[CANCER RESEARCH 53, 2715-2718, June 15, 1993]

Advances in Brief

DNA Hypermethylation Is Associated with 17p Allelic Loss in Neural Tumors[1]

Michele Makos,[2] Barry D. Nelkin, Victoria R. Chazin, Webster K. Cavenee, Garrett M. Brodeur, and Stephen B. Baylin

Oncology Center [M. M., B. D. N., S. B. B.], Department of Medicine [S. B. B.], and Human Genetics Program [M. M., S. B. B.] of the Johns Hopkins Medical Institutions, Baltimore, MD; The Ludwig Institute for Cancer Research [V. R. C.], Department of Medicine and Center for Molecular Genetics, University of California, San Diego, CA; and the Department of Pediatrics, Washington University, School of Medicine, and the Pediatric Oncology Group, St. Louis, MO [G. M. B.]

Abstract

It has long been debated whether the accumulation of allelic losses in tumors involves the selection of cells which have stochastically lost chromosomal regions or whether there is, inherent to the neoplastic state, a process which predisposes to genetic instability. Changes in DNA methylation are commonly seen in human tumors and can alter chromosome structure. We now have examined specific types of primary neural tumors which allow us to determine relationships between abnormal DNA hypermethylation and allelic loss. In primary brain tumors which frequently lose chromosome 17p (30–50%) even in the earliest stages, we now show that 84% (21 of 25) exhibit hypermethylation at locus D17S5, on 17p. However, in primary neuroblastomas, a tumor type which does not lose chromosome 17p, no regional hypermethylation is observed. These data suggest that on chromosome 17p, regional D17S5 hypermethylation constitutes a molecular change which is associated with genetic instability.

Introduction

Abnormal DNA methylation patterns are characteristic of neoplastic cells (1, 2). Widespread areas of genomic hypomethylation (1, 3) coexist with regional increases in DNA methylation (1, 2, 4, 5) at early stages of tumor progression. We have shown that the chromosomal locations of DNA hypermethylation in lung and colon cancers are not random but may occur in areas thought to harbor tumor suppressor genes (4, 5). During colon cancer progression, a normally unmethylated CpG-rich region (D17S5; Ref. 6) of chromosome 17p, recognized by the probe YNZ22, becomes increasingly methylated (5). This change begins in noncultured colonic polyps, which are benign precursors to colon carcinoma (7), before 17p losses have appeared (5). These data suggested that regional hypermethylation could be associated with a predisposition for allelic losses of chromosome 17p in colon cancer.

The above hypothesis concerning regional hypermethylation and allelic loss leads to several predictions, which we have now examined directly. If regional DNA hypermethylation is associated with predisposition to chromosomal loss in tumors, then the methylation abnormality should: (*a*) be present predominantly in those tumor types in which specific allelic losses commonly occur and infrequent in tumor types in which these losses are rarely seen; (*b*) have a higher frequency in tumors predisposed to the chromosome loss than the actual incidence for reduction to homozygosity; (*c*) potentially involve both chromosomes at risk for structural changes; and (*d*) coincide with or precede the timing of reduction to homozygosity during defined stages of tumor progression.

To test these predictions, we have examined a series of primary neural tumors, which either do or do not exhibit loss of chromosome 17p regions. All stages of human brain tumors, derived from cells of astrocytic lineage, from premalignant stages to cancers, and from medulloblastomas arising in the cerebellum, are frequently (30–50%) reduced to homozygosity for chromosome 17p, including the D17S5 region and the *p53* tumor suppressor gene (8–19). The remaining *p53* allele is often (60–70%) simultaneously mutated in those tumors with 17p allelic loss (12, 13, 15, 16, 19). However, reduction to allelic homozygosity of the D17S5 region can occur separately (12, 13, 15, 16, 19) from loss or mutation of the *p53* gene, which has led to the suggestion that there are other tumor suppressor genes on 17p, distal to *p53* (12, 13, 15, 16, 19). In contrast to brain tumors, neuroblastomas rarely if ever demonstrate loss of the D17S5 or other 17p regions (20). In the present study, we have compared the methylation status of D17S5 in human brain tumors and neuroblastomas to determine if there is an association between DNA hypermethylation and chromosome 17p loss in neural tumors.

