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Change in Methylation Status of DNA of Remaining Allele in Human Lung Cancers with Loss of Heterozygosity on Chromosomes 3p and 13q

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Loss of heterozygosity at several chromosomal loci is frequently observed in human cancers and loss of one allele is supposed to affect expression of the gene(s) in the remaining allele. As DNA methylation is known to be closely related to gene expression in vertebrates, we are interested in the methylation status of the regions of alleles remaining after loss of their counterparts. In this work we investigated the methylation status of DNA from human lung carcinoma in which heterozygosity was lost at 3p and 13q and found that the remaining allele at these loci was preferentially demethylated. In contrast, tumor DNAs without allele loss tended to retain highly methylated states. These results suggest that in tumors, change of the DNA methylation status is closely related with allele loss, and *vice versa*.

Key words: Human lung cancer — DNA methylation — Allelic deletion

There is accumulating evidence of frequent loss of heterozygosity at specific chromosomal loci in a variety of human tumors, suggesting unmasking of an inactivated tumor suppressor gene(s) by removal of the corresponding normal allele.[1,2] In vertebrates, many lines of evidence indicate that the control of gene expression is related with the extent of methylation of cytosine residues in CpG sequences of DNA.[3–5] We are interested in the methylation status in a remaining allele after loss of its counterpart. In all types of human lung cancer, including small cell carcinoma, losses of heterozygosity at loci on the short arm of chromosome 3 (3p) and on the long arm of chromosome 13 (13q) have frequently been observed.[6–10] In this work, we analyzed DNAs from normal and cancerous portions of surgical specimens of human lung cancer using polymorphic DNA probes for loci on 3p and 13q, and compared the methylations of *Hpa*II recognition sequences at these loci in DNAs showing loss of heterozygosity and normal DNAs.

Surgical specimens of human lung cancer were obtained from patients in the National Cancer Center Hospital, Tokyo. High-molecular-weight DNA was prepared from both cancerous and normal portions of the tissue by the method of Blin and Stafford.[11] Specimens of DNA were digested with restriction endonuclease *Msp*I or *Hpa*II and the digests were subjected to Southern blot analysis.[12] The DNA probes used in this work to detect restriction fragment length polymorphisms (RFLPs) are summarized in Table I. Each probe was labeled with ^{32}P by the method of Feinberg and Vogelstein[13] using random primers and [α-^{32}P]dCTP as a radioactive substrate.

Both *Msp*I and *Hpa*II recognize the unmethylated nucleotide sequence CCGG and cleave DNA at this recognition site. However, when the 5-position of the second cytosine residue is methylated, this sequence is still cleaved by *Msp*I, but not *Hpa*II. When DNA of individuals who are heterozygous at this locus is digested with *Msp*I and probed by a DNA fragment from the *D3S2* locus, three fragments, 2.9-kb fragment from allele 1 and 1.6- and 1.3-kb fragments from allele 2, are produced. Representative results are shown in Fig. 1A. RFLP analysis of *Msp*I digests revealed loss of allele 1 and allele 2 in tumor DNAs from patients 1 and 2, respectively, while heterozygosity was retained in tumor DNA from patient 3. The residual amounts of signals for alleles 1 and 2 in the tumor DNAs in which these alleles were lost might be due to DNA from normal cells present in the tumor specimens. On digestion with *Hpa*II, DNAs from cancerous portions, but not normal portions, of the lungs of patients 1 and 2 gave smaller fragments, suggesting that after allele loss the amount of methylated cytosine residues in CpG sequences in the remaining allele in the *D3S2* locus was greatly reduced. On the other hand, the region of the *D3S2* locus of the tumor DNA from patient 3, which did not show allele loss, retained a highly methylated status and therefore could not be cleaved to smaller fragments by *Hpa*II digestion (Fig. 1A).

To determine whether the hypomethylation in the remaining allele was restricted to a particular chromosomal locus, we also analyzed the regions of the *D13S2* and *D13S4* loci in the DNAs of patients. Figure 1B shows results obtained for the *D13S2* locus. *Msp*I diges-

tion of DNA revealed that the three patients were constitutionally heterozygous and that the cancer DNAs from patients 1 and 2, but not patient 3 showed loss of heterozygosity. When the DNAs were digested with *Hpa*II, only the tumor DNAs from patients 1 and 2 were cleaved completely and gave a fragment with the size expected from the remaining allele, while the DNAs from normal tissues of patients 1 and 2 and the DNAs from both normal and cancerous portions of the lung of patient 3 retained their large sizes. These results again indicate that allele loss in the region of the *D13S2* locus was associated with change from highly methylated status to a hypomethylated status, while this region of the tumor DNA showing no allele loss remained highly methylated. Hypomethylation of the remaining allele at the *D3S2, D13S2* and *D13S4* loci in these tumor DNAs was not due to overall undermethylation of DNA in tumor cells. For example, demethylation of tumor DNA retaining heterozygosity was not observed when DNAs from patients 1 and 2 in Fig. 1A were analyzed at the *MET* locus (7q31-q32) and at the *D9S7* locus (9q), respectively, and DNA from patient 1 in Fig. 1B was analyzed at *MET, PALB* (18q11.2-12.1) and *D15S1* (15q14-q21) loci (data not shown).

