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Hyper-methylation of *RIZ1* tumor suppressor gene is involved in the early tumorigenesis of hepatocellular carcinoma

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Summary. The retinoblastoma protein-interacting zinc finger gene *RIZ1* is a putative tumor suppressor gene, and the inactivation of the *RIZ1* is frequently found in tumors through a loss of mRNA expression. In order to understand the role of *RIZ1* inactivation in the tumorigenesis of hepatocellular carcinoma (HCC), we detected the *RIZ1* promoter methylation status in 39 HCCs using a methylation specific PCR (MSP) method, and carried out LOH study with marker P704. We also assessed the associations between the methylation status and clinicopathological parameters, tumor size, tumor differentiation, and fractional allelic loss (FAL). The results showed that the *RIZ1* promoter methylated both in advanced tumors (>3 cm), (18/31, 58.0%) and in early tumors (<3 cm), (4/8, 50.0%). There were 54.6% (12/22) tumors with hyper-methylation in the low FAL group and 45.5% (10/22) in the high FAL group. Moreover, the DNA methylation of the *RIZ1* promoter was found not only in the poorly differentiated tumors (12/22, 54.6%), but also in the well differentiated tumors (10/22, 45.5%). Among the 22 HCCs (22/39, 56.4%) that showed hyper-methylation at the *RIZ1* promoter region, 3 cases showed biallelic methylation. Interestingly, one case showed hyper-methylation on one allele and a loss of heterozygosity (LOH) on the other allele. In other words, 4 HCCs showed the biallelic inactivation of the *RIZ1*. These results suggest that the inactivation of the *RIZ1* by DNA methylation at its promoter region is involved in the tumorigenesis of HCC, particularly in the early stage of disease.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common human cancers in Asia and Africa (Simonetti et al., 1991). However, the molecular mechanisms of hepatocarcinogenesis are largely unknown. Recent reports have shown that there is a frequent LOH on chromosomes 1p, 4q, 6q, 8p, 10q, 13q, 16p, 16q, 17p, and gain on chromosomes 1q, 5p, 5q, 6p, 7q, 8q, 17q, and 20q in HCC (Piao et al., 1998a,b, 1999a,b; Fang et al., 2000; Chang et al., 2002; Zhang et al., 2005; Midorikawa et al., 2006). It has also been reported that a putative tumor suppressor gene, *RIZ1*, is located within the center of minimal deleted region at 1p (Fang et al., 2000, 2001). It raises the possibility that *RIZ1* may be a candidate of the tumor suppressor genes (TSG) at this region. The recent observation of frequent frameshift mutations of the *RIZ1* in gastrointestinal tumors with microsatellite instability (MSI) has provided strong evidence that the *RIZ1* may play a role as a TSG in these tumors (Chadwick et al., 2000; Piao et al., 2000a; Tokumaru et al., 2003). However, the mutational analysis with primary tumors without MSI phenotype could not reveal any mutations that inactivate *RIZ1*'s function (Fang et al., 2000). Recently, a study of the *RIZ1* promoter region has shown that a hyper-methylation at its CpG islands is associated with gene silence in the tumor cell lines and some primary tumors (Du et al., 2001). However, previous studies did not answer some critical questions such as 1) Is DNA methylation at promoter regions of *RIZ1* involved in

most HCCs? 2) Is it involved in the early stage of tumorigenesis or does it contribute to the tumor progression? 3) What is the tumor biology of hyper-methylation of the *RIZ1* gene? To further evaluate the role of the *RIZ1* inactivation in the tumorigenesis of HCC, we detected the *RIZ1* promoter methylation status in 39 HCC by using a MSP assay and LOH of *RIZ1* with P704. We also assessed the associations between the methylation status and the clinico-pathological parameters including tumor size, tumor differentiation, and FAL.

Materials and methods

Tissues and DNA extraction

A total of 39 surgically resected primary hepatocellular carcinomas from Yonsei University College of Medicine, Seoul, Korea were included in this study: 26 (66.7%) cases were positive for HbsAg and 2 (5.1%) cases were positive for anti-HCV Ab; 8 cases (20.5%) were small HCCs (<3 cm) and 31 (79.5%) cases were advanced (>3 cm) HCCs; 23 (59.0%) HCCs had cirrhosis; 20 (51.3%) HCCs were well differentiated (grade I to II), and 19 (48.7%) HCCs were poorly differentiated (grade III to IV), according to the Edmondson and Steiner (Edmondson and Steiner, 1954) grading system. The cell lines used in this study were obtained from the American Type Culture Collection (ATCC). Genomic DNA was extracted as described previously (Piao et al., 1998a).

