Jpn. J. Cancer Res. 87, 1210–1217, December 1996

Aberrant DNA Methylation on Chromosome 16 Is an Early Event in Hepatocarcinogenesis

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In order to clarify the significance of DNA methylation in both earlier and later stages of hepatocarcinogenesis, the DNA methylation state on chromosome 16, on which loss of heterozygosity (LOH) has frequently been detected in human hepatocellular carcinomas (HCCs), was examined. DNA from primary HCCs and tissues showing chronic hepatitis and liver cirrhosis, which are considered to be precancerous conditions, was analyzed by digestion with methylation-sensitive and non-sensitive restriction enzymes. DNA hypermethylation at the D16S32, tyrosine aminotransferase (TAT) and D16S7 loci and hypomethylation at the D16S4 locus were detected in 18%, 58%, 20% and 48% of examined HCCs, respectively. Aberrant DNA methylation occurred more frequently in advanced HCCs than in early HCCs. Moreover, DNA hypermethylation at the D16S32, TAT and D16S7 loci was frequently observed in chronic hepatitis and liver cirrhosis. The incidence of DNA hypermethylation was higher than that of LOH (42% at the TAT locus). These data suggest that DNA hypermethylation might predispose the locus to allelic loss. Aberrant DNA methylation is a significant change which may participate in the early developmental stages of HCCs.

Key words: Loss of heterozygosity — Hepatocellular carcinoma — Multistage carcinogenesis

The majority of hepatocellular carcinomas (HCCs) are associated with infection by hepatitis B virus (HBV) or hepatitis C virus (HCV), and clonal expansion of hepatocytes is initiated during the regeneration process after damage by hepatitis viruses or other carcinogenic factors.[1,2] Chronic hepatitis and subsequent liver cirrhosis are widely considered to be precancerous conditions, which correspond to an early stage of hepatocarcinogenesis. Small nodular lesions with structural abnormalities develop in these precancerous conditions and are considered to be early HCCs.[3,4] Within these nodules, advanced HCCs often emerge as nodule-in-nodule lesions.[4]

Corresponding to the stages of malignant progression, frequent allelic losses on specific chromosomes, including 1, 4q, 5q, 8p, 11p, 13q, 16p, 16q and 17p, are observed, indicating that dysfunctions of diverse tumor suppressor genes located on these chromosomes are involved in the development of HCCs.[5–12] Indeed, mutations of the *p53* and *RB* tumor suppressor genes have been noted during the progression of HCCs.[13–16] Loss of heterozygosity (LOH) on chromosome 16 correlates with clinicopathological parameters, that is, it is frequently detected in HCCs which are poorly differentiated, larger in size, and with metastasis, whereas it is not detected in HCCs at earlier stages, indicating that LOH on chromosome 16 may be involved in the progression of HCCs.[7] Previously reported genetic alterations other than LOH on chromosome 16 also occur in the later stages of HCCs and few events responsible for the earlier processes of hepatocarcinogenesis have been identified to date.

Alteration in DNA cytosine methylation is one of the most consistent molecular changes in human cancers.[17,18] The total level of DNA methylation is generally lower in cancer cells than in normal cells.[19,20] However, a normal to high level of DNA methyltransferase expression is usually seen in cancer cells.[21,22] Hypermethylation on 11p in lung cancer and lymphocytic leukemia,[23] on 17p in colon,[24] renal[25] and ovarian[26] cancers and neural tumors[27] and on 3p in lung[24] and breast[28] cancers has been reported, whereas 11p15 is hypomethylated in lung cancer.[29] DNA methylation may play roles in carcinogenesis in three ways: (a) DNA cytosine methylation facilitates gene mutation via a unique mechanism, i.e., 5-methylcytosine is deaminated to thymine;[30,31] (b) DNA methylation occurs frequently in clusters of CpG dinucleotides near regulatory region of genes[23,32] and affects the transcription of specific genes;[33,34] (c) aberrant DNA methylation may be associated with allelic loss, as reported on 17p.[24–27]

In order to clarify the significance of aberrant DNA methylation in hepatocarcinogenesis, we assessed aberrant CpG methylation on chromosome 16 in both primary HCCs and precancerous conditions.

