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Original Research

GESDAV

Hypomethylation of long interspersed nuclear element-1 is involved in the early tumorigenesis of hepatocellular carcinoma

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ABSTRACT

Objective: Hypomethylation of long interspersed nucleotide elements 1 (LINE-1) promoter has been reported in many cancer types including pancreatic endocrine tumors, colon cancers, and stomach carcinomas. Hypomethylation of LINE-1 is also frequently observed in hepatocellular carcinoma (HCC). However, it still unknown how LINE-1 is involved in the hepatocarcinogenesis. **Methods:** The level of LINE-1 promoter methylation in 28 HCC was detected by a methylation specific polymerase chain reaction (MSP) at CpG site seven of LINE-1 promoter region (gene bank: X58075). A fractional allelic loss (FAL) of these tumors was evaluated by a combination of loss of heterozygosity with microsatellite markers D4S1545, D4S2920, D8S264, D8S1752, D16S498, D16S514, and p53. The associations between the level of LINE-1 hypomethylation status and the clinico-pathological parameters were finally observed. The clinicopathological parameters were included hepatitis B virus (HBV) status, cirrhosis, tumor size, tumor differentiation and FAL. **Results:** High level of hypomethylation of LINE-1 promoter was found both in the advanced tumors (>3 cm), (7/18, 39%) and in the early tumors (<3 cm), (6/10, 60%). Moreover, the high level of deoxyribonucleic acid hypomethylation at the LINE-1 was found in both poorly differentiated tumors (8/13, 61.5%) and well-differentiated tumors (5/15, 33.3%). The mean value of FAL in 28 HCCs was 0.535. All cases were divided according to FAL score: Low FAL (FAL < 0.535) and high FAL (FAL > 0.535). There were 41% (7/17) tumors with a high level of hypomethylation in the low FAL group and 54.5% (6/11) tumors with a high level of hypomethylation in the high FAL group. No associations between the level of hypomethylation of LINE-1 and HBV infection, age, sex, and cirrhosis were found. **Conclusions:** These results are strongly suggested that the hypomethylation of LINE-1 plays a role in the hepatocarcinogenesis; moreover, the hypomethylation of LINE-1 occurs not only in the progression of HCC, but also in the early stage of HCC tumorigenesis.

KEY WORDS: Global hypo-methylation, hepatocellular carcinoma, long interspersed nuclear element-1

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human cancers and a leading course of death in Asia and Africa [1,2]. It has been shown that chromosomal instability, but not microsatellite instability, is associated with HCC tumorigenesis [3-5]. However, the molecular mechanisms of hepatocarcinogenesis remain largely unknown [6].

Global hypomethylation of cytosine residues in the promoter regions has been found in various tumor types [7,8]. One of the frequently analyzed genes for global hypomethylation is a long interspersed nucleotide elements 1 (LINE-1), because it is the most abundant in the genome and makes up about 17% of genome [9-11]. It has been postulated that the hypomethylation of LINE-1 could re-express of this gene and, which in turn, may change the expression level of oncogenes and may facilitate chromosomal instability in various tumors [12-16]. Moreover, a hypomethylation of LINE-1 is consistently observed in many cancers including ovary, prostate, pancreatic, colonic, and stomach carcinomas [17-21]. These observations suggest that the LINE-1 hypomethylation is one of the common events in variety of cancer types and may reflect global hypomethylation of tumor deoxyribonucleic acid (DNA). Furthermore, it has been reported that LINE-1 hypomethylation was associated with poor prognosis in colorectal cancer [19], leukemia [20], ovarian cancer [17], and non-small cell lung cancer [21] patients. In HCC, the LINE-1 hypomethylation has been also observed [22-24]. In addition, serum LINE-1 hypomethylation is reported to correlate with hepatitis B-surface antigen (HBsAg) status, large tumor size, and advanced tumor stage; therefore, it could be used as a poor prognostic marker for HCC [23,24].

However, the previous studies did not answer some critical questions such as (1) Is hypomethylation at promoter regions of LINE-1 involved in the early stage of tumorigenesis or does it contribute to the tumor progression? (2) What is the relationship between the level of hypomethylation of the LINE-1 and chromosomal instability? To further characterize the role of the hypomethylation of LINE-1 in the tumorigenesis of HCC, we detected the LINE-1 promoter methylation status and fractional allelic losses (FAL) in 28 HCC. We also assessed the associations between the level of LINE-1 hypomethylation status and the clinic pathological parameters including hepatitis B virus (HBV) status, cirrhosis, tumor size, tumor differentiation, and FAL.

MATERIALS AND METHODS

Tissues and DNA Extraction

A total of 28 surgically resected primary HCC from Yonsei University College of Medicine, Seoul, Korea, were included in this study. There were 5 (18%) HCCs from female patients, and 23 (82%) were from male patients. The age of patients was arranged from 17 to 66 years old (mean: 49.7). 19 (68%) patients were positive for HbsAg; 2 (7%) patients were negative for HbsAg; and 7 (25%) patients were with unknown etiology. 10 (36%) cases had small HCCs (tumor size <3 cm) and 13 (46%) patients had advanced HCCs (>3 cm). 18 (64%) HCCs had Cirrhosis. 15 (54%) HCCs were well differentiated (Grade I-II), and 13 (46%) HCCs were poorly differentiated (Grade III-IV), according to the Edmondson and Steiner [25] grading system. The clinicopathological features of each tumor have shown in Table 1. Genomic DNA was extracted as described previously [26].

Table 1: Clinicopathological features, methylation of LINE-1, and FAL in 28 HCCs

Number	Sex	Age	Cirrhosis	Size	HBV	DF	ME-L1	FAL
1	M	61	+	12.0		W	82.70	0.17
2	M	41	+	8.0	+	P	63.25	1.00
3	F	62	+	1.2	+	W	77.19	0.40
4	M	49	-	15.0	+	P	65.60	1.00
8	M	50	+	6.5	+	W	60.00	0.17
10	M	51	+	12.0	+	P	45.03	0.83
11	M	66	-	9.0	+	P	66.84	1.00
13	M	42	+	5.0	+	W	71.20	0.00
14	M	38	+	3.5	+	P	56.91	0.17
17	M	51	+	3.5	+	P	33.75	1.00
19	M	66	+	5.5	-	W	41.70	0.50
20	M	63	+	2.0	-	P	46.86	0.40
21	F	25	-	5.0	+	W	79.70	0.33
22	M	41	-	2.3	+	W	63.70	0.40
23	M	54	-	2.0		W	53.73	0.57
24	M	49	+	2.0	+	P	50.58	0.80
25	M	44	+	1.2		W	59.62	0.40
26	M	63	-	2.5	+	P	40.00	0.50
27	M	46	+	2.5		W	56.69	0.50
28	M	59	-	6.0		W	58.64	0.14
29	F	63	+	3.0	+	W	64.79	0.33
33	M	53	+	3.5	+	W	53.51	0.50
34	F	51	+	8.0	+	P	23.26	0.60
35	M	50	-	14.0	+	W	63.20	1.00
36	F	54	-	8.0		P	57.33	0.60
37	M	43	-	2.5		W	36.24	0.50
38	M	39	+	4.0	+	P	73.15	0.17
39	M	17	+	4.0	+	P	69.43	1.00

DF: Tumor differentiation, W: Grade I-II, P, Grade III-IV according to the Edmondson and Steiner grading system, ME-L1, The percentage of methylation at promoter CpG site seven of LINE-