feinberg and Charles Gehrke (Feinberg *et al*., 1988), we compared colon adenocarcinomas and adjacent normal mucosa, which contains the cell type that gives rise to the malignancy. Significant reductions of 10 and 8% in the average genomic m^5C levels were observed in the cancers and benign polyps compared with the normal mucosa. Furthermore, all of these tumors were less methylated than the adjacent, non-tumorous tissue. Therefore, how early DNA hypomethylation can be detected during tumorigenesis may depend on the type of tumor as well as the individual tumor sample.

In collaboration with Emerich Fiala, we have recently shown that about 60% of Wilms tumors, a pediatric cancer, were globally hypomethylated in their DNA compared to all five analysed normal postnatal somatic tissues (Ehrlich *et al*., submitted). Also, 17 out of 19 ovarian epithelial carcinomas examined displayed global DNA hypomethylation relative to microdissected ovarian epithelial cystadenomas (L Dubeau, E Fiala, and M Ehrlich, unpublished data). Our results on the ovarian tumors are similar to those from an earlier study (Cheng *et al*. 1997) in which the ovarian carcinomas had on the average 25% less m^5C in their DNA than did the cystadenomas, which had not been microdissected. Wilms tumors and ovarian epithelial carcinomas are well suited for this kind of analysis

Table 1 Comparison of global DNA methylation levels in various tumours and normal somatic tissues as well as in normal *vs* ICF brain

Tissue origin of DNA samples	*No. of samples*[a]	*% C residues methylated* $m^5dC/(m^5dC+dC)\times100$ *Mean*[b]	*Range*[b]	*Percentage distribution of various tissue samples according to the* m^5C *content of the DNA*[c] *<3.3% C methylated*	*3.3–3.5% C methylated*	*>3.5% C methylated*
Various normal postnatal somatic tissues	15	3.7	3.5–4.1	0[d]	14	86
Various benign tumors	21	3.7	3.2–4.3	10	14	76
Various primary malignancies	62	3.4	1.4–4.1	27	26	47
Various secondary malignancies	20	3.2	2.7–4.3	60	0	40
Normal cerebrum	2	4.06±0.16 (18)	–	NA[e]	NA	NA
Normal cerebellum	3	4.04±0.03 (6)	–	NA	NA	NA
ICF syndrome brain	1	3.80±0.03 (4)	–	NA	NA	NA

[a]15 types of normal tissues from 2–7 individuals each and a large variety of tumors were analysed by HPLC with 2–4 determinations for each sample. The number of HPLC determinations for the brain samples is given in parentheses. Because of the extreme rarity of the ICF syndrome, which is associated with *DNMT3B* mutations, only one ICF tissue was available. [b]Most of these data are adapted from previously published results (Gama-Sosa *et al*., 1983b; Tuck-Muller *et al*., 2000). The standard deviation is shown for analyses of only one tissue type. The range is given only for data from a variety of tissue types. [c]The percentage distribution refers to the percentage of different types of normal tissues, benign tumors, primary malignancies, or secondary malignancies displaying the indicated range of C methylation. This table is adapted from previously published results (Gama-Sosa *et al*., 1983b). As explained in the text, hypomethylation in tumors may be underestimated for many samples. [d]Term placenta, the only tested normal somatic tissue with <3.3% of its C residues methylated (i.e., 3.2%), is not included in this table because it is not postnatal and its largely trophoblastic origin is probably responsible for its low m^5C content.[e]NA, not applicable; only one type of tissue was analysed for these samples

because they are mostly composed of tumor cells with relatively small amounts of normal cells. Therefore, unlike many types of tumors, such as breast cancers, the percentage of normal cells is not expected to vary widely between samples.

These findings on overall genomic hypomethylation in human malignancies are in accord with early studies of rat hepatocellular carcinomas (Lapeyre and Becker, 1979; Lu *et al*., 1983). Those investigations showed that the m^5C content of DNA from hepatocellular carcinomas was about 20–40% less than that of normal liver. Furthermore, these results could not simply be explained by increased cell cycling. This is consistent with our comparison of overall genomic methylation in various normal human tissues (Ehrlich *et al*., 1982).

Variety of cancers displaying DNA hypomethylation

Global genomic hypomethylation has been found in many types of human cancer, including prostate metastatic tumors *vs* normal prostate (Bedford and van Helden, 1987); leukocytes from B-cell chronic lymphocytic leukemia *vs* normal leukocytes (Wahlfors *et al*., 1992); hepatocellular carcinomas *vs* matched non-hepatoma liver tissue (Lin *et al*., 2001); and cervical cancer or high-grade dysplastic cervical lesions *vs* normal cervical tissue or low-grade dysplasia of the cervix (Kim *et al*., 1994). An exception is a report of higher levels of global DNA methylation in breast cancer (Hakkarainen *et al*., 1996) in a comparison of acid-hydrolyzed DNA from seven lobular carcinomas and that from 10 benign breast tumors. A possible complicating factor in the latter study is that DNA samples that were digested to the free bases by acid might have had varying amounts of RNase degradation products from contaminating RNA, which could influence the results. For this reason, hydrolysis of DNA to deoxynucleosides for m^5C determination is preferable unless the DNA samples are carefully evaluated for lack of contaminating RNA or ribo-oligonucleotides. In the latter study, a small, but not statistically significant, increase in global m^5C levels was observed in ductal carcinomas. In contrast, two other large-scale analyses of breast ductal carcinomas found significant global hypomethylation relative to normal breast tissue or benign breast lesions by analysis of patterns of *Hpa*II- or *Msp*I-digested DNA or in an *in vitro* methyl acceptor assay with *Sss*I methyltransferase (Bernardino *et al*., 1997; Soares *et al*., 1999). In summary, global deficiencies in the genomic m^5C content in various cancers *vs* control tissues are seen very much more often than overall increases in m^5C levels in DNA, despite the frequent findings of localized hypermethylation in all the above types of cancers.