MATERIALS AND METHODS

Tissue samples and DNA preparation Fifty-two HCCs, including multicentric HCCs, and corresponding non-

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cancerous tissues were obtained from surgically resected materials of 44 patients (H1 to H44), who underwent partial hepatectomy at the National Cancer Center Hospital, Tokyo, Japan. Ten lesions were pathologically classified as early HCCs, which are composed of well differentiated cancer cells and retain underlying liver structure.[3,4] The other 42 lesions were classified as advanced HCCs.[3,4] The lesions were graded from I to III, according to Edmondson's criteria for histological grading.[35] Data were also available regarding the presence or absence of involvement of portal vein and intrahepatic metastasis at the time of surgery. Histological findings compatible with chronic hepatitis and liver cirrhosis were observed in non-cancerous liver tissues of all 44 HCC cases. Additionally, liver tissues with no remarkable histological findings were obtained from surgically resected materials of four patients with liver metastatic lesions of primary colonic cancer (C1 to C4) and were subjected to the same analyses for comparison. High-molecular-weight DNA was isolated from fresh tissue samples by phenol-chloroform extraction and dialysis.[36]

CpG methylation on chromosome 16 DNA probes for chromosome 16, D16S32 (16 pter to p13),[37] D16S4 (16q22.1),[38] TAT (tyrosine aminotransferase, 16q22.2)[39] and D16S7 (16q24.3),[40] were used.[41] The methylation state was assessed by digesting DNA with *Msp* I and *Hpa* II, which cut at the sequence CCGG. *Hpa* II does not cut when the internal cytosine is methylated.[42] High-molecular-weight DNA (5 μg) was digested for 24 h with 10 units of either *Msp* I or *Hpa* II per microgram of DNA. The DNA fragments were separated by electrophoresis, transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled DNA probes.

LOH on chromosome 16 A polymorphic marker on chromosome 16, HP 0.4, which is a 400-bp subclone obtained by *Hin*d III-*Pst* I digestion of the TAT gene and which reveals a two allele restriction fragment length polymorphism in DNA cut with *Msp* I,[39] was used. High-molecular-weight DNA (5 μg) was digested for 24 h with 10 units of *Msp* I per microgram of DNA. The DNA fragments were separated by electrophoresis, transferred to nitrocellulose membranes, and hybridized with a ^{32}P-labeled DNA probe.

Statistics The relationship between the incidence of aberrant methylation on chromosome 16 and clinicopathological parameters was analyzed by using the χ^2-test.

RESULTS

CpG methylation on chromosome 16 in HCCs Hybridization patterns of DNA digests from normal liver tissue samples (C1 to C4) without chronic hepatitis or liver cirrhosis, which were obtained from patients with liver metastatic lesions of primary colonic cancer, were almost identical to each other. Although faint bands with larger size appeared in *Hpa* II digests, *Hpa* II patterns were similar to *Msp* I patterns at the D16S32, TAT and D16S7 loci in the normal liver tissue samples, indicating that genomic DNA is normally unmethylated or only slightly methylated in these regions (Figs. 1A, C and D; lanes C1 and C2). Multiple bands with larger size were detected in *Hpa* II digests compared to *Msp* I digests at the D16S4 locus in the normal liver tissues, indicating that this region is normally methylated (Fig. 1B; lanes C1 and C2).

Hybridization patterns could be clearly identified at the D16S32, D16S4, TAT and D16S7 loci in 50, 44, 50 and 45 HCCs, respectively. In accordance with previously described criteria,[23] the methylation state of each locus examined was judged from the *Hpa* II digestion pattern of the HCC samples compared with the *Msp* I digestion pattern of the same HCCs, and both the *Hpa* II and *Msp* I digestion patterns of the normal liver tissue samples C1 to C4 (Fig. 1).