Table II summarizes results obtained on DNAs from 24 patients with lung carcinoma, including those shown in Fig. 1. Heterozygosity detected by RFLP analysis was not frequent, and therefore the number of informative DNA samples was limited, but the results clearly indicate that more than 75% of the tumor DNAs with allele loss tended to be undermethylated at the corresponding

Table I. DNA Probes Used to Detect RFLP

Probe	Locus	Chromosomal location	Allele	Polymorphic fragment (kb)
pHF12-32	*D3S2*	3p21-p14	1	2.9
			2	1.6, 1.3
p9D11	*D13S2*	13q22	1	15.0
			2	10.5
p1E8	*D13S4*	13q22-q31	1	10.1
			2	7.4

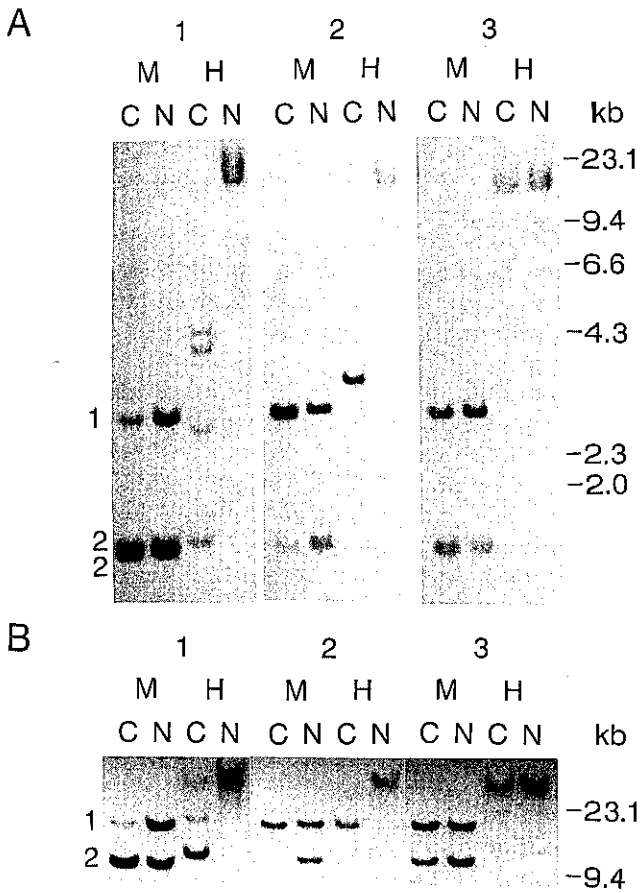

Fig. 1. Methylation status of normal and tumor DNAs at the *D3S2* locus (A) and at the *D13S2* locus (B). Samples of 2 μg of high-molecular-weight DNA of cancerous (C) and normal (N) portions of lung carcinoma from the same patient were digested with restriction endonuclease *Msp*I (M) or *Hpa*II (H). The fragments produced were separated by electrophoresis in 0.7% agarose gel and transferred to a nylon membrane (GeneScreen Plus, DuPont). Southern blots were hybridized to the ^{32}P-labeled DNA probes shown in Table I. Numbers 1 and 2 on the left indicate the observed alleles. Because of the relatively small numbers of cleavage sites, the viscosity of the reaction mixture due to high-molecular-weight DNA was not significantly reduced upon digestion with *Hpa*II, and so the mobility of *Hpa*II fragments was affected. Therefore, the positions of *Hpa*II fragments differ slightly from those of the corresponding *Msp*I fragments, especially those of patient 1 in (A) and (B).

Table II. Methylation Status of the Remaining Allele in Human Lung Carcinomas with Allele Loss

Locus	No. of tumors analyzed	No. of tumors with constitutional heterozygosity	No. of tumors with demethylated allele / No. of tumors with loss of heterozygosity	No. of tumors with demethylated allele / No. of tumors retaining heterozygosity
D3S2	24	12	7/8	1/4
D13S2	24	10	2/2	2/8
D13S4	24	13	3/4	2/9

region of the remaining allele. In all patients who were heterozygous at these loci, DNAs from normal portions of the lung were highly methylated. In contrast to the loci of tumor DNAs that showed allele loss, those of more than 75% of the tumor DNAs analyzed that retained heterozygosity also retained a highly methylated status at these loci.

Considerable heterogeneity has been observed in the DNA methylation patterns of human and animal tumors and cultured cell lines, suggesting that the methylation status is not as rigorously controlled in tumor cells as in their normal counterparts.[14] DNAs of primary human cancers have been found to show increase, decrease or no change in the degree of methylation depending on the technique and system used, the tumors analyzed and the genetic locus examined. The 5-methylcytosine contents of DNA from various human tumors,[15] human neoplastic prostate[16] and colonic neoplasia[17] were found to be significantly lower than those of DNA from benign tumors or normal tissues. Similarly, there are reports of hypomethylation of tumor DNA at particular chromosomal loci, such as the *HRAS1* locus in colon carcinoma and small cell carcinoma of the lung[18] and the *GH, HBG, CGH* and *CRYG* loci in colon carcinomas.[19, 20] In contrast, increased methylation has been observed in DNA from human lung carcinomas and lymphomas.[21]

In this study, we demonstrated extensive demethylation of DNA in the regions of the remaining alleles after loss of their counterparts in human lung carcinomas. The physiological significance of the hypomethylation remains to be clarified. However, preferential loss of methylation at loci remaining after allelic deletion might influence expression of the gene(s) present in the corresponding region and might be involved in some stage of tumor development.

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