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HYPOMETHYLATION OF ras ONCOGENES IN PRIMARY HUMAN CANCERS

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SUMMARY: We have examined the methylation status of two cellular oncogenes, c-Ha-ras and c-Ki-ras, in primary human carcinomas and the adjacent analogous normal tissues from which the tumors derived. The c-Ha-ras gene was hypomethylated in six of eight carcinomas, including five colonic adenocarcinomas and one small cell lung carcinoma, when compared to adjacent normal tissues. The c-Ki-ras gene was hypomethylated to a lesser extent in two colonic adenocarcinomas. This is the first demonstration of alterations in methylation of cellular oncogenes in human cancer.

INTRODUCTION: Abnormal gene expression is a hallmark of cancer (1-5), and alterations in DNA methylation, heritable by cell progeny, appear to play a crucial role in the regulation of gene expression (6-7). Thus, it has been suggested that alterations in DNA methylation could underlie some aspects of malignancy (8-10). In an examination of DNA methylation in primary human tumor tissues, we found substantial hypomethylation of growth hormone and globin genes of human cancer cells when compared to adjacent analogous normal cells from the same patients. These alterations, which were readily detectable by nucleic acid hybridization, were present in two histologic types of cancer and were progressive in a metastatic lesion (10).

Several lines of evidence suggest that the human cellular homologues of animal oncogenic viruses play a role in human cancer (11-15). In particular, the cellular homologue of the Harvey murine sarcoma virus, c-Ha-ras, from a human bladder carcinoma, and the cellular homologue of the Kirsten murine sarcoma virus, c-Ki-ras, from human lung and colon carcinomas, can transform mouse NIH 3T3 cells (16-18) and, in at least one case, a point mutation in c-Ha-ras is responsible for the transforming property (19,20). In the present

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study we examined whether alterations in methylation of these oncogenes occur in primary human cancer tissues.

METHODS: Human tissues were removed from surgical patients in accordance with institutional guidelines. Patients 1-7 had grade C-D adenocarcinoma of the colon (21); patient 8 had a small cell carcinoma of the lung. Normal colonic mucosa was stripped from adjacent colon just outside the histologically proven tumor margin, and the normal lung tissue was removed from the contralateral lung. Tissues were frozen in liquid nitrogen, then pulverized and the DNA extracted as described elsewhere (22). The DNA was cleaved with a 50-fold excess of restriction endonuclease, and DNA digests (5 ug per lane as assessed by a fluorometric assay [23]) were electrophoresed on agarose gels at 70V for 4h, then transferred to nitrocellulose by the modification of Southern's (24) procedure described by Wahl et al (25). pBR322 plasmids containing an insert of v-Ki-ras (HiHi3; ref. 26) and a genomic insert of c-Ha-ras (pEJ; ref. 27) were grown using E. coli strain HB101 as host, and plasmid DNA was isolated by standard techniques (28,29). The v-Ki-ras insert in HiHi3 was purified by electrophoresis and labeled with [^{32}P]dCTP by a procedure described elsewhere (30). pEJ DNA was labeled with [^{32}P]dCTP by nick-translation (31). The probes were hybridized to the filters for 36-60h and then washed as described (32). The autoradiographs were exposed for 2-5 days using Kodak XAR-5 film with DuPont Lightning Plus intensifying screens (33,34).

RESULTS: The technique we used is based on the fact that certain restriction endonucleases (e.g., HpaII and HhaI), which cleave at sites containing the sequence 5'-CG-3', do not function if the internal cytosine residue is methylated (35,36). Cytosine is the only significantly modified base in mammalian DNA (37), and the two-base sequence 5'-CG-3' can be heritably methylated (38-40). Thus, enzymes that discriminate between methylated and unmethylated CG sequences are sensitive indicators of methylation (35,36). In contrast, the HpaII isoschizomer MspI is not sensitive to methylation of the internal cytosine residue and can thus be used for comparison (41).