Aberrant CpG methylation was observed in 32 out of 52 HCCs. Chromosomal loci involved in aberrant CpG methylation in the 32 HCCs are illustrated in Fig. 2. Hypermethylation at the D16S32, TAT and D16S7 loci, at which DNA from normal liver tissues was unmethylated or only slightly methylated, was detected in 18%, 58% and 20% of analyzed HCCs, respectively (Table I). Hypomethylation at the D16S4 locus, at which DNA from normal liver tissues was methylated, was detected in 48% of analyzed HCCs (Table I). Correlations between clinicopathological parameters and aberrant DNA methylation are summarized in Table II. The incidence of hypermethylation at any of the D16S32, TAT or D16S7 loci in advanced HCCs was significantly higher than that in early HCCs ($P<0.01$) and correlated significantly with the histological grade ($P<0.05$). Hypermethylation was detected more frequently in HCCs that were associated with portal vein involvement and intrahepatic metastasis than in HCCs without them (Table II). The incidence of hypomethylation at the D16S4 locus in advanced HCCs was significantly higher than that in early HCCs ($P<0.05$). Hypomethylation was detected more frequently in HCCs with malignant histological grades or with portal vein involvement and intrahepatic metastasis than in HCCs without them (Table II). There was no significant difference in the incidence of aberrant DNA methylation between cases with HBV or HCV infection and cases without it (Table II).

CpG methylation on chromosome 16 in chronic hepatitis and liver cirrhosis As in HCC samples, the methylation state in chronic hepatitis and liver cirrhosis was judged in comparison with that of the normal liver tissues C1 to C4 (Fig. 1). CpG hypermethylation was detected even in chronic hepatitis and liver cirrhosis at 36 (shown by