DNA methylation analysis

It has been demonstrated that a hyper-methylation of the specific CpG islands at the promoter regions is associated with the *RIZ1* silenced (Du et al., 2001). Therefore, the results of methylation specific PCR would indirectly indicate the expression status of the *RIZ1* in a tested sample. For this experiment, genomic DNA was treated with bisulfite according to the procedure described by Herman et al. (1996) and Du et al. (2001). We used the primer set RP291MF (5'-TGG TGG TTA TTG GGT GAT GGT-3') and PR291MR (5'-GCT ATT TCG CCG ACC CCG ACG-3') for the amplification of the methylated DNA. For the amplification of unmethylated DNA we used the primer set RP291UF (5'-TGG TGG TTA TTG GGT GAT GGT-3') and RP291UR (5'-ACT ATT TCA CCA ACC CCA AGA-3'). PCR was performed as described previously (Du et al., 2001).

Detection of LOH at RIZ1 locus

In order to detect LOH at the *RIZ1*, we used the PCR-LOH method with P704 marker. The primers for this marker were P704-F (5'-GGC AGT GTC TTT CCA CAA AG-3') and P704-R (5'-CTT GAG GTC ACA GGC AAC AT-3'). PCR was performed as described previously (Fang et al., 2001).

Assessment of fractional allelic losses (FAL)

FAL is one of the valuable parameters that show the extent of genomic damage in the tumor cells. For the evaluation of the value of FAL in each tumor, we detected LOH with the following microsatellite markers: D4S1545 (4q), D4S2920 (4q), D8S264 (8p), D8S1752 (8p), D16S498 (16q), D16S514 (16q), and p53 (17p). These markers showed a high LOH frequency in our previous HCC studies (Piao et al., 1998a,b, 1999a,b; Chang et al., 2002) and we believed that a combination LOH at these loci would represent a relevant data of genomic damage index in HCC. PCR reactions and LOH interpretation were carried out as described previously (Piao et al., 2000b).

Statistical analysis

Methylation status was assessed for associations with clinicopathological parameters, including HBV infection, cirrhosis, tumor size, tumor differentiation and FAL. All the analyses were performed using the SAS software.

Results

Methylation of the RIZ1 promoter in tumor tissues with methylation specific PCR and LOH at RIZ1 locus

A methylation specific PCR method has been previously developed to distinguish methylated and unmethylated DNA at *RIZ1* promoter regions (Du et al., 2001). The specific CpG islands methylation used in the primers for MSP are associated with *RIZ1* silencing. Therefore, the detection of methylated PCR products by MSP is an approach to assess the expression of the *RIZ1*. Previously, it has been observed that the tumor cell lines HepG2 and Huh1 are hyper-methylated at the CpG island of the *RIZ1*, whereas MCF7 and HCT116 are unmethylated (Du et al., 2001). Therefore, we used HepG2 and Huh1 as methylation positive controls and MCF7 and HCT116 for the negative controls for each experiment. We detected a hyper-methylation of *RIZ1* in 22 out of 39 HCCs (56.4%). Among 8 small tumors (<3 cm), 50.0% (4/8) of the cases showed hyper-methylation, whereas 58.0% (18/31) of cases showed hyper-methylation in the large tumors (>3 cm). Among 22 cases that showed hyper-methylation of *RIZ1*, 3 cases (13.6%, case # 12, 19, and 28) showed a biallelic methylation pattern and the remainder showed a mono-allelic methylation pattern. To evaluate the status of the other allele in cases that showed mono-allelic methylation in the *RIZ1* gene, we observed the LOH in tumors with the P704 marker. Interestingly, one case (case # 16) showed hyper-methylation on one allele and a loss of heterozygosity (LOH) on the other allele with the P704 marker (Fig. 1). Among 4 cases that showed a

