[CANCER RESEARCH 53, 2719-2722, June 15, 1993]

*Advances in Brief*

# Regional DNA Hypermethylation at D17S5 Precedes 17p Structural Changes in the Progression of Renal Tumors[1]

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## Abstract

**In a preceding paper for brain tumors, we demonstrate a tight association between regional hypermethylation at locus D17S5 of chromosome 17p and allelic loss of this chromosome. Because 17p allelic losses occur at the earliest stages of brain tumors, the exact temporal relationship between this event and the hypermethylation could not be elucidated. In renal cancers, two linked structural changes on chromosome 17p, allelic loss and *p53* gene mutations, generally occur late in progression. We now show that D17S5 hypermethylation is tightly coupled to both of these genetic changes in late stage renal tumors. However, the methylation change is the only one of the 17p abnormalities which occurs at a high incidence in early-stage renal cancers (hypermethylation, 50%; 17p allelic loss, 13%; *p53* mutations, 0%). Our results firmly suggest that D17S5 regional hypermethylation precedes the appearance of the consistent 17p genetic changes in renal cancers, suggesting that this event either marks, or may even cause, chromatin changes which predispose to genetic instability.**

## Introduction

In an accompanying study of neural tumors (1), and in an earlier study of colon tumor progression (2), we have associated abnormal DNA hypermethylation with genetic changes on chromosome 17p. However, the early appearance of both of these changes in brain tumor progression (for a discussion, see Ref. 1) and the observation that the two events occur in close temporal proximity after the transition from benign colon polyps to colon carcinomas (2, 3) do not permit the timing between these DNA changes to be delineated. This is an important point, since changes in DNA methylation have been associated with chromatin alterations (4), which perhaps could lead to genetic instability in cancers. Human renal cancers provide a model in which to explore directly this timing question, because loss of chromosome 17p alleles and mutation of the tumor suppressor gene *p53* are infrequently detected at early stages of this tumor (5, 6) as compared to other neoplasms. In addition, established cultures of late-stage renal cancers provide an excellent system in which to determine the relationship between 17p structural changes and DNA methylation, since over one-half (52%) of these cultures have no 17p deletions and no detectable *p53* gene mutations, while 48% have at least one, and frequently both (6).

In the present study, we have used an approach identical to that in the accompanying paper (1), in which we compare, in DNA from normal renal tissue and samples of fresh and cultured renal cancers, the methylation status of *Not*I restriction sites in the D17S5 region (see Fig. 1 of Ref. 1) of chromosome 17p. The results have been compared to the allelic status of the same D17S5 region (7) and other 17p loci (6, 8) and to the presence of point mutations in the *p53* tumor suppressor gene. Our findings suggest that the D17S5 methylation abnormality is associated with 17p chromatin alterations in human renal cancers and actually precedes these two events.

## Materials and Methods

**Renal Tumor Samples and Cell Cultures.** All fresh renal cancers and adjacent normal renal tissue were obtained at the time of surgery and clinically staged exactly as described previously (6, 8, 9). The tumor tissue sections were prepared by histological analysis, such that normal tissue was separated from the cancers as much as possible and DNA was prepared as previously described (8). The efficiency of the separation of normal from tumor tissue was documented, as discussed in "Results," by the consistent detection of chromosome 3p allelic loss in the cancer DNA (8). The established cultures of late-stage, clinical cancers and paired normal renal tissue from each patient are those described in detail in Ref. 6.

**Determination of 17p Methylation and Allelic Status.** The D17S5 allelic status was determined by both *Bam*HI and *Msp*I restriction analyses of the highly polymorphic region detected by probe YNZ22 exactly as previously described in the accompanying paper (1). Other 17p probes specific for restriction length polymorphisms were also utilized (6, 8). The *Not*I and *Not*I/*Bam*HI restriction analysis assessing the methylation status of the D17S5 region was also performed as previously described (1, 2).

***p53* Gene Mutation Analysis.** All samples have been assessed, as previously described (6), for *p53* gene mutations in exons 5–9 by single-strand conformational polymorphism and by sequencing of polymerase chain reaction-amplified products, in the regions where 95% of the mutations are thought to occur(10).