Materials and Methods

Tissue Preparation. DNA from surgically removed brain tumors, adjacent normal brain, and neuroblastomas was prepared exactly as previously described (8, 9, 20)

Determination of 17p Allelic Status. We used *Bam*HI restriction analysis (21) and a polymerase chain reaction analysis, with primers flanking the D17S5 variable number of tandem repeats region (22), to document the D17S5 allelic status of the samples in Figs. 2 and 3. We also used polymerase chain reaction analysis, with primers flanking the more centromeric 17p locus, D17S261, and the proximal 17q, D17S250 region, to observe reduction to homozygosity at other chromosome 17 regions (23).

D17S5 Methylation Status. The methylation-sensitive restriction enzyme *Not*I was used to determine the methylation status of the D17S5 region exactly as previously described (5) and as outlined in Fig. 1. Double digests with *Not*I and *Bam*HI were also used to determine the methylation status of *Not*I site 2.

Results

D17S5 Hypermethylation Is Observed in All Stages of Primary Astrocytomas and Medulloblastomas. The CpG-rich region (6), D17S5, recognized by probe YNZ22 (Fig. 1), is unmethylated at the *Not*I restriction sites in this region (Fig. 1) in normal brain (Fig. 2), as we have found in other normal tissues (5). In studies using a probe specific for the region between *Not*I site 2 and the far left *Bam*HI site (Fig. 1), with *Bam*HI and *Not*I double digestions, we determined that *Not*I site 2 remained unmethylated in all neural tumors examined (presence of a predicted 600-base pair fragment in all samples tested; data not shown). However, DNA from 17 of 20 (85%) primary brain tumors, representing each stage of tumor progression along the astrocytic cell lineage (8, 24), exhibited extensive methylation at *Not*I sites 3–6 (see Fig. 2, *A*, low-grade astrocytoma; *B*, intermediate grade anaplastic astrocytoma; *C*, high-grade glioblastoma multiforme; and legend to Fig. 2, which describes band patterns specific for the methylated *versus* unmethylated status of *Not*I sites 3–6). In addition, in 4 of 5 (80%) cerebellar medulloblastomas these same *Not*I sites were shown to be methylated (Fig. 2*D*).

Received 3/25/93; accepted 5/4/93.

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[1] Supported by NIH Grant R01-CA43318.

[2] To whom requests for reprints should be addressed, at 424 N. Bond Street, Baltimore, MD 21231.

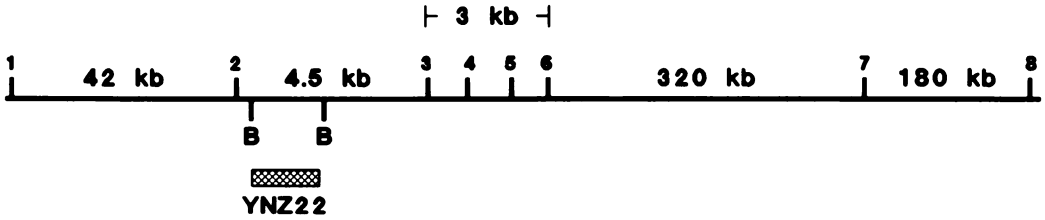

Fig. 1. Strategy for analyzing allelic and methylation status of the region detected by YNZ22. The *hatched bar* represents the position of the YNZ22 probe (note that the figure is not drawn to scale). A variable number of tandem repeats, flanked by *Bam*HI sites (*B*), renders the region detected by YNZ22 polymorphic in 86% of the population (21). The *Not*I restriction sites used to assess the methylation status of the D17S5 region are numbered *1–8*. The recognition sequence for the restriction enzyme *Not*I contains 2 CpG dinucleotides. If these cytosines are unmethylated, as we have found in all normal human tissues tested (5), *Not*I produces a polymorphic 4.5–5.0-kilobase fragment detected by YNZ22. Since *Not*I site 2 appears not to be methylated in the examined neural tumors, the presence of a >20-kilobase band on conventional Southern blot gels is due to the sequential methylation of *Not*I sites 3–6 (Ref. 5; and Figs. 2 and 3). Fragment sizes between >20 kilobases and 6 kilobases, previously seen in colon adenomas (5) and in some neural tumors in this study, are due to sequential methylation of sites 3, 4, and/or 5 but not sites 2 or 6.