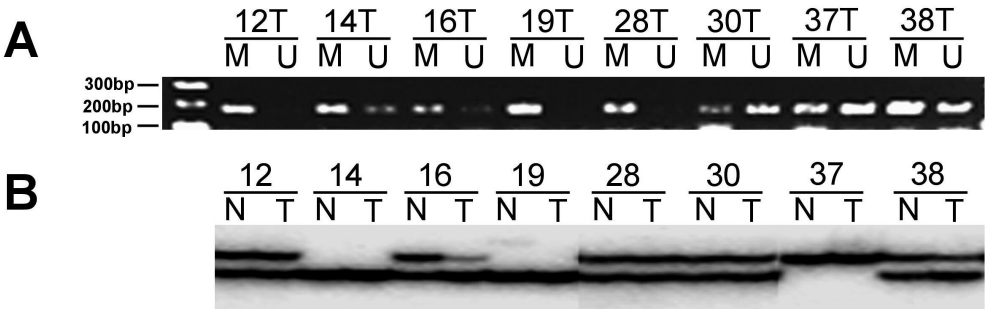

Fig. 1. MSP of *RIZ1* gene and LOH with P704. **A.** MSP analysis with primary HCC. Case numbers are shown at the top with matched DNA methylation (M) and unmethylation (U) PCR products. A biallelic hyper-methylation of RIZ1 was detected in tumors 12, 19, and 28 and the rest of the cases showed a monoallelic methylation. **B.** LOH with marker P704. Case numbers are shown at the top with matched normal (N) and tumor (T). The tumor of case 16 showed LOH with marker P704. This tumor also had a monoallelic hyper-methylation with MSP analysis shown in figure A.

Table 1. Clinicopathological features of 39 HCCs with and without RIZ1 hyper-methylation.

Hypermethylation of RIZ1	Tumor Size (cm)		Differentiation[a]		FAL[b]	
	<3	>3	Well	Poor	<0.563	>0.563
Present	4 (10%)	18 (46%)	10 (26%)	12 (31%)	12 (31%)	10 (26%)
Absent	4 (10%)	13 (33%)	9 (23%)	8 (21%)	8 (21%)	9 (23%)

[a]Differentiation: tumor differentiation; Well: grade I-II, Poor: grade III-IV according to the Edmondson and Steiner grading system (Edmondson and Steiner, 1954); [b]FAL: fractional allelic loss; FAL for each tumor was defined as the number of markers with LOH divided by the number of informative markers. The markers used were D4S1545, D4S2920, D8S264, D8S1752, D16S498, D16S514, and p53 (some of these data have been presented in the paper Piao, et al., 1997, 1998b, 1998c, 1999a, 1999b, 2000a,b).

biallelic inactivation of the *RIZ1*, 3 cases were well differentiated HCC.

FAL in HCCs

Together with our previous studies (Piao et al., 2000b), an overall FAL in each of these tumors was calculated on the basis of the combined LOH status with microsatellite markers D16S498, D16S514, D4S1545, D4S2920, D8S264, D8S1752, and p53. FAL for each tumor was defined as the number of markers with LOH divided by the number of informative markers examined.

Association between Hyper-methylation of RIZ1 and clinico-pathological parameters

For the evaluation of the biological significance of hyper-methylation of the *RIZ1* in the hepatocarcinogenesis, we assessed the associations between the hyper-methylation status of RIZ1 and clinico-pathological parameters. The results showed that the RIZ1 promoter hyper-methylated both in the advanced tumors (>3 cm), (18/22, 81.8%) and in the early tumors (<3 cm), (4/22, 18.2%). Moreover, the DNA methylation of the RIZ1 promoter was found not only in the poorly differentiated tumors (12/22, 54.6%), but also in the well differentiated tumors (10/22, 45.5%) (Table 1). For the assessment of association between methylation status and FAL, we calculated the mean of FAL in 39 HCCs to be 0.563. All cases were then divided into two groups: low FAL (FAL<0.563) and high FAL (FAL>0.563). When the cases were divided into these two groups according to the FAL score, there were 54.6% (12/22) tumors with hyper-methylation in the low FAL group and 45.5% (10/22) in the high FAL group (Table 1). No associations between hyper-methylation of RIZ1 and HBV infection and cirrhosis were found.