## Results

**D17S5 Hypermethylation Is Associated with 17p Allelic Loss in Renal Tumors.** In contrast to the completely unmethylated status of all the *Not*I sites in the D17S5 region in all other normal tissues we have previously studied (1, 2), we found that *Not*I sites 3 or 3 and 4 (Fig. 1 in the accompanying paper) are partially methylated in DNA from normal renal tissue from patients with and without renal cancer. This methylation is reflected by either the presence of three *Not*I restriction bands (see Fig. 1, *Sample 3N*) or two widely spaced *Not*I bands (Fig. 1, *Sample 2N*), rather than the usual one or two closely spaced bands seen in other normal tissues (see all figures in the accompanying paper). Comparison of *Bam*HI and *Not*I digests for normal renal *versus* other tissues (data not shown) revealed that the three *Not*I bands in renal tissues reflect partial methylation of two YNZ22 alleles differing in size by 0.4 kilobases or more, and the two *Not*I bands are associated with methylation of two *Bam*HI alleles which are of identical size or which differ in size by <0.4 kilobases.

When compared to the above *Not*I digestion patterns for normal kidney, we found that, as in neural tumors (1), regional D17S5 hypermethylation occurs in all renal cancers tested which have lost 17p alleles. All 11 renal cancers (one fresh tumor, 10 cultures) which have lost one or more 17p loci (6, 8) exhibit D17S5 regional hypermethylation on the remaining 17p chromosome (Fig. 1). In contrast, of 20

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

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[1] Supported by NIH Grant R01-CA43318.

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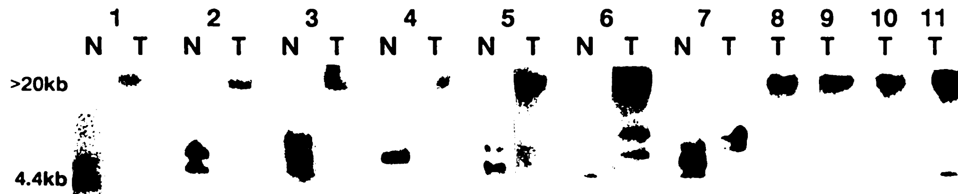


Fig. 1. Methylation status of YNZ22 *NotI* sites in DNA from fresh and cultured renal tumors which have lost one copy of chromosome 17p, including region D17S5. Each of the renal tumors (*T*) shown has lost 17p alleles, at one or more loci, always inclusive of the D17S5 region. Sample 1 represents DNA from the same patient, including normal renal tissue (*N*) and a noncultured early-stage renal tumor (*T*). Pairs 2–7 are cultures of late-stage renal tumors (*T*) compared to normal renal tissue from each patient (*N*), while *Lanes 8–11* represent cultured tumor DNA only. *p53* gene mutations were found in tumors 2, 5–7, 9, and 11 and were not detected in 1, 3, 4, 8, and 10 (6). Eight tumors (samples 1–5, 8–10) show extensive methylation, reflected by the complete loss of normal restriction fragments at 4.5–5.0 kilobases and hybridization of only a >20-kilobase fragment. Samples 6 and 11, besides hybridization at >20 kilobases, also show smaller abnormal bands around 8.0 kilobases. Sample 7 is the only tumor which does not have any hybridization at >20 kilobases. However, this DNA still has an increased methylation pattern compared to the corresponding normal kidney sample. All renal tumors examined in Figs. 1 and 2 were unmethylated at *NotI* site 2 (Fig. 1, previous paper), indicating that the abnormal hypermethylation was occurring at *NotI* sites 3–6.

tumors (9 fresh cancers and 11 cultures) which have not lost D17S5 alleles (6, 8), only 10 demonstrated abnormal hypermethylation (Fig. 2*A*). The remaining 10 samples either had the identical (Fig. 2*B*) or a decreased (Fig. 2*C*) methylation pattern. The hypomethylation pattern is reflected by the lower-molecular-weight bands in the tumor DNA as compared with the matched normal renal tissue (Fig. 2*C*). All of our data revealing relationships between allelic loss and 17p methylation status are summarized in Fig. 3.