The overall incidence of 84% for D17S5 regional hypermethylation in brain tumors exceeds the known high frequency (30–50%; Refs. 8–19) of chromosome 17p and D17S5 loss in these tumor types. The extensive hypermethylation of this region in low-grade astrocytomas also coincides with the described early timing of chromosome 17p loss in astrocytic tumor progression (8, 10, 11, 15, 17). Furthermore, we studied the allelic status in a subset of the brain tumors in each progression stage (data not shown) and found that the extensive hypermethylation of this region occurred not only in cases in which one allele was already lost (Fig. 2, *A*, *Lane 3T*; *B*, *Lanes 1T* and *3T*) but even in those tumors in which both alleles were retained (Fig. 2, *A*, *Lanes 1T, 2T, 4T–6T; C, Lanes 1T, 2T, 4T; D, Lane 2T*).

Finally, we determined that none of the tumors which had a normal D17S5 methylation pattern had evidence for loss of chromosome 17 alleles. Each of these tumors was informative for one or more chromosome 17 loci, and none had lost alleles at D17S261 (17p12–11.1) or D17S250 (17q11.2–12). These tumors were unfortunately either noninformative for D17S5 (Fig. 2, *C, Lane 8T; D, Lane 5T*) or the germline tissue was unavailable (Fig. 2*C*, *Lane 7T*).

D17S5 Hypermethylation Is Not Observed in Primary Neuroblastomas. Findings for the methylation and allelic loss status of the D17S5 region in neuroblastoma are in stark contrast to those above for brain tumors. Recently, it was reported that 0 of 20 noncultured neuroblastomas had lost the D17S5 region (20). In the 12 primary tumors we examined here, ranging from the lowest to highest stage of malignancy (20), 11 were informative by *Bam*HI restriction analysis (Fig. 1) for the allelic status of D17S5, and each of these neuroblastomas retained both alleles (data not shown). Furthermore, 0 of these 12 primary neuroblastomas exhibited the extensive methylation abnormality observed in brain tumors for the D17S5 region (Fig. 3*A*). Only one neuroblastoma (Fig. 3*A*, *Lane 8*) showed minor methylation changes involving *Not*I sites 3–5 (Fig. 1).