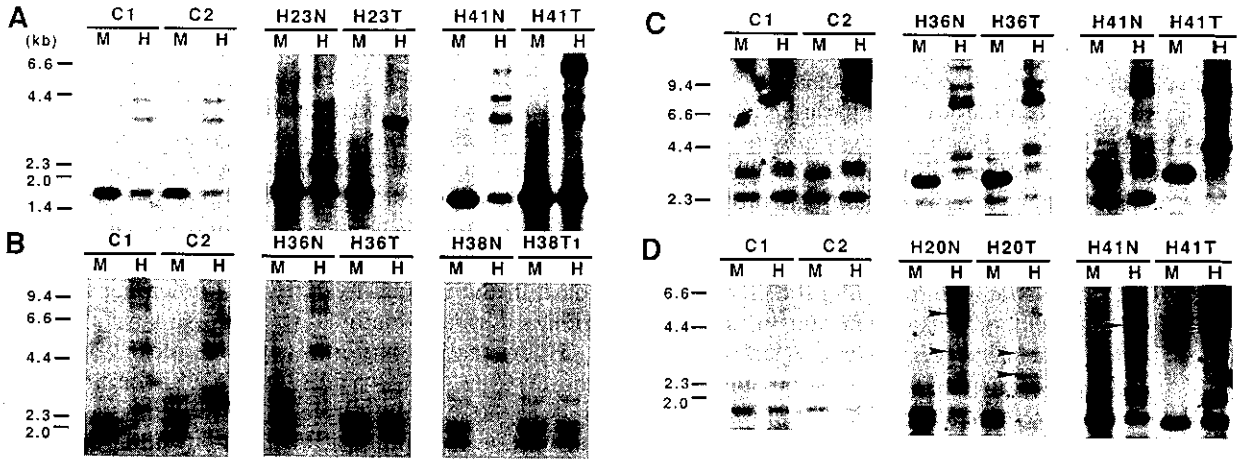

Fig. 1. CpG methylation on chromosome 16 in primary HCCs and in chronic hepatitis and liver cirrhosis. Normal liver tissues without chronic hepatitis or liver cirrhosis (C1 and C2), as well as cancerous tissues (T) and corresponding tissues showing chronic hepatitis and liver cirrhosis (N) of primary HCC cases (H20, H23, H36, H38 and H41) were examined. High-molecular-weight DNA was digested with either *Msp* I (M) or *Hpa* II (H), subjected to electrophoresis, transferred to nitrocellulose membranes, and hybridized with DNA probes for the D16S32 (A), D16S4 (B), TAT (C) and D16S7 (D) loci. A, Multiple bands with larger size, which were not obvious in the C1 and C2 digests, appeared in the *Hpa* II digest of H23N. In the *Hpa* II digest of H23T, the intensity of the 1.8 kb band, which was a major band in the C1 and C2 digests, was decreased and that of a band with larger size was increased. Although the *Hpa* II pattern of H41N seemed to be the same as those of C1 and C2, the intensity of the bands with larger size was increased in H41T. The *Msp* I digests of H23 and H41 indicated that differences in the *Hpa* II patterns of H23N, H23T and H41T compared to those of C1 and C2 were not simply due to a polymorphism. DNA from H23N, H23T and H41T was hypermethylated at the D16S32 locus. B, Although the *Hpa* II patterns of H36N and H38N seemed to be the same as those of C1 and C2, 1.8 and 2.1 kb bands, which were not detected in *Hpa* II digests of C1, C2, H36N and H38N, were seen in the *Hpa* II digests of H36T and $H38T_1$. DNA from HCC samples of both cases was hypomethylated at the D16S4 locus. C, In H36N, H36T and H41T, 2.4 and 3 kb *Hpa* II-digested bands, which were characteristic of normal tissues, were reduced and the intensity of the bands with larger size was increased. Although the 2.4 and 3 kb bands remained, the intensity of the bands with larger size was increased in H41N. DNA from H36N, H36T, H41N and H41T was hypermethylated at the TAT locus. In lane M of H41T, the absence of the 2.4 kb band may be due to the loss of one allele at the TAT locus, although analysis using HP 0.4 failed to detect LOH in this case (Fig. 2). D, In lane H of H20T, a 1.8 kb band, which was one of the major bands in C1 and C2, was absent. In lane H of H20N, H20T, H41N and H41T, the intensity of the bands with larger size (arrowheads) was increased. DNA from H20N, H20T, H41N and H41T was hypermethylated at the D16S7 locus.

closed circles in Fig. 2) out of the 47 loci, in which HCC samples showed hypermethylation. Hypermethylation at the D16S32, TAT and D16S7 loci was not detected in chronic hepatitis or liver cirrhosis from cases which did not show hypermethylation in HCC samples. Hypomethylation at the D16S4 locus was not detected in chronic hepatitis or liver cirrhosis from any of the HCC cases.

LOH on chromosome 16 in HCCs The relationship between aberrant DNA methylation and LOH was examined. It was previously reported that no polymorphic bands were detected with the TAT probe, which revealed the highest incidence of aberrant DNA methylation in the present analysis, in DNA cut with nine different restriction enzymes.[39] Therefore, we used HP 0.4, a *Hin*d III-*Pst* I fragment of the TAT gene, as a polymorphic marker and informative polymorphic bands were obtained for 31 HCCs. In accordance with previously described criteria,[7] when the hybridization intensity of one allele in the cancerous tissue was markedly less than that of the other allele, taking into consideration the ratio of intensity of the two alleles in the corresponding non-cancerous tissue, it was judged as indicating LOH* (Fig. 3). LOH at the TAT locus was detected in 13 (42%, shown by obliques in Fig. 2) out of 31 informative HCCs. In 12 of 13 HCC cases with LOH at the TAT locus, hypermethylation at the same locus was detected not only in HCCs, but also in chronic hepatitis and liver cirrhosis (Fig. 2).

* Although incomplete loss of DNA fragment indicating one allele is usually attributed to DNA from contaminating non-cancerous cells, the possibility of allelic gain is not completely excluded. Therefore, some investigators prefer to use the term allelic imbalance instead of LOH.[43, 44]

DISCUSSION

The present findings imply that changes in DNA methylation are significant in hepatocarcinogenesis, since: (a) DNA hypermethylation on chromosome 16 occurred even in chronic hepatitis and liver cirrhosis, which are considered to be precancerous conditions and (b) aberrant DNA methylation was detected more frequently in advanced HCCs than in early HCCs.

We previously reported that LOH on chromosome 16 is a late event in multistage hepatocarcinogenesis.[7,8] However, gene alterations associated with LOH on chromosome 16 in HCCs have not been identified to date. Hypermethylation on 17p is associated with LOH on the same chromosome region in human cancers.[24–27] Therefore, we assumed that aberrant DNA methylation on chromosome 16 may have significance in hepatocarcinogenesis. The present study shows that the D16S32, TAT and D16S7 loci are hot spots of DNA hypermethylation in HCCs, and that the D16S4 locus is frequently hypomethylated in HCCs. The incidence of aberrant DNA methylation in advanced HCCs is significantly higher than that in early HCCs, indicating that aberrant DNA methylation on chromosome 16 may play a role in the progression of HCCs.