Discussion

Recently, frequent frameshift mutations of the *RIZ1* have been observed in gastrointestinal tumors with microsatellite instability (MSI) (Chadwick et al., 2000; Piao et al., 2000a; Tokumaru et al., 2003). These types of mutations would eliminate the carboxy-terminal region of the *RIZ1* product that has a protein-binding activity towards the PR domain of *RIZ1*. Thus, the mutations would result in a disruption of its normal function. In addition, a mouse gene-knockout model has shown that mice with *RIZ1* inactivation are susceptible to tumors compared with a control group (Steele-Perkins et al., 2001). These data strongly indicate that *RIZ1* could be a tumor suppressor gene. However, despite the fact that *RIZ1* has been found within the minimal deleted region at 1p36 in HCC, no somatic mutations that inactivate the *RIZ1* have been observed so far in primary tumors without the MSI phenotype (Fang et al., 2000, 2001). This raises the possibility that there is another

mechanism of inactivation of the *RIZ1* in the primary tumors without the MSI phenotype if *RIZ1* plays a role as one of the putative tumor suppressor genes in these tumor types. A study of the *RIZ1* promoter region has shown that the *RIZ1* promoter is highly methylated in the cell lines without *RIZ1* expression, and un-methylated in cell lines with *RIZ1* expression. Moreover, a 5-Aza-dC treatment of those *RIZ1* hyper-methylated cell lines, which do not express *RIZ1*, allow the cell lines to re-express the *RIZ1*, suggesting that a hyper-methylation of the *RIZ1* promoter region is the mechanism of down regulation of the *RIZ1* (Du et al., 2001). Later the specific CpG islands that are associated with down regulation of the *RIZ1* are identified (Du et al., 2001). These specific CpG islands are very useful for the design of the primers for the MSP assay of the *RIZ1*. A preliminary study with the MSP assay in breast and liver cancers has shown that hyper-methylation of *RIZ1* is involved in these tumor types (Du et al., 2001). But whether hyper-methylation of *RIZ1* is involved in most HCCs and whether it plays a role in early or later stage of tumorigenesis remains to be determined. To address these issues, we carried out a MSP study with 39 primary HCCs. The result showed that 22 out of 39 HCCs (56.4%) showed DNA hyper-methylation at the promoter region of *RIZ1*. Among the 22 HCCs (22/39, 56.4%) that showed hyper-methylation at its promoter region, 3 cases showed biallelic methylations. Interestingly, one case showed a hyper-methylation on one allele and a loss of heterozygosity (LOH) on the other allele. In other words, 4 HCCs showed a biallelic inactivation of the *RIZ1*. These results suggest that the inactivation of the *RIZ1* by the DNA methylation at its promoter region is involved in the tumorigenesis of HCC.

It has been postulated that tumorigenesis is a multiple process in which it reflects the genetic alterations that drive the transformation of normal cells into tumor cells. Furthermore, the accumulation of genetic alterations in the process contributes to tumor progression and metastasis (Piao et al., 1997; Fodde et al., 2001). For the evaluation of the accumulated genetic alterations in a tumor, a variety of methods have been used, including FAL and the genomic damage index (Piao et al., 1997, 1998a, 2000b). In the allelotype studies FAL has been widely used for the evaluation of accumulated genomic damage in different tumor types, including in HCC (Piao et al., 1997, 1998a, 2000b). In this study we used the microsatellite markers, which have showed a high frequency of LOH in our previous deletion mapping studies in HCC, for the evaluation of FAL in a tumor. A combination of LOH results at these loci in a tumor represents a relevant picture of genomic damage index in each tumor in our collection. When 39 HCCs were divided into two groups according to the FAL score (FAL<0.563 and FAL>0.563), there were 54.6% (12/22) tumors with hyper-methylation in the low FAL group and 45.5% (10/22) in the high FAL group. More than half of the tumors with low FAL exhibited *RIZ1* hyper-methylation, indicating that the *RIZ1* is inactivated before the occurrence of an extensive genomic damage, suggesting that the inactivation of *RIZ1* might play a role in the early stage of malignant transformation. On the other hand, 45.0% of high FAL tumors showed *RIZ1* hyper-methylation, suggesting that the *RIZ1* remains in its inactivation state during the process of tumor progression. This interpretation is also supported by the observation that the DNA methylation of the *RIZ1* promoter region was found not only in the poorly differentiated tumors (54.6%), but also in the well differentiated tumors (45.5%). This notion is also strengthened by the results that the *RIZ1* promoter is equally hyper-methylated in both the advanced tumors (>3 cm), (58.0%) and in the early tumors (<3 cm), (50.0%). Moreover, 3 out of 4 cases that showed the biallelic inactivations of the *RIZ1* belong to the well differentiated group of HCCs. Taken together our results strongly suggest that the inactivation of the *RIZ1* plays a role in the hepatocarcinogenesis, particularly when it is involved in the early stage of malignant transformation from normal to tumor cells.

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