DNA hypermethylation in human cancer: extent, frequency, and types of cancers

Cancer-associated localized hypermethylation has usually been described for CpG islands, DNA sequences of about 1–2 kb that are (C+G)-rich and do not have the typical underrepresentation of CpG seen in vertebrate genomes (a much lower CpG frequency than predicted for a random sequence of that base composition). When they overlap promoters, CpG islands are usually, but not always (e.g. Lethe *et al*., 1998), constitutively unmethylated in normal tissues. Frequent and extensive hypermethylation of certain CpG islands is observed in a very wide variety of cancers (Figure 1). In a study of methylation of almost 1200 unselected CpG islands in 98 primary cancers (breast, colon, brain, head and neck, testicular, neuroectodermal tumors and acute myeloid leukemia) by restriction landmark genomic scanning (RLGS) of

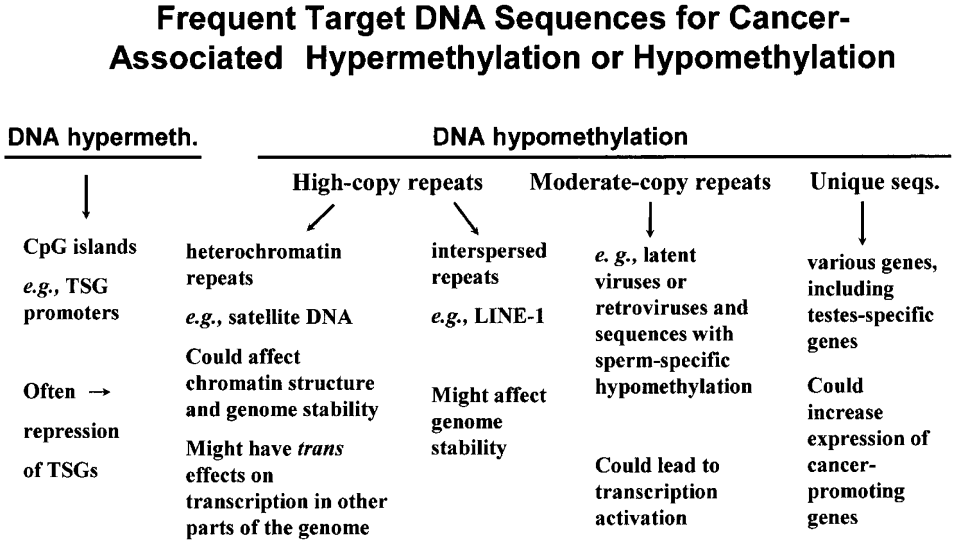

Figure 1 A summary of the most frequent types of sequences affected by cancer-specific DNA hypermethylation or hypomethylation. TSG, tumor suppressor gene

*Not*I/*Eco*RV fragments, it was estimated that an average of ~600 of ~45 000 CpG islands in the genome are hypermethylated in cancer *vs* control tissue with a maximum of ~4500 and a minimum of 0 (Costello *et al*., 2000). Among the tumors with the highest frequency of CpG island hypermethylation were colon cancers. Testicular cancers had the lowest frequency of this hypermethylation. Hypermethylation of 12 CpG islands at the 5′ ends of tumor suppressor genes was investigated in a methylation-sensitive PCR analysis of DNA from 600 cancers, including most of the types of tumors mentioned above plus kidney, bladder, esophageal, pancreatic, and liver cancers. Cancer-associated hypermethylation was found in most tumors of a given type in at least a few of these CpG islands (Esteller *et al*., 2001). In both of these studies, the pattern of which CpG islands are most often hypermethylated varied with the kind of cancer (Costello *et al*., 2000; Esteller *et al*., 2001).

How do cancer-associated DNA hypomethylation and hypermethylation differ in the types of sequences affected?

Types of sequences affected by cancer-associated hypermethylation

An RLGS search for cancer-hypermethylated sequences indicated that most of the affected sequences in non-small cell lung cancers were CpG islands (Dai *et al*., 2001). However, the results were probably influenced by the use of the CpG methylation-sensitive *Not*I for the DNA digestion because *Not*I preferentially cleaves in CpG-rich regions of the mammalian genome. Cancer-associated hypermethylation is seen in CpG islands distant from the 5′ end of the gene (Dai *et al*., 2001; Nguyen *et al*., 2001a) as well as in those that straddle the transcription start site. Therefore, the observed prevalence of cancer-linked hypermethylation in 5′ CpG islands may be due to an ascertainment bias. Cancer-associated hypermethylation occurs not only in genes with a known or likely role in carcinogenesis, but also in genes not implicated in cancer, such as, *MYOD* at its 5′ CpG island (Issa, 2000). Hypermethylation of ribosomal DNA genes was found in breast cancer (Yan *et al*., 2000), but reports of cancer-associated hypermethylation of repeated sequences in human cancer are rare.

Hypomethylation of highly repeated, interspersed DNA sequences in cancer

Hypomethylation has been observed very often in DNA repeats in diverse cancers (Figure 1). The phenomenon of repeat-induced gene silencing, which has been seen in mammals as well as plants and fungi (Garrick *et al*., 1998), is probably related to the finding that mammalian DNA repeats tend to be highly methylated in postnatal somatic tissues. The repeats that display tumor-associated hypomethylation include endogenous retrotransposons. Retrotransposons or retroviral-derived elements can have their transcription upregulated *in vivo* by DNA demethylation. This was concluded from studies of *Dnmt1* knockout mouse embryos, interspecies mammalian hybrids, and mice with an inherited epigenetically controlled phenotype whose expression is regulated by a genetically linked retrotransposon (IAP) (Walsh *et al*., 1998; O'Neill *et al*., 1998; Morgan *et al*., 1999). Also, there is evidence for frequent activation of expression of full-length transcripts from retrotransposons in certain types of murine cancer (Dupressoir and Heidmann, 1997).

Several studies have demonstrated cancer-associated hypomethylation of LINE-1 (or L1), a highly repeated, interspersed human retrotransposon that is up to 6 kb in length. LINE-1 repeats constitute about 15% of the human genome, but of the $\sim 4 \times 10^5$ copies of LINE-1 elements in the human genome, only about 30–60 are estimated to be competent for transposition (Sassaman *et al*., 1997). There have been occasional reports of cancer-associated retrotransposition-like insertions involving LINE-1 sequences (Miki *et al*., 1992; Morse *et al*., 1988), and they may mobilize cellular RNAs at low frequencies (Wei *et al*., 2001). Their activation can also lead to transcriptional interference involving neighboring genes (Whitelaw and Martin, 2001). However, retrotransposition of endogenous elements is implicated in disease much less frequently for humans than for mice (Kazazian and Moran, 1998).