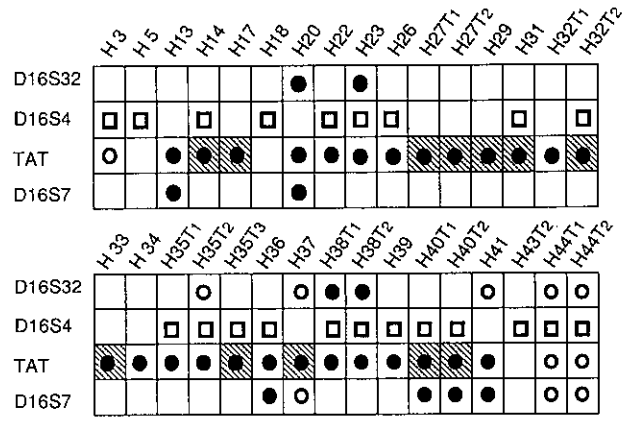

Fig. 2. Map of the regions showing aberrant DNA methylation in primary HCC cases. Thirty-two HCC lesions in which aberrant DNA methylation was detected at one or more of the four loci examined are presented. Hypermethylation of DNA from cancerous tissues (open circles) and from both cancerous and corresponding cirrhotic tissues (closed circles), and hypomethylation of DNA from cancerous tissues (open squares), are indicated. No case showed hypomethylation of DNA from corresponding cirrhotic tissues. Simultaneous detection of hyper- or hypo-methylation patterns at different chromosomal loci in the same DNA samples of most cases excludes the possibility of incomplete *Hpa* II digestion. LOH at the TAT locus is indicated by obliques. Informative polymorphic bands were not obtained in H5, H13, H18, H20, H22, H36, H38 and H39 out of these 32 HCCs in analysis of LOH at the TAT locus.

Although genetic alterations that accumulate during multistage hepatocarcinogenesis have been well described,[5–16] few significant events of the earlier stages are known. Perhaps the most striking finding of the present study is that frequent hypermethylation at the D16S32, TAT and D16S7 loci, compared to normal liver tissues without chronic hepatitis or liver cirrhosis, was detected even at the stage of chronic hepatitis and liver cirrhosis. Since the molecular weight of *Hpa* II-digested DNA fragments in HCCs is higher than that in precancerous conditions and the intensity of bands with larger size is increased in HCCs compared with precancerous conditions, as shown in Fig. 1A, the degree of methylation seems to increase further during the progression from a precancerous condition to an HCC in at least some cases. The detection of DNA hypermethylation in both the precancerous conditions and advanced HCCs suggests that this process might be involved in an early developmental stage of HCCs.

The hot spot of hypermethylation detected in the present study is in accordance with a previously reported hot spot of LOH on chromosome 16 in HCCs. Several aspects of our data suggested that hypermethylation might predispose the locus to allelic loss, although hypermethylation may not be the only cause of LOH. Firstly, the incidence of hypermethylation at the TAT locus (58%) exceeded that of LOH (42%). Secondly, DNA hypermethylation was observed in chronic hepatitis and liver cirrhosis, in which LOH was not detected. Most cases with LOH in HCCs also showed hypermeth-

Table I. Incidence of Aberrant DNA Methylation on Chromosome 16 in HCCs

Marker locus	Localization	Number of tumors		
		Analyzed	Hypermethylation detected (%)	Hypomethylation detected (%)
D16S32	pter-p13	50	9 (18)	0 (0)
D16S4	q22.1	44	0 (0)	21 (48)
TAT	q22.2	50	29 (58)	0 (0)
D16S7	q24.3	45	9 (20)	0 (0)

Table II. Association of Aberrant DNA Methylation on Chromosome 16 with Clinicopathological Parameters of HCCs

Parameter			Number of tumors			
			Analyzed	Hypermethylation[a] detected (%)	Analyzed	Hypomethylation[b] detected (%)
Early HCC[3,4]			10	1 (10)	9	1 (11)
Advanced HCC			42	28 (67)[c]	35	20 (57)[d]
Edmondson's grade	I[35]		13	2 (15)	11	3 (27)
	II		21	13 (62)[d]	17	8 (47)
	III		18	14 (78)	16	10 (63)
Portal vein involvement		negative	33	16 (49)	31	13 (42)
		positive	19	13 (68)	13	8 (62)
Intrahepatic metastasis		negative	33	16 (49)	29	13 (45)
		positive	19	13 (68)	15	8 (53)
HBV infection	negative		20	12 (60)	19	11 (58)
	positive		32	17 (53)	25	10 (40)
HCV infection	negative		18	8 (44)	17	6 (35)
	positive		29	18 (62)	23	15 (65)
	undetermined		5	3 (60)	4	0 (0)
Total cases			52	29 (56)	44	21 (48)

a) At any of D16S32, TAT and D16S7. *b*) At D16S4. *c*) $P<0.01$. *d*) $P<0.05$.