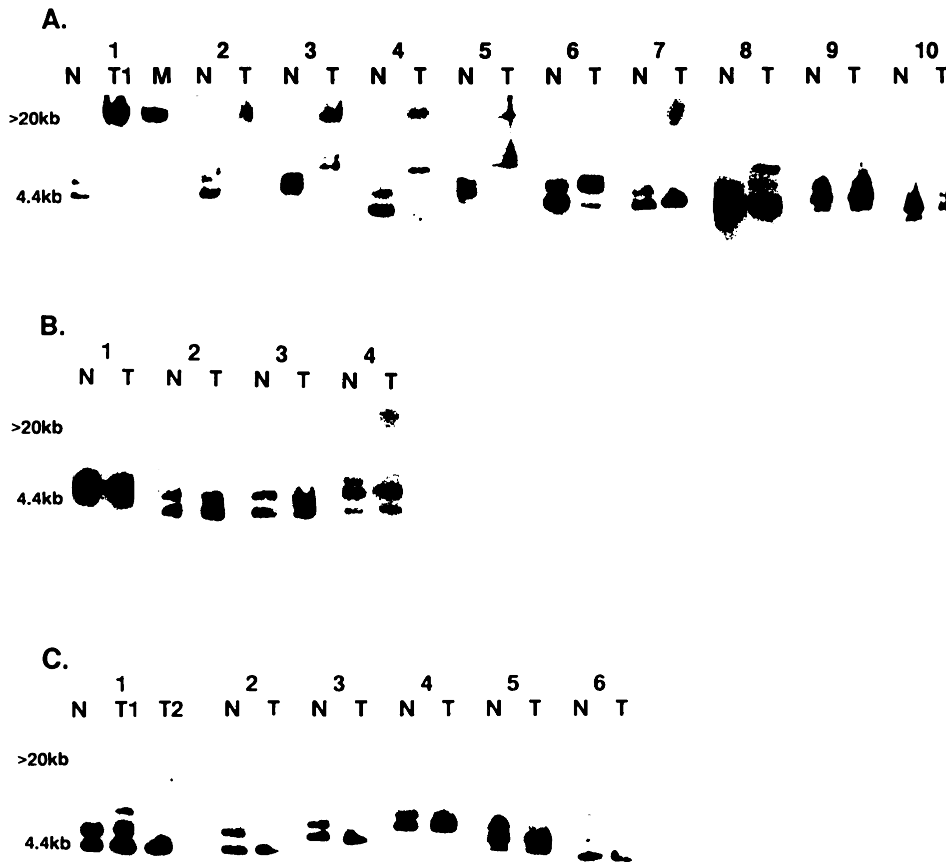


Fig. 2. Methylation status of the D17S5 region in DNA from cultured and fresh renal tumors which have retained both copies of chromosome 17p. Each tumor DNA is heterozygous for one or more polymorphic regions, always including D17S5 (6, 8). *A*, tumors with hypermethylation of the D17S5 region. DNA sample pairs 1–6 represent cultured late-stage renal tumors (*T*), samples 7, 9, and 10 contain DNA from early-stage renal tumors, and sample 8 is a noncultured late stage renal tumor, positioned next to the corresponding normal (*N*) renal tissues. Sample 1 also contains DNA from an adrenal metastasis (*M*) originating from the original renal tumor (*T*). Tumors *1T* and *1M* are the only samples which have retained both copies of the D17S5 region but which have one mutated *p53* allele (6). Samples 1 and 2 are the only 2 allele tumors which exhibit the same extensive methylation pattern, as seen in the tumors which have lost 17p alleles (Fig. 2), indicated by hybridization at >20 kilobases, and no other lower-molecular-weight bands. Samples 3–5 have hybridization at >20 kilobases but also exhibit abnormal bands of 8.0 kilobases. Samples 6–10 exhibit hypermethylated *NotI* fragments between 4.5 and 8.0 kilobases. The sample pair in 7 contains a tumor (*T*) which appears to have extensively methylated one allele (note >20-kilobase band) but not the other (note the one restriction fragment in the normal 4.5–5.0-kilobase region). *B*, tumors with methylation patterns in the D17S5 region identical to those in corresponding normal tissue. Sample 1 represents DNA from a cultured, late-stage tumor with no *p53* gene mutation (*T*) compared to DNA from the corresponding normal kidney (*N*). Samples 2–4 are early-stage, fresh tumors (*T*) and also do not have *p53* gene mutations. The methylation patterns seen in the tumor of each of these samples are identical to those in the corresponding normal tissue. *C*, tumors with hypomethylation of the D17S5 region. DNA samples 1–4 are from cultured late-stage renal tumors which do not have *p53* gene mutations (6). Sample 1 contains DNA from the original renal cancer (*T1*) and a metastatic lesion removed at a later time (*T2*). Sample 5 contains DNA from a noncultured, early-stage tumor (*T*), and sample 6 is a late-stage renal tumor (8). The normal samples (*N*) all show the partially methylated 4.5–5.0-kilobase bands, whereas all tumors labeled *T* show a decrease in the methylation of *NotI* sites in this region. Tumor *T1* shows a slight increase in methylation relative to the normal kidney, suggesting that heterogeneity for D17S5 methylation patterns can exist between primary and metastatic lesions from a single renal cancer.