In the present study, we compared the methylation patterns of cellular oncogenes in DNA purified from primary human cancers and adjacent analogous normal tissues from the same patients. After cleavage with HpaII, HhaI, or MspI, the DNA samples were electrophoresed and transferred to nitrocellulose filters by the method of Southern (24,25) and were then hybridized to [^{32}P]-labelled c-Ha-ras and c-Ki-ras probes. In normal colon and lung, the cellular oncogene c-Ki-ras appeared fully methylated, since in all normal tissues examined a single band of DNA >24 kbp in size was seen on Southern hybridization after HpaII or HhaI digestion, while 5 fragments of 2.5 to 6 kbp were obtained

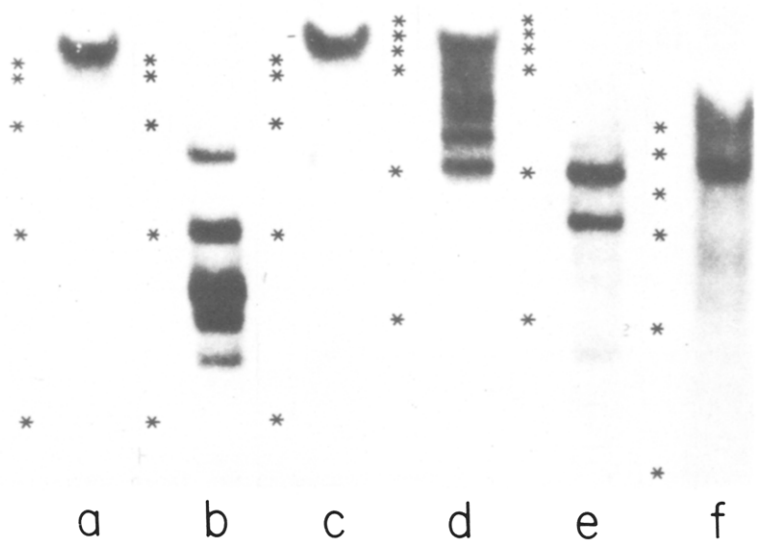

Figure 1 - Methylation patterns of c-Ha-ras and c-Ki-ras in normal colon of patient 1. Asterisks to the left of each lane in all figures represent molecular weight markers (HindIII-digested bacteriophage lambda DNA), of sizes 24, 9.5, 6.7, 4.4, 2.0 kbp, and in cases where there are six asterisks, 0.57 kbp, from top to bottom, respectively). c-Ki-ras: HpaII (lane a), MspI (lane b), HhaI (lane c). c-Ha-ras: HpaII (lane d), MspI (lane e), HhaI (lane f). Similar patterns were observed in all normal tissues studied.

with MspI (Figs. 1a-c). In contrast, c-Ha-ras yielded several discrete fragments on HpaII or HhaI digestion, although fragments <2 kbp were seen in the normal tissues only after treatment with MspI (Figs. 1d-f). Thus, c-Ki-ras appears fully methylated and c-Ha-ras partially methylated in all normal tissues studied.

When we compared the c-ras hybridization patterns of normal and cancerous tissues, the most pronounced difference we observed was a striking decrease in c-Ha-ras methylation at HhaI sites. There were four or five HhaI fragments detectable in all human DNA samples tested; these fragments were >24, 9, 3.7, 3 and 2.2 kbp in size (Fig. 2). In normal colon and lung, bands representing the two largest fragments (>24 and 9 kbp) were the most intense on the autoradiograms. In six of the eight tumors, this pattern was reversed, with the 3.7, 3, and 2.2 kbp bands more intense than the >24 and 9 kbp bands, indicating a decrease in c-Ha-ras methylation at HhaI sites. Two controls showed that these differences were not due to incomplete digestion of DNA from