LINE-1 hypomethylation was observed in chronic lymphocytic leukemia *vs* normal mononuclear blood cells (Dante *et al*., 1992), urinary bladder carcinomas compared to normal bladder (Jurgens *et al*., 1996), hepatocellular carcinomas *vs* non-tumorous 'normal' or cirrhotic tissue (Takai *et al*., 2000), and prostate carcinomas *vs* normal prostate and other normal tissues (Santourlidis *et al*., 1999). In the prostate carcinoma study, the important question of the percentage of tumor cells in the cancer samples was addressed by microscopic examination of paraffin-embedded sections of tumor adjacent to the frozen sample used for DNA isolation. The samples ranged from having ~10–90% carcinoma cells. The tumor DNAs with the highest levels of hypomethylation of LINE-1 sequences all had high percentages of tumor cells, but some of the tumor specimens that consisted of mostly cancer cells showed no detectable LINE-1 hypomethylation (Santourlidis *et al*., 1999). In a chemically induced mouse hepatoma, LINE-1 hypomethylation was also seen (Ushijima *et al*., 1997). That study involved methylation-sensitive representational difference analysis (MS-RDA) to survey *Hpa*II-sensitive fragments in the tumor *vs* normal liver, with confirmation of the results by Southern blotting (Ushijima *et al*., 1997). LINE-1 hypomethylation was the only alteration seen repeatedly in the MS-RDA banding patterns from different tumors. Hypomethylation, including of single-copy DNA sequences, was observed more frequently than hypermethylation. In these studies of murine and human tumors, it was not demonstrated that hypomethylation of LINE-1 sequences increased the use of these repeats as

transcription units in cancer, but it has been hypothesized that this hypomethylation might sometimes lead to the retrotransposition of the very small percentage of retrotransposition-competent copies of LINE-1 (Santourlidis *et al*., 1999).

Of even higher copy number than the LINE-1 repeats in the human genome are the Alu repeats (size, ~0.3 kb; copy number, $>10^6$), which also can be mobilized, thus leading occasionally to cancer-associated gene insertions (Rothberg *et al*., 1997; Schichman *et al*., 1994). These repeats constitute >10% of the genome. Frequent loss of methylation from the normally highly methylated Alu repeats in human cancer is a possibility, but this has not been investigated in more than a few tumors, which were from the germline (Rubin *et al*., 1994). Analysis of such tumor samples is complicated by the hypomethylated state of Alu repeats in normal sperm compared to postnatal somatic tissues (Rubin *et al*., 1994).

Hypomethylation of moderately repeated DNA sequences in cancer

Another class of human retrotransposons that is usually highly methylated and may become hypomethylated specifically in association with oncogenesis are endogenous retroviruses, especially, the HERV-K family. While there are only about 30–50 full-length HERV-K sequences in the human genome, there are an estimated 10 000 solitary long terminal repeats (LTRs) from HERV-K (Leib-Mosch *et al*., 1993). At least some of the solitary LTRs can drive reporter gene expression as determined from an HERV-K LTR-containing transgene (Casau *et al*., 1999). Expression levels from the transgene were high in adult mouse testes and very low or undetectable in other tissues as determined by RT–PCR and Northern blotting. In the previously mentioned study of urinary bladder cancers (Florl *et al*., 1999), cancer-associated hypomethylation of the HERV-K sequences was seen by Southern blotting with an HERV-K *gag* probe. Although analysis of the presence of HERV-K transcripts in these bladder cancers was not reported, evidence for demethylation of HERV-K *gag* sequences and correlated expression of Gag at the protein level in human testicular tumor samples was provided in a small-scale study involving Southern and Western blotting (Gotzinger *et al*., 1996). Also HERV-K *env* transcripts were detected by RT–PCR in 25 out of 55 breast cancer tissues and not in any of the 35 breast tissue samples from cancer-free patients, although DNA methylation was not assayed in that study (Wang-Johanning *et al*., 2001). Expression of HERV-K *env* was seen in 7 out of 40 samples of tissue adjacent to the cancer that were judged normal by gross pathology, but that probably contained some malignant cells as determined histologically. The presence of these transcripts specifically in breast tissue from the cancer patients was confirmed by RNA *in situ* hybridization and Northern blot analysis. The transcripts included HERV-K *env* RNA capable of encoding the HERV-K envelope protein. Moreover, high-level expression of HERV-K *gag* and *env* RNA is associated with germ cell and trophoblastic tumors (Herbst *et al*., 1996). That *env* RNA was detected in most assayed term placental samples (Wang-Johanning *et al*., 2001) may be related to much of term placental tissue being of trophoblast origin and placental DNA displaying global genomic hypomethylation (Ehrlich *et al*., 1982). With respect to methylated integrated viral DNA sequences, a number of reports examining latent human DNA viruses and the proviral form of exogenous retroviruses indicate that, sometimes, these can be activated by demethylation of critical control elements (Gutekunst *et al*., 1993; Mikovits *et al*., 1990; Robertson, 2000; Takacs *et al*., 1998; Tanaka *et al*., 1995).

Several types of non-viral, moderately repeated DNA sequences have been shown to be hypomethylated in certain cancers. These include sequences tandemly repeated at a small number of different regions of the genome. For example, a 9-kb DNA repeat (NBL1) in the short arm of the acrocentric chromosomes, the pericentromeric region (vicinity of the centromere) of chromosomes 3 and 4, and subtelomeric locations was hypomethylated in some human neuroblastomas as determined by RLGS on tumor DNA *vs* blood DNA followed by Southern blot analysis (Thoraval *et al*., 1996a). Also hypomethylated in many neuroblastomas was another non-satellite repeat (NBL2,1.2-kb) located in the centromeric regions of chromosomes 9, 13, 14, and 21 (Thoraval *et al*., 1996b). This sequence is about 60% homologous to the *Sst*I repeat, a moderately repeated DNA sequence found in tandem copies at 4q31 and 19q13 (Epstein *et al*., 1987). In another RGLS study (Nagai *et al*., 1999), a variant CpG-rich *Sst*I repeat that was present in tandem 1.4-kb repeats was found to be hypomethylated in 75% of hepatocellular carcinomas relative to apparently normal liver from the same patients. The repeat was also hypomethylated in sperm compared with various normal somatic tissues. Therefore, this sequence displays the type of sperm-specific hypomethylation that we previously defined for a heterogeneous group of CpG-rich sequences in human DNA (Zhang *et al*., 1985, 1987). Moreover, part of the sequence displaying cancer- and sperm-specific hypomethylation is 70% homologous to one of the sequences that we previously cloned on the basis of its sperm-specific hypomethylation (Sp-12; Accession number X07490) (Nagai *et al*., 1999; Zhang *et al*., 1987). Another of the sperm-specific hypomethylated sequences (Sp23; Accession number X07493) that we previously isolated (Zhang *et al*., 1987) is implicated in cancer-linked hypomethylation. It is part of the non-transcribed intergenic spacer for the 45S ribosomal RNA gene repeat. In 5 out of 10 analysed Wilms tumors, it was hypomethylated compared with a variety of normal postnatal somatic tissues (Qu *et al*., 1999b). A different portion of this rDNA non-transcribed spacer was found to be undermethylated in one of two examined human lung cancers relative to

adjacent 'normal' tissue in another study (Shiraishi *et al*., 1999). The authors of the latter study propose that there may have been spreading of hypomethylation from the relatively unmethylated 45S rDNA repeat to the intergenic spacer during carcinogenesis.