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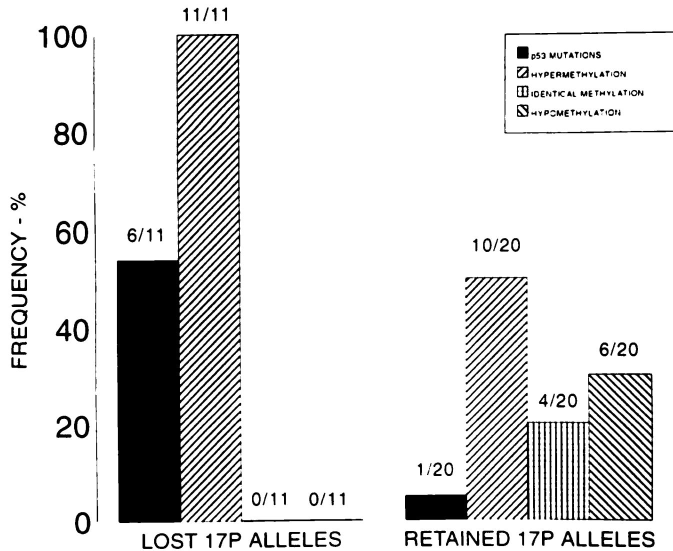


Fig. 3. Relationships between 17p allelic status, *p53* gene mutations, and methylation of *NotI* sites in the D17S5 region. DNA samples from fresh and cultured renal cancers have been grouped for those which have lost or retained chromosome 17p alleles. The frequency for D17S5 hypermethylation and *p53* mutations is compared within the two groups, and the actual number of samples positive for a given change over the number tested is given *above each frequency bar*.

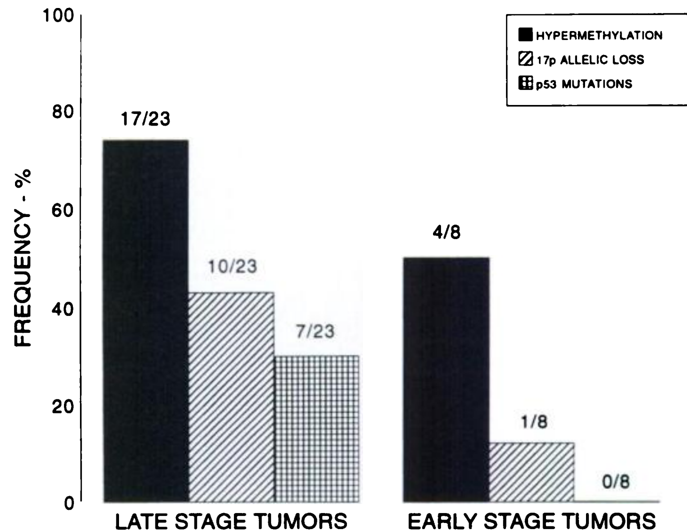


Fig. 4. Frequency of *p53* mutations, D17S5 hypermethylation, and 17p allelic losses as a function of tumor stage. The clinical stage of all renal cancers examined and of those from which culture lines were established was determined as previously described (6, 8, 9). The number of samples showing a given change over the number of samples tested is given at the *top of each frequency bar*.