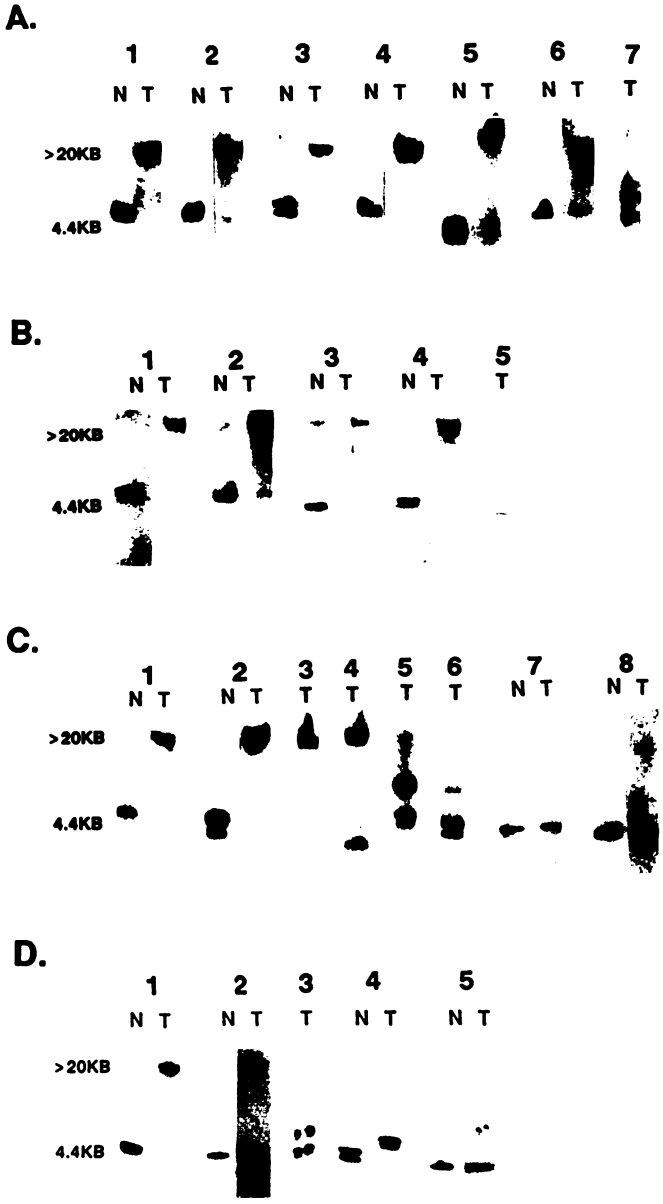

Fig. 2. Methylation status of the D17S5 region in DNA from brain tumors. *A*, low-grade astrocytomas. All normal brain samples (*Lanes 1N, 2N, 3N, 4N, 5N, 6N*) from patients with tumor exhibit the 4.5–5.0-kilobase polymorphic *Not*I fragments, as expected if *Not*I sites 2–6 (Fig. 1) are unmethylated. All 7 noncultured, low-grade tumors exhibit abnormal methylation of the YNZ22 region. Six of the tumors showed extensive methylation of *Not*I sites 3–6, as indicated by complete loss of the 4.5–5.0-kilobase bands and gain of a hybridization signal at >20 kilobases (*Lanes 1T, 2T, 3T, 4T, 5T, 6T*). One tumor (*Lane 7T*) exhibited abnormal methylation which was not as extensive as in the previous samples, shown by the multiple bands of <8.0 kilobases. These bands are produced when *Not*I sites 3–5 are methylated (see Fig. 1). *B*, intermediate-grade (anaplastic) astrocytomas. DNA from the normal brain of each patient exhibits the polymorphic 4.5–5.0-kilobase fragments (*Lanes 1N, 2N, 3N, 4N*). In the tumors, 4 of 5 samples show extensive abnormal methylation of *Not*I sites 3–6, as indicated by the loss of the normal 4.5–5.0-kilobase bands, and the gain of hybridization signal at >20 kilobases. Only tumor sample 5T shows the normal unmethylated pattern for this probe. *C*, high-grade astrocytomas (glioblastoma multiforme). DNA from normal brain samples again shows an unmethylated pattern. Six of eight of the high-grade tumors show abnormal methylation, indicated by both hybridization at >20 kilobases (*Lanes 1T, 2T, 3T*, and *4T*) and at 8.0 kilobases (*Lanes 4T, 5T, 6T*). Samples 7T and 8T show no methylation, since the fragments produced in the tumors are identical in size to the normal tissue DNA. *D*, medulloblastomas. In this brain tumor, 4 of 5 tumors show some degree of abnormal methylation when compared to corresponding normal tissue. Samples 1T and 2T show the same extensive methylation of *Not*I sites 3–6 (>20-kilobase band), as was seen in the majority of astrocytomas. Samples 3T and 4T also show abnormal methylation, with the formation of 6.0–8.0-kilobase bands, indicating that *Not*I sites 3–5 (Fig. 1) are methylated. Only tumor 5T has no methylation when compared to adjacent normal tissue.