Hypomethylation of satellite DNA in cancer

Satellite DNAs are tandem, high-copy-number repeats, composed of variations of oligonucleotide sequences and are usually found in constitutively heterochromatic chromosome regions. We observed hypomethylation at *Bst*BI sites in the main DNA component of the centromere (satellite α; Satα) in chromosome 1 (Chr1) or in the unusually long region of juxtacentromeric (centromere-adjacent) heterochromatin of Chr1 and Chr16 (satellite 2; Sat2) in 40–90% of human breast adenocarcinomas, ovarian epithelial carcinomas, and Wilms tumors (Narayan *et al*., 1998; Qu *et al*., 1999a,b). Hypomethylation in these studies was defined in comparison with normal postnatal somatic tissues, all of which are highly methylated in these sequences. Hypomethylation of Chr1 Sat2 and of Chr16 Sat2 was usually concordant, which is consistent with their sequence homology. However, undermethylation of Chr1 Sat2 was also paralleled by hypomethylation of the above-described heterologous rDNA spacer in the acrocentric chromosomes' p-arms in the ten Wilms tumors analysed for methylation of both repeats (Qu *et al*., 1999b). In human hepatocellular carcinomas, the frequent hypomethylation of Sat2 DNA (Saito *et al*., 2001; Wong *et al*., 2001) was highly concordant with that of Sat3, the main DNA component of the long juxtacentromeric heterochromatin of Chr9 (Saito *et al*., 2001). Sat2, Sat3, and Satα DNA sequences are normally hypomethylated in human sperm compared with a wide variety of normal postnatal somatic tissues just as the rDNA spacer sequence is (Qu *et al*., 1999b; Zhang *et al*., 1987). Moreover, in mice it has been shown that both oocyte and sperm DNAs are hypomethylated in satellite sequences (Chapman *et al*., 1984). Therefore, hypomethylation of various DNA sequences in both cancers and germ cells seems to be a common theme.

We recently extended our initial Wilms and ovarian tumor studies to demonstrate that most of these cancers are hypomethylated in Satα (centromeric) sequences throughout the genome's chromosomes and not just in Chr1 (Ehrlich *et al*., submitted). We have obtained similar results for ovarian epithelial carcinomas (L Dubeau and M Ehrlich, unpublished results), indicating that the hypomethylation of Wilms tumor DNA is cancer-specific and not just related to the embryonic nature of the tissue from which these pediatric cancers arise. Furthermore, the global DNA demethylation, including satellite DNA demethylation, that occurs from the one-cell stage through the morula stage is reversed by genome-wide remethylation throughout the early murine embryoblast by 6.5–7.5 d.p.c. (Chapman *et al*., 1984; Oswald *et al*., 2000; Razin and Kafri, 1994). Therefore, the highly methylated state of satellite DNA in tissues other than extraembryonic tissues is established much before the appearance of metanephric blastema, the embryonic precursor tissue for Wilms tumor formation. Although there are methylation changes later in embryogenesis, they are much less wide-scale (Razin and Kafri, 1994). It is possible that during oncogenesis there was selection for rare cells in the untransformed precursor tissue that had pre-existing hypomethylation of satellite DNA. However, this type of caveat is generally applicable to examples of cancer-linked DNA methylation changes.

Our findings on cancer-associated centromeric satellite DNA hypomethylation are consistent with the above-described hypomethylation of moderately repeated, non-satellite sequences that hybridize to the centromeric region of a few chromosomes (Thoraval *et al*., 1996a,b). The importance of satellite DNA hypomethylation is suggested by the highly significant relationship between it and global m^5C deficiency in Wilms tumors as well as in ovarian epithelial tumors (Qu *et al*., 1999a; Ehrlich *et al*., submitted). Therefore, one of the important consequences of global DNA hypomethylation that may favor oncogenesis might be the strongly associated satellite DNA hypomethylation.

Hypomethylation of single-copy genes in cancer

To study whether cancer-linked hypomethylation is found in DNA sequences of various degrees of repetitiveness, we examined methylation levels in randomly fragmented DNA fractionated by reassociation kinetics using DNA from a human gastric adenocarcinoma that had an abnormally low global m^5C content (Gama-Sosa *et al*., 1983b). Hypomethylation was present in highly repetitive, middle repetitive, and single-copy DNA fractions. Correspondingly, many reports indicate that part of the cancer-associated hypomethylation occurs in single-copy gene regions (Figure 1). Some of these genes have no apparent relationship to carcinogenesis. For example, the genes encoding growth hormone, γ-globin, γ-crystallin, and α-chorionic gonadatropin displayed hypomethylation at *Hpa*II or *Hha*I sites in almost all of 12 examined colon adenocarcinomas and seven polyps relative to adjacent apparently normal colon tissue in a Southern blot analysis with cDNA or gene region probes (Goelz *et al*., 1985). Frequent hypomethylation of the γ-globin gene and less frequent undermethylation of the parathyroid hormone and catalase genes was seen in breast and colon adenocarcinomas (Ribieras *et al*., 1994). Hypomethylation within certain gene regions in cancer might have an effect on gene expression but is less likely to do so than when the undermethylation is in the promoter or known enhancer sequences. However, it is possible that hypomethylation within gene regions is sometimes coordinated with hypomethylation in upstream transcription regulatory sequences.

Several tumor- or proliferation-associated genes have been found to be hypomethylated in human cancers. For example, cancer-associated hypomethylation concordant with expression was observed for the *pS2* gene (Martin *et al*., 1997), which encodes a pleiotropic factor implicated in the control of cell proliferation. Normal stomach tissue and five breast adenocarcinomas that transcribe *pS2* were hypomethylated at *Hpa*II sites at the 5′ end of the gene relative to seven non-expressing breast carcinomas and normal, non-expressing breast and endometrium. In another study, methylation of the CpG-rich promoter of the *HOX11* proto-oncogene in three T-cell lymphoblastic leukemia samples, a normal bone marrow sample, a sample from a patient in remission, a leukemic T-cell line, and a control T-cell line was compared by genomic sequencing. This promoter was hypomethylated in the three at-diagnosis leukemic samples and the leukemic cell line, all of which expressed the gene, and not in the control cell line, the normal bone marrow, or the sample from the patient in remission, all of which were non-expressing (Watt *et al*., 2000).