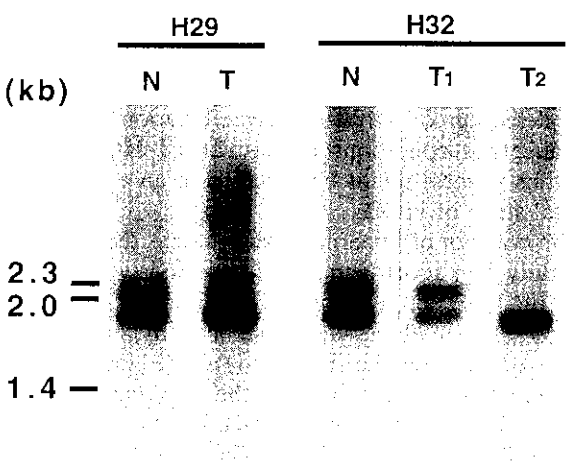

Fig. 3. LOH at the TAT locus in primary HCCs. High-molecular-weight DNA was digested with *Msp* I, subjected to electrophoresis, transferred to nitrocellulose membranes, and hybridized with the DNA probe, HP 0.4. LOH at the TAT locus was detected in H29T and H32T2, but not in H32T1.

ylation in chronic hepatitis and liver cirrhosis. Further study is needed to elucidate the relationship between aberrant DNA methylation and LOH at other chromosomal loci and in cancers from other organs.

Recently, it has been discovered that some tumor suppressor genes, including *RB*,[45] *VHL*,[46] *p15* [47] and *p16*,[48,49] and the E-cadherin invasion suppressor gene[50] are inactivated by reduced expression due to CpG methylation. A new candidate tumor suppressor gene, *HIC-1*, has been isolated by molecular analysis of a DNA site which is hypermethylated in cancer cells.[51] Participation of hypermethylation on chromosome 16 in human hepatocarcinogenesis may not only predispose the chromosome to DNA instability and allelic loss, but also alter the transcription of specific genes in the affected chromosome region.

In addition to the hypermethylation at the three loci, CpG methylation at the D16S4 locus was frequently reduced, indicating that the DNA methylation state on chromosome 16 is regionally disturbed in HCCs. Moreover, activation of some oncogenes, including *ras*[29,52] and c-*myc*,[53] due to DNA hypomethylation has been reported. Hypomethylation at the D16S4 locus, by itself, may play a role in hepatocarcinogenesis by activating specific genes. Hypomethylation at the D16S4 locus was distinct from the hypermethylation at the three other loci examined and was not detected at the stage of chronic hepatitis and liver cirrhosis. However, this change was observed in an early HCC (H3 in Fig. 2), suggesting that hypomethylation in this region is also an early event, in contrast to the majority of previously reported genetic alterations in multistage hepatocarcinogenesis.

Recently, it was reported that a reduction in the DNA (cytosine-5) methyltransferase (EC 2.1.1.37) activity, due to heterozygosity of the DNA methyltransferase

gene and the DNA methyltransferase inhibitor 5-aza-deoxycytidine, resulted in suppression of Apc^{MIN}-induced intestinal neoplasia.[54] Correction of aberrant DNA methylation may provide a new strategy for suppressing the progression of multistage hepatocarcinogenesis in hepatitis virus carriers suffering from chronic hepatitis and liver cirrhosis. Moreover, a few cases of spontaneous regression of human cancers have been reported.[55] Some step of carcinogenesis involving non-mutational genetic alterations may be reversible. Aberrant DNA methylation might become a target for cancer prevention and therapy designed to reverse certain steps of multistage carcinogenesis.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for the Second-Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.

(Received September 30, 1996/Accepted October 18, 1996)

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