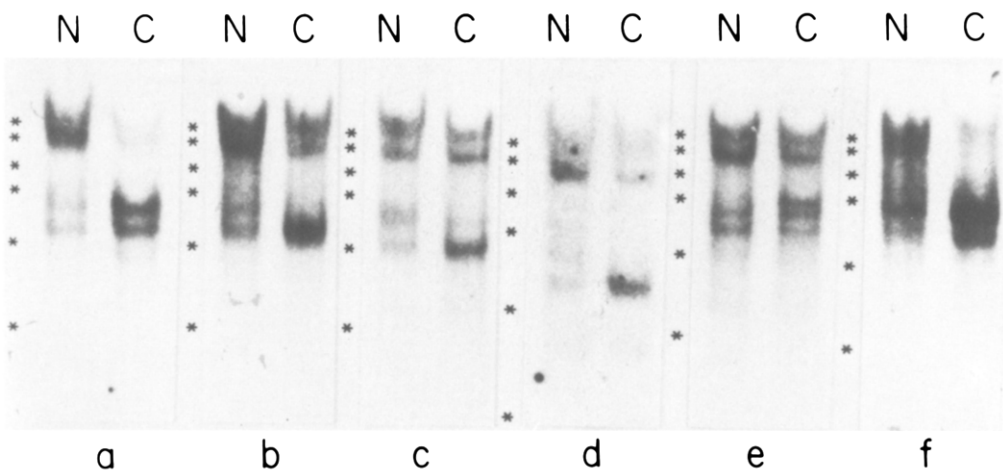

Figure 2 - Methylation patterns of HhaI-digested DNA from normal tissues (N) and carcinomas (C) hybridized with c-Ha-ras probe: Lanes a through e represent colon tissue from patients 2, 3, 4, 5, and 6, respectively, and lane f represents lung tissue from patient 8. Asterisks are as described in the legend to Figure 1.

the normal tissues. First, before digestion with restriction enzymes, all DNA samples used in the study had a size >24 kbp. In each case the DNA was cleaved with a 50-fold excess of restriction endonuclease, as assessed by the amount required to digest PBR322 included in a matched duplicate digest of the human DNA. Second, the digestion patterns of each DNA sample were identical to those obtained when only 1/10 the enzyme concentration was used, showing that a limit digest had been obtained. A less pronounced difference in methylation was observed in c-Ha-ras at the HpaII sites; in three of the eight patients, these sites were slightly hypomethylated in the cancer DNA when compared to the normal (Figs. 3a-c).

In contrast to the relative hypomethylation of c-Ha-ras in the tumor tissues compared to adjacent normal tissues, only two colon carcinomas showed alterations in methylation of c-Ki-ras when compared to adjacent normal tissues, and these alterations were slight (Figs. 3d-e). The other six tumor samples remained fully methylated at c-Ki-ras when compared to the adjacent normal tissues.

Parenthetically, we observed considerable polymorphism of the base sequence 5'-CCGG-3' at several sites in the c-Ha-ras gene, as seen by a compari-

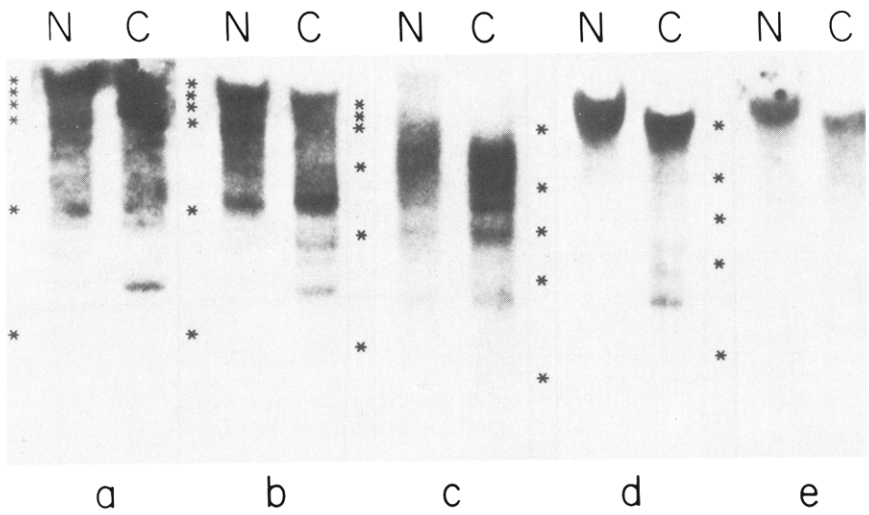

Figure 3 - Methylation patterns of HpaII-digested DNA from normal tissues (N) and carcinomas (C). c-Ha-ras: patient 4 (lanes a), patient 5 (lanes b), patient 8 (lanes c). c-Ki-ras: patient 4 (lanes d), patient 2 (lanes e). Asterisks are as described in the legend to Figure 1.

son of normal DNA samples digested with MspI (Fig. 4). No differences in MspI digests were detected between the normal and cancerous tissues of each patient.