Genomic sequencing of the promoter of *MN/CA9*, a tumor antigen gene, from 13 renal cell carcinomas and normal kidney tissue revealed tumor-linked hypomethylation of 6 CpGs out of 10 (Cho *et al*., 2001). Hypomethylation at one of these sites (GCGC) was correlated with expression of the gene. A caveat of such correlative results is that hypomethylation of transcription control sequences can sometimes be an effect, rather than a cause, of transcriptional activation. In the above study, evidence was provided for a causative role for the cancer-associated hypomethylation in gene expression. A plasmid containing the *MN/CA9* promoter driving reporter gene expression was methylated *in vitro* with *Hha*I methylase or *Sss*I methylase before being used for transient transfection of a renal cell carcinoma cell line. Remarkably, even though there is only a single *Hha*I site (the above-mentioned GCGC) compared with many *Sss*I sites (CpG) in the promoter region, both enzymes gave equally strong inhibition of reporter gene expression. More testing is needed for cause-and-effect relationships between cancer-associated promoter/enhancer hypomethylation and cancer-linked expression of genes, e.g. transfection experiments with *in vitro* methylation of reporter plasmids that mimics *in vivo* methylation patterns and controls for the effects of methylation of vector and reporter sequences.

In the third exon of c-*MYC*, hypomethylation of *Hpa*II sites was observed in colorectal cancers and hepatocellular carcinomas compared to the corresponding normal tissues (Sharrard *et al*., 1992; Shen *et al*., 1998). Although the relationship of this hypomethylation to transcription remains to be demonstrated, it might be associated with the elevated c-*MYC* expression often observed in rapidly proliferating cells. A study of murine tumors induced by chlorinated acetic acids gave evidence for an association between DNA hypomethylation and c-*Myc* or c-*Jun* expression. Hypomethylation of the c-*Myc* and c-*Jun* promoters at *Hpa*II sites and increased expression of the corresponding gene at the RNA and protein levels was seen in liver tumors relative to liver from untreated mice or non-tumorous liver from treated mice (Tao *et al*., 2000).

Some tumor marker genes display germ cell-specific and cancer-associated expression and can be activated by DNA demethylating agents. A group of genes, including members of the *MAGE*, *LAGE*, and *GAGE* families, encode tumor-specific antigens presented by Class I major histocompatibility proteins and are expressed in testes and certain types of cancers with no detectable expression in a wide variety of normal postnatal somatic tissues (De Backer *et al*., 1999; De Smet *et al*., 1996; Lethe *et al*., 1998). Expression of these genes can be induced in non-expressing cancer cell lines or normal cell lines by treatment with 5-azadeoxycytidine, and methylation of Ets motifs in the promoter of *MAGE-1* interferes with binding of an Ets family transcription factor (De Backer *et al*., 1999; De Smet *et al*., 1996; Lethe *et al*., 1998). The *MAGE-1* promoter is hypomethylated in sperm and *MAGE-1*-expressing cell lines, but not in non-expressing cell lines and leukocytes (De Smet *et al*., 1996; Serrano *et al*., 1996) Unmethylated promoter sequences were found for three *MAGE* family genes in lung cancers and, to a lesser extent, in surrounding tissue, but lung from non-cancer patients was not examined (Jang *et al*., 2001). Expression was significantly correlated with promoter hypomethylation. In ~40% of breast cancers, hypomethylation at the 3′ end of *TSP50*, a protease-like gene expressed specifically in testes, was seen (Yuan *et al*., 1999). Hypomethylation in breast cancer and testes relative to normal somatic tissues was demonstrated by cleavage with *Hpa*II (at unmethylated CCGG sites) and *Msp*I (specifically at unmethylated GGCCGG sites).

Another group of genes that often have disruptions in their DNA methylation and expression patterns in cancer are imprinted genes (Malik and Brown, 2000). Imprinted genes are characterized by parent-of-origin specific expression, maintenance of which is crucial to normal development (Tycko, 2000). DNA methylation acts as a primary genomic signal for monoallelic expression, such that in the majority of cases, a methylated allele is switched off and an unmethylated allele switched on. Across a cluster of imprinted genes, differential methylation of alleles is focused at CpG islands known as differentially methylated regions (DMRs). The best studied pathological model of the consequences of disrupted imprinting is the Beckwith-Wiedemann syndrome (BWS) (Tycko, 2000). Aberrant imprinting of *IGF2* at 11p15.5 is a key factor in the development of this overgrowth syndrome. *IGF2* encodes a mitogen and fetal growth promoting protein, which is one of the most frequently dysregulated genes in human cancers (Schofield *et al*., 2001). Evidence indicates that its overexpression may contribute to the early stages of tumorigenesis (Malik and Brown, 2000). In some cases of BWS, a tumor-predisposing syndrome, there is paternal uniparental disomy of

11p15.5 (as a somatic mosaic) that results in an extra copy of the paternally transmitted, active *IGF2* gene replacing the maternally inherited, inactive copy of this gene. Similarly, cancers frequently display relaxation of imprinting (sometimes called loss of imprinting) of imprinted genes, including *IGF2*. When imprinting is relaxed, the inactive imprinted allele becomes activated to give biallelic, and increased, expression. In most cancers displaying relaxation of imprinting, imprinting is altered by one of two pathways (Tycko, 2000). One is the loss of heterozygosity (LOH) of imprinted genes, i.e., the loss of maternal or paternal alleles. LOH can occur by mitotic recombination, deletion, or chromosome loss. When LOH involves mitotic recombination and the loss of the inactive allele is accompanied by the reciprocal gain of an extra copy of the active allele, relaxation of imprinting can result. Because of differential allelic DMR methylation, LOH usually gives changes in the DNA methylation pattern, but just because of selective loss of maternal or paternal alleles. The other pathway for relaxation of imprinting involves LOH-independent alterations resulting in alteration of normal allele-specific methylation patterns. It is usually due to abnormal *de novo* methylation at an imprint-associated DMR, such as hypermethylation of the DMR immediately upstream of *H19*, which acts as a chromatin insulator preventing *IGF2* expression only when unmethylated (Sasaki *et al*., 2000). *H19* and *IGF2* genes are part of a 1-Mb imprinted domain including *KvLQT1* and *CDKN1C* (see below). The *H19* upstream DMR makes *H19* and *IGF2* reciprocally expressed at a given 11p15.5 locus. Hypermethylation of this DMR leads to relaxation of imprinting of *IGF2* (biallelic expression instead of monoallelic expression) and loss of expression of *H19* in a variety of cancers (Takai *et al*., 2001; Tycko, 2000).

Recently, several examples of cancer-related hypomethylation of the normally hypermethylated allele of an imprinted gene have been reported, although it remains to be demonstrated how much this hypomethylation contributes to changes in allele-specific expression. More than one DMR in the *H19*/*IGF2* region (Ishihara *et al*., 2000; Sullivan *et al*., 1999) might play a role in imprinting because hypermethylation of the *H19* upstream DMR (methylation of the normally unmethylated maternal allele) does not suffice for relaxation of imprinting (Cui *et al*., 2001). Another DMR was found to overlap exons 2 and 3 in human *IGF2* (Sullivan *et al*., 1999). This gene is paternally expressed and maternally repressed in normal kidney. *IGF2* displays maternal allele-specific methylation in normal kidney that was absent in the four examined LOH-negative Wilms tumors which exhibited biallelic expression of *IGF2*; however, methylation of the *H19* upstream DMR was not examined in this study (Sullivan *et al*., 1999). Decreased DNA methylation by homozygous *Dnmt1* knockout, which leads to embryonic death, results in activation of only the normally silent paternal allele of *H19* (Li *et al*., 1993). This suggests that if *IGF2* DMR methylation helps to silence the maternal allele, demethylation of this DMR may not suffice to activate it.

Hypomethylation of the paternally transmitted *H19* upstream DMR (which is normally paternally methylated) was seen in two of six analysed bladder cancers (Takai *et al*., 2001). The authors were unable to test whether this hypomethylation resulted in relaxation of imprinting because of the lack of heterozygous markers in the appropriate samples. However, examination of somatic cell hybrids suggests that this promoter hypomethylation results in activation of expression (Takai *et al*., 2001), which is consistent with results from the *Dnmt1* knockout study (Li *et al*., 1993). Hypomethylation of the promoter region of *H19* in the paternally transmitted allele was associated with relaxation of imprinting in a few LOH-negative tumors in a study of lung cancer (Kondo *et al*., 1995). Frequent hypomethylation of the maternally methylated DMR in the intron downstream of exon 10 of *KvLQT1* was seen in Wilms tumors (Tycko, 2000) and hepatocellular carcinomas (Schwienbacher *et al*., 2000). This intronic DMR may be an alternative imprint regulatory element for the *H19*/*IGF2*-containing imprinted domain working through an antisense transcript from the paternally expressed allele (Smilinich *et al*., 1999). In a study of hepatocellular carcinoma and adjacent non-tumorous liver without LOH in the *CDKN1C*/*IGF2* region, evidence was found for tumor-specific hypomethylation of this *KvLQT1* DMR being associated with gain of imprinting (reduced expression of one allele) and, sometimes, loss or relaxation of imprinting for *CDKN1C* or *IGF2* (Schwienbacher *et al*., 2000).

There is another gene domain on 11p that appears to be imprinted and is frequently hypomethylated in Wilms tumors. At 11p13 an antisense gene, *WT1-AS*, overlaps the Wilms tumor suppressor gene *WT1*. In normal kidney, *WT1-AS* displays allele-specific expression and differential methylation in the antisense regulatory region immediately upstream of the antisense promoter (Malik *et al*., 2000). In contrast, sense *WT1* is biallelically expressed in kidney. Unlike normal kidney, eight out of 10 studied LOH-negative Wilms tumors had only the unmethylated form of the allele and so apparently lost methylation from the maternally inherited allele, as detected by genomic sequencing and Southern blot analysis. One of the tumors displaying this hypomethylation was informative for allele-specific expression and shown to have biallelic expression of *WT1-AS*, unlike the adjacent, histologically normal kidney. Recently this analysis has been extended to include Wilms tumor pre-malignant lesions known as nephrogenic rests. These also display hypomethylation and biallelic expression, suggesting that relaxation of imprinting at this locus is an early event in tumorigenesis (K Malik, personal communication). Therefore, demethylation of the maternal allele during tumorigenesis might lead to activation of its expression. Interestingly, tumors that do have LOH in this region preferentially lose the silent, hypermethylated maternal allele. These results suggest a role in Wilms tumor formation for hypomethylation of the *WT1-AS*

upstream regulatory region, upregulation of *WT1-AS* expression, and, perhaps also, increased *WT1* sense expression (Malik *et al*., 2000).

Cancer-associated hypermethylation of promoter regions will probably be involved in gene dysregulation in tumors more often than cancer-associated hypomethylation because these regions are usually constitutively unmethylated in normal tissues. Nonetheless, as illustrated by the above-mentioned studies, it is likely that there are many unelucidated examples of cancer-associated gene-region hypomethylation that affect gene expression. Impediments to their discovery are that studies designed to look for alterations in methylation in gene regions in cancer are usually set up specifically to detect increased methylation, generally involve non-quantitative PCR techniques, and usually do not employ multiple types of control tissue. The cancer-related hypermethylation of CpG islands overlapping the 5′ ends of tumor suppressor genes can often be analysed by non-quantitative methods. These assays are designed to detect methylation of multiple CpGs, and those islands tend to have very little or no methylation in normal tissues (although not always, and this could lead to erroneous conclusions). In collaboration with Peter Laird, we quantitatively analysed DNA from 31 Wilms tumors *vs* five types of normal tissues for hypermethylation at 13 CpG-rich regions by MethyLight, a bisulfite modification-dependent real-time PCR assay (Eads *et al*., 2000; Ehrlich *et al*., submitted). Almost all of the assayed regions were standard types of CpG islands, several of which displayed significant hypermethylation in the Wilms tumors. We found an unexpected example of cancer-associated hypomethylation at the 5′ end of *MTHFR* (methylene tetrahydrofolate reductase). This region is CpG-rich, but not as much as in a typical island. The MethyLight assay revealed that there were varying levels of methylation of the 5′ region of *MTHFR* in five different normal tissues, but nonetheless there was a statistically significant cancer-related undermethylation of this region ($P<0.0005$). The only previous report on methylation of *MTHFR* described this CpG-rich region as highly methylated in both normal and malignant esophageal tissue (Eads *et al*., 2001). *MTHFR* hypomethylation might be only a marker of Wilms tumors or it might contribute to altered DNA metabolism by affecting the expression of the gene and, hence, the pools of dNTPs and S-adenosylmethionine, the donor for methylating C residues in DNA. This hypomethylation probably would have been missed if the analysis for methylation had not been quantitative and had not included a variety of normal tissue DNAs.

Is cancer-associated DNA hypomethylation just a prelude to or byproduct of cancer-linked DNA hypermethylation?

In DNA from breast adenocarcinomas, we found no apparent relationship between the often-observed hypermethylation of the 5′ CpG island of the E-cadherin gene and hypomethylation of Chr1 Sat2 (Narayan *et al*., 1998). In our recent study of Wilms tumors, 31 of the samples were analysed for DNA hypermethylation as well as hypomethylation. Four of the CpG islands examined for hypermethylation were hypermethylated in about 30 to 90% of the tumors compared to various postnatal tissues (Ehrlich *et al*., submitted). We found no significant positive or negative association between hypermethylation of any of the studied CpG islands and global DNA hypomethylation or satellite DNA hypomethylation (Ehrlich *et al*., submitted). A negative association between CpG island methylation and global or satellite DNA hypomethylation would have been predicted if tumors with much CpG island hypermethylation were less likely to also display DNA hypomethylation. A positive association might have been seen if CpG island hypermethylation preceded and provoked global genomic hypomethylation of *vice versa*. The absence of any such relationship suggests that tumor-associated DNA hypomethylation contributes to carcinogenesis or tumor progression separately from aberrant DNA hypermethylation and its attendant silencing of tumor suppressor genes.

Does cancer-associated DNA hypomethylation contribute to karyotypic instability?

The above-described highly methylated state of many retrotransposons and endogenous retroviruses in normal tissues, their frequent hypomethylation in cancer, and the repressive effects of DNA methylation on their promoter function suggest that hypomethylation of these types of sequences in cancer could contribute toward karyotypic instability, which facilitates tumor progression. Another type of repeated DNA sequence whose hypomethylation in cancer may foster chromosome instability is satellite DNA. As described above, cancers often display hypomethylation of the normally highly methylated satellite DNA sequences, including centromeric Satα and juxtacentromeric Sat2, both of which are in the pericentromeric heterochromatin. Sat2, which is found mostly in Chr1 and Chr16, is hypomethylated in all studied tissues and cell cultures from patients with the ICF syndrome (immunodeficiency, centromeric region instability, facial anomalies) (Jeanpierre *et al*., 1993; Tuck-Muller *et al*., 2000), a rare recessive disease. This hypomethylation is usually associated with mutations specifically affecting the catalytic domain of *DNMT3B*, the DNA methyltransferase 3B gene (Hansen *et al*., 1999; Okano *et al*., 1999; Xu *et al*., 1999). While Sat2 sequences are not the only ones that are hypomethylated in ICF cells (Kondo *et al*., 2000), there is only a small percentage decrease in DNA methylation in ICF genomes. We showed that DNA from the one assayed uncultured ICF tissue, ICF brain DNA, had only about 7% less m^5C than normal brain DNA samples (Table 1) (Tuck-Muller *et al*., 2000). Much of this hypomethylation is in repeated DNA sequences (Kondo *et al*, 2000). Sat2 and, sometimes, adjacent centromeric regions of Chr1 and

Chr16 are specifically targeted for almost all of the rearrangements seen in mitogen-stimulated ICF lymphocytes or untreated ICF lymphoblastoid cell lines (LCLs) (Jeanpierre *et al*., 1993; Tuck-Muller *et al*., 2000). In detailed studies of G-banded chromosomes of ICF LCLs from five unrelated patients with *DNMT3B* mutations, we found that decondensation of the Chr1 juxtacentromeric Sat2-rich region was the most frequent anomaly, and that this decondensation was often seen at the break-points of whole-arm deletions or multiradial chromosomes (three or more Chr1 and/or Chr16 arms fused in the pericentromeric region) (Ehrlich *et al*., 2001; Tuck-Muller *et al*., 2000). These results and our findings that 5-azadeoxycytidine or 5-azacytidine treatment of normal lymphoid cultures induces chromosome rearrangements identical to those of ICF lymphoid cells (Hernandez *et al*., 1997; Ji *et al*., 1997) suggest that DNA hypomethylation predisposes to heterochromatin decondensation, which in turn, facilitates recombination between repeated DNA sequences.

Pericentromeric rearrangements of Chr1 and Chr16 are overrepresented in diverse types of cancers, including hepatocellular carcinomas and Wilms tumors, and generally lead to gains of 1q and/or losses of 16q (Brito-Babapulle and Atkin, 1981; Le Baccon *et al*., 2001; Mitelman *et al*., 2001). In a study of hepatocellular carcinomas by comparative genomic hybridization, 23 out of the 36 tumors examined showed 1q gain; 22 of these displayed Chr1 Sat2 hypomethylation at *Bst*BI sites (Wong *et al*., 2001). In the above-mentioned study of 35 Wilms tumors involving Southern blotting and karyotype analysis, we also saw overrepresentation of Chr1 rearrangements in the pericentromeric region, all of which were accompanied by 1q gain, but only five of the tumors had pericentromeric rearrangements of Chr1 (Ehrlich *et al*., submitted). Our examination of karyotypic changes by LOH analysis of Chr16 or karyotype analysis are consistent with hypomethylation of Chr1 Sat2, Chr16 Sat2, and general centromeric hypomethylation favoring pericentromeric chromosome rearrangements, including isochromosome formation, in *cis* (Ehrlich *et al*., submitted; Qu *et al*., 1999b), although our data are not as convincing about this relationship in Wilms tumors as those from the hepatocellular carcinoma study. From this Wilms tumor study, we could detect no relationship of hypomethylation of centromeric or juxtacentromeric satellite DNA and aneuploidy (Ehrlich *et al*., submitted). This is in agreement with the lack of aneuploidy in ICF cells, which always display juxtacentromeric hypomethylation of Chr1 and Chr16 Sat2 DNA (Jeanpierre *et al*., 1993; Tuck-Muller *et al*., 2000), although they usually do not exhibit centromeric hypomethylation (Miniou *et al*., 1997; Tuck-Muller *et al*., 2000).

Hypomethylation of DNA sequences other than satellite DNAs may also predispose to chromosome recombination. In support of the postulated involvement of abnormal DNA hypomethylation in aberrant recombination, Chen *et al*. (1998) recently showed in murine embryonic stem cells that global DNA hypomethylation induced by homozygous knockout of the main DNA methyltransferase gene (*Dnmt1*) increased abnormal DNA recombination and, thereby, mutagenesis at *Hprt* and *tk* genes. However, using different stably transfected murine embryonal stem cells, Chan *et al*. (2001) unexpectedly found that homozygous knockout of *Dnmt1* decreased gene loss and point mutagenesis from a chimeric *tk-neo* transgene. The differences between these studies might be due to chromosome position effects. Analysis of another model system, an interspecies hybrid of mammalian origin, provided more evidence for DNA undermethylation favoring chromosome instability. In the sterile hybrid obtained from two species of kangaroos, genome-wide hypomethylation was accompanied by several types of chromosomal rearrangements including elongation of the centromeres from one of the parents and various translocations (O'Neill *et al*., 1998, 2001). The centromeric extension resulted from amplification of a retroviral element in the centromeric region and the amplified element was hypomethylated.