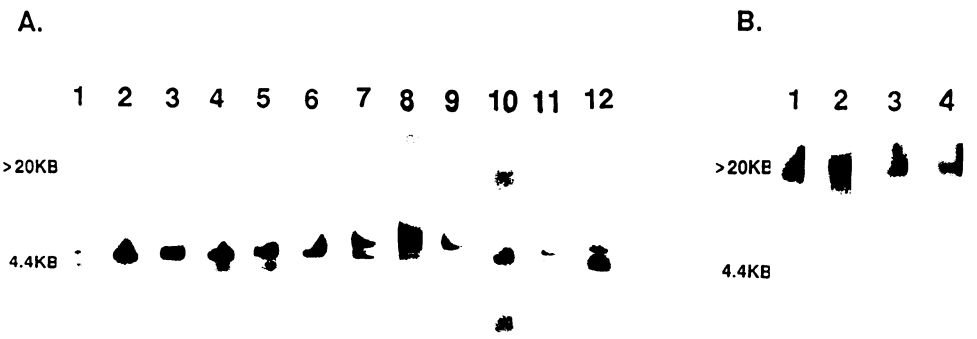

Fig. 3. Methylation status of the D17S5 region in DNA from primary neuroblastomas and tumor-derived cell lines. *A*, primary neuroblastomas. DNA from 12 primary neuroblastomas does not show the extensive methylation patterns seen in the brain tumors in Fig. 2, indicated by the absence of a hybridization signal at >20 kilobases and strong hybridization at the 4.5–5.0-kilobase range. *Lane 8* contains DNA from a tumor which does show partial methylation involving only *Not*I sites labeled *3–5* in Fig. 1. The allelic status for the D17S5 region was examined by *Bam*HI restriction as outlined in Fig. 1, and 11 of 12 of these primary neuroblastomas were informative and showed heterozygosity at this locus (data not shown). *B*, neuroblastoma cell lines. All four established cell lines exhibit the extensive methylation of *Not*I sites 3–6 characteristic of most noncultured brain tumors (Fig. 2). There is virtually no hybridization signal at the 4.4-kilobase marker, with most of the hybridization at the >20-kilobase unresolvable range. Although we were unable to allelotype the corresponding normal tissue from these cell lines, all four were homozygous for the D17S5 region, as determined by *Bam*HI restriction analysis, which detects 86% heterozygosity in the normal population (21).

Studies of cultured neuroblastomas further emphasize the association between D17S5 hypermethylation and 17p allelic loss. It has been reported that normally unmethylated CpG-rich regions have increased methylation in cultures of immortalized (25) and transformed (4) cells. We examined cultures of neuroblastomas to determine if this CpG-rich region would also become hypermethylated and if hypermethylation would again correlate with 17p allelic status. Four of four neuroblastoma cell lines exhibited extensive methylation of the *Not*I sites 3–6 (Fig. 3*B*). Furthermore, unlike the primary neuroblastomas, at least some of these cell lines appeared to have lost D17S5 alleles, since none were heterozygous for this locus (data not shown), which is polymorphic in 86% of the population (21).

Discussion

In summary, our current data associating regional hypermethylation of D17S5 and 17p allelic loss in neural tumors are consistent with the hypothesis that the neoplastic process involves mechanisms which mark chromosomes for risk of reduction to allelic homozygosity. Our findings for the 17p region suggest that in neoplastic cells either: (*a*) there are molecular events which predispose to the loss of 17p regions which also result in DNA hypermethylation or (*b*) the DNA hypermethylation changes, themselves, predispose to allelic loss. While both of these possibilities must be the focus of future studies, our data demonstrating that both copies of D17S5 alleles are methylated in brain tumors which have not yet lost regions of chromosome 17p, and our findings in the following study of human renal cancers (26) provide evidence that DNA hypermethylation precedes 17p allelic loss.

Whichever of the two above possibilities is eventually established, the molecular basis for the association we now report between changes in DNA methylation and chromosome structure in tumors will probably relate to the building body of evidence that methylation of CpG-rich regions can either result from, or cause, changes in chromatin structure (for a review, see Ref. 27). Examples include changes associated with inactivation of the X-chromosome during embryogenesis (28), hypermethylation of the genetically unstable region involved with the fragile X syndrome (29), and the assumption of a transcriptionally closed chromatin configuration by transfected genes which are methylated, *in vitro* (27), or by endogenously hypermethylated genes in immortalized (25) or neoplastic cells (4). The hypermethylation associated with X-chromosome inactivation (28) and with the abnormality in the fragile X syndrome (29) are correlated with delayed DNA replication of the involved regions (30). Such delays have been proposed to increase chromosome fragility (31), which could play a key role in predisposition to genetic instability and chromosome loss during tumor progression. In the paper which follows, we describe the timing sequence for D17S5 hypermethylation and 17p structural changes, which strengthens the probability that abnormal hypermethylation highlights chromatin changes which are critical for increased genetic instability in tumor progression.

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