DISCUSSION: We previously reported an alteration in methylation of growth hormone and globin genes of human cancers when compared to the normal tissues from the same patients from which the tumors derived (10). We extend this observation in the present study to two human cellular oncogenes that have been proposed to play a role in the development of at least some human cancers (16-20). We observed substantial alterations in the methylation patterns in six of eight patients, with colon or lung carcinomas, at the cellular oncogene c-Ha-ras. These alterations in methylation did not appear to be entirely random, in that c-Ha-ras was hypomethylated more frequently in cancers when compared to adjacent normal tissues, than was c-Ki-ras; and within the c-Ha-ras regions, HhaI sites were much more frequently affected than were HpaII sites. Nonrandomness in hypomethylation in cancer tissues was also observed in the analysis of other genes from these patients. For example, in human colon cancer, growth hormone related genes and the gamma-globin gene were more

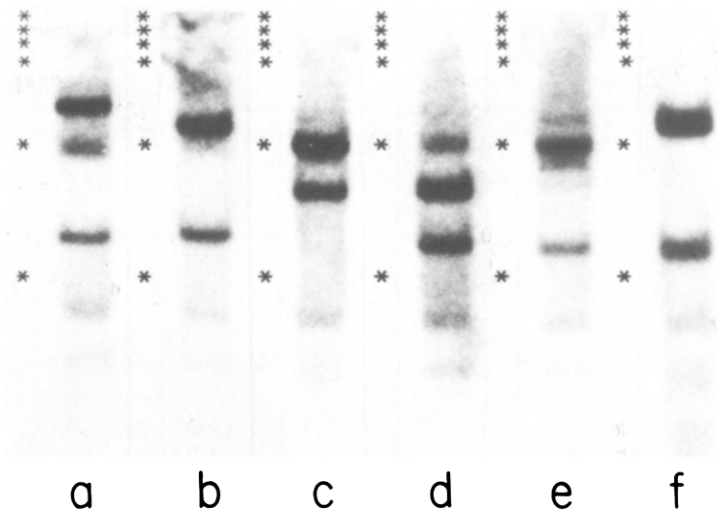

Figure 4 - Methylation patterns of MspI-digested DNA from normal tissues hybridized with c-Ha-ras probe (normal and cancer were identical in each case): patient 4 (lane a), patient 3 (lane b), patient 1 (lane c), patient 7 (lane d), patient 5 (lane e), patient 8 (lane f). This demonstrates marked polymorphism of c-Ha-ras among the patients.

frequently hypomethylated than were alpha-globin genes (ref. 10 and unpublished data).

The observations reported here are of interest for three reasons. First, they support the observation that methylation alterations are widespread in the genomes of some human cancers (10). Second, it has been shown by many investigators that methylation plays a role in gene expression (6,7). In particular, it has been noted that experimentally induced demethylation of non-expressed genes can result in the expression of those genes (42-44). One could speculate that a defect in methylation of genes in human cancer could lead to the abnormal expression of these genes. By this argument, the hypomethylation of a growth hormone-related gene might result in ectopic hormone production; the hypomethylation of a ras-related oncogene might result in the development of some tumorigenic properties, etc. Obviously, further investigation will be required to determine if the methylation changes we observed are associated with expression of the genes, and to determine the sequence of events (i.e., whether hypomethylation precedes the expression of these genes).

Third, the results presented here raise the possibility that differences in methylation might play a role in the ability of some human tumor DNA samples to cause transformation of fibroblasts. Several investigators have shown that methylated genes will not be expressed upon DNA mediated gene transfer or microinjection (39,40,45,46). In those tumor cells containing a transforming homologue of an oncogene (16-20), the loss of methylation of these genes might be a critical event in allowing the experimenter to detect transforming activity.

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