More studies are needed to elucidate the types of DNA sequences and cells that are more susceptible to rearrangements when the DNA is hypomethylated and the extent of the contribution of DNA hypomethylation to karyotypic instability in cancer. However, there is a mechanistic basis for DNA hypomethylation favoring recombination, especially at repeated DNA sequences. The significant association of global DNA hypomethylation with satellite DNA hypomethylation in human cancer (Ehrlich *et al*., submitted; Qu *et al*., 1999a) and the frequent hypomethylation of both tandem and interspersed repeated DNA sequences in cancer (see above) suggest that these repeated sequences are playing a special role in carcinogenesis when undermethylated. Repeated DNA sequences should be more recombinogenic than unique sequences, but normally in eucaryotes such recombination is largely repressed. The repression may involve a more heterochromatic structure making the DNA sequences less accessible for recognition of interchromosomal and intrachromosomal DNA homology. For example, the very repetitious satellite DNAs are located in constitutive heterochromatin and are often highly methylated. DNA methylation has been linked to histone hypoacetylation and heterochromatinization (Garrick *et al*., 1998; Nguyen *et al*., 2001b). Therefore, it is likely that loss of DNA methylation favors recombination between repeated DNA sequences partly by loosening their packaging in chromatin.

Does cancer-associated DNA hypomethylation lead to altered gene expression that favors oncogenesis? Cis and possible trans effects

Cis *effects*

Among the previously mentioned cancer or proliferation-associated genes for which increased mRNA levels

were correlated with hypomethylation in the promoter or the 5′ end of the gene were *pS2*, *HOX11*, and *MN/CA9* (Cho *et al*., 2001; Martin *et al*., 1997; Watt *et al*., 2000). Also, hypomethylation in transcription control regions of imprinted genes (see above) may promote carcinogenesis by increasing expression of paternally expressed imprinted genes, which usually contribute to increased cell growth. There is evidence that cancer-associated hypomethylation of proto-oncogene promoters is correlated with activation of expression (Tao *et al*., 2000). While more studies are needed to determine if hypomethylation of retrotransposons or endogenous retroviruses in human cancer causes increased transcription, there is evidence, as mentioned above, that abnormal DNA hypomethylation in model mammalian systems is associated with upregulation of expression of some of these elements (Morgan *et al*., 1999; O'Neill *et al*., 1998; Walsh *et al*., 1998).

Possible trans *effects*

The very high frequencies at which cancer-linked hypomethylation of DNA repeats is seen, its lack of a positive or negative association with CpG island hypermethylation, and its association with global DNA hypomethylation suggest that this hypomethylation aids tumorigenesis. Most of these repeats do not function as transcription units or do so only very poorly *in vivo* (e.g., satellite DNAs and LINE-1 repeats). In particular, the above-described cancer-associated satellite DNA hypomethylation (including Satα, Sat2, and Sat3) is unlikely to have a *cis* effect on transcription unless the hypomethylation spreads from these heterochromatic sequences into neighboring euchromatin. However, this satellite DNA hypomethylation might promote tumorigenesis by interfering with negative regulation of gene expression in *trans*. There are precedents for centromeric heterochromatin interacting with early lymphogenesis genes and the β-globin locus in a manner that downregulates expression of those genes either directly or by acting as a reservoir for transcription control proteins that preferentially bind to them as well as to certain gene promoters (Cobb *et al*., 2000; Francastel *et al*., 2001; Sabbattini *et al*., 2001). In parallel, it has been suggested that the hypomethylation of juxtacentromeric heterochromatin of ICF syndrome lymphoid cells interferes with normal downregulation of certain genes by disrupting heterochromatin-euchromatin interactions in *trans* (Ehrlich *et al*., 2001). Therefore, centromeric and/or juxtacentromeric satellite DNA hypomethylation in tumors might be a new source of gene dysregulation in cancer from hypomethylated constitutive heterochromatin, perhaps in conjunction with cancer-linked alterations in expression from elsewhere in the genome.

Outlook and applications

While there is only a poor understanding of the pathways that lead to cancer-associated hypomethylation of some sequences and hypermethylation of others, there are some clues as to the types of sequences generally affected. For cancer-linked DNA hypermethylation, it seems to be usually CpG islands, and for hypomethylation, it is often, but not only, repeated DNA sequences. Furthermore, sequences that are known to be specifically undermethylated or expressed in germ cells or their precursors are often hypomethylated in cancer (Figure 1, satellite DNA, certain moderate-copy repeats, and testes-specific genes). Evidence suggests that this hypomethylation of DNA in cancer is not a consequence of or a prelude to hypermethylation in cancer. The hypomethylation in repeated DNA sequences may play special roles in carcinogenesis, such as, increasing karyotypic instability or affecting expression of genes indirectly. While there are some cancer-related genes with evidence for expression-linked hypomethylation in cancer, the number of studies looking for such associations and testing the effects of altering methylation experimentally has been very limited compared to analysis of repression-linked hypermethylation of tumor suppressor genes in cancer.

DNA hypermethylation is emerging as an important diagnostic and prognostic tool (see other articles in this issue). DNA hypomethylation analysis may also be useful in detecting cancer and managing the disease. Hypomethylation of repeated or single-copy DNA sequences has been significantly correlated with disease progression for some tumors (Qu *et al*., 1999a; Santourlidis *et al*., 1999; Shen *et al*., 1998). Furthermore, a number of studies have shown significant decreases in global DNA methylation levels with progressing tumor stage, tumor grade, or various other indicators of poor prognosis (Lin *et al*., 2001; Shen *et al*., 1998; Soares *et al*., 1999), and global DNA hypomethylation in cancer is significantly associated with hypomethylation of certain DNA repeats (Ehrlich *et al*., submitted; Qu *et al*., 1999a). In addition, for some types of cancer, DNA hypomethylation is seen as an early indicator of tumorigenesis (Feinberg *et al*., 1988; Qu *et al*., 1999a; Ribieras *et al*., 1994). Therefore, assays for DNA hypomethylation may become a clinically useful addition to hypermethylation analyses of CpG islands.

Studies of cancer-associated DNA hypomethylation sound a note of caution in the current development of cancer therapies aimed at decreasing DNA methylation in a non-targeted manner. As for cancer-associated DNA hypermethylation, cancer-linked DNA hypomethylation is generally unlikely to suffice for carcinogenesis. For example, normal placental DNA has an overall deficiency in m^5C and hypomethylation of satellite DNA relative to normal postnatal somatic tissues. Furthermore, children with the ICF syndrome, a very rare DNA methylation-deficiency and chromosome breakage syndrome, have not been reported to have cancer. Nonetheless, the evidence described above suggests a frequent contributory role of cancer-linked genomic hypomethylation to tumorigenesis or tumor progression. Therefore,

while suppressing CpG methylation may have a short-term beneficial effect in cancer treatment, it might help speed tumor progression from residual cancer cells.

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