**D17S5 Hypermethylation Is also Associated with *p53* Gene Point Mutations.** We also found a correlation between D17S5 regional hypermethylation and detected *p53* gene point mutations in renal cancers. Six of 11 renal tumors with 17p allelic loss had *p53* mutations (Fig. 3). Each of these tumors also had D17S5 regional hypermethylation (Fig. 1, *Samples 2, 5–7, 9, 11*). In addition, there was one tumor (Fig. 3) which, although it retained both 17p alleles, had a *p53* gene point mutation. It also exhibited extensive 17p hypermethylation (Fig. 2*A*, *Sample 1*). Thus, in renal cancers, D17S5 regional hypermethylation is associated not only with chromosome 17p allelic loss but also with *p53* gene mutations.

**D17S5 Hypermethylation Precedes 17p Allelic Loss and *p53* Mutations.** Perhaps the most striking feature of the present study is that several aspects of our data strongly suggest that D17S5 hypermethylation precedes both 17p allelic loss and *p53* gene mutation in renal cancer. First, not one of the 12 tumors that had either *p53* gene mutations, 17p allelic loss, or both lacked D17S5 hypermethylation (Fig. 1 and Fig. 2*A*, *Sample 1*). Second, the incidence of D17S5 hypermethylation exceeds that of 17p allelic loss and detected *p53* mutations at all stages of renal tumors (Fig. 4). In this regard, there were 9 examples of hypermethylated tumors, which did not have 17p allelic loss or *p53* gene mutations (Fig. 2*A*, *Samples 2–10*). Furthermore, in at least 5 tumors that retained heterozygosity for chromosome 17p and were hypermethylated (Fig. 2*A*, *Lanes 1–5*), the methylation change was present on both D17S5 alleles, since no normal *NotI* bands were detected. Fourth, as predicted from previous studies (5), we found little evidence in 8 early-stage renal tumors of 17p structural changes (Fig. 4). Yet one-half of these 8 tumors (Fig. 4) exhibited D17S5 hypermethylation, although the pattern was less extensive than in most late-stage tumors (compare Fig. 1 to Fig. 2*A*, *Samples 7, 9, 10*). Our inability to detect 17p structural alteration in these early-stage primary tumors was not due to infiltrating normal cells, since we were able to detect the most frequent genetic change seen in early-stage renal cancer, allelic loss on chromosome 3p (5, 9, 11–13), in 6 of 7 tumors tested (8). Finally, the one early-stage tumor which did have a 17p structural change, reduction to allelic homozygosity for 17p, exhibited the extensive methylation pattern on the remaining 17p allele (Fig. 1, *Sample 1*), as was seen in most of the single-allele, late-stage tumors (Fig. 1, *Samples 2–11*).

## Discussion

Our present data for renal cancers, together with our previous studies of colon (2) and brain tumors (1), establish that D17S5 hypermethylation is tightly coupled to 17p deletions and *p53* gene mutations in human cancers. Our results in renal cancer strongly suggest that this hypermethylation precedes the other two events. If so, hypermethylation either plays a direct role in causing chromatin changes which predispose to chromosome 17p structural alterations or marks an event(s) which places chromosomes at risk for genetic instability. We do not yet know the precise mechanisms which underlie the association between hypermethylation and 17p structural changes. In the preceding study of brain tumors(1), we discussed the evidence that methylation of normally unmethylated CpG-rich areas can both result from and cause changes in chromatin structures (14–18). One known result of this interaction is that methylated DNA replicates later than unmethylated DNA (15, 19). Such delays have been proposed, by others (20), to render chromosomal regions more susceptible to genetic instability. The allelic losses we have studied might be the consequences of such changes. The association of *p53* mutations with methylation changes occurring in a region distal to this gene is intriguing. It is possible that these two events are linked only because, as some have hypothesized, 17p allelic losses select for tumor cells with *p53* gene mutations (3). However, we cannot rule out the possibility that the regional hypermethylation itself highlights chromatin changes which predispose to both 17p allelic loss and *p53* gene mutations.

In summary, our current findings for renal tumors and the accompanying data for brain cancers (1) emphasize the probability that distinct chromosome regions undergo increasing pressure for genetic instability as tumors progress. Altered DNA methylation patterns appear to be one molecular change that marks such predisposition events, and it will be important to determine the precise mechanism between this DNA modification and the chromatin changes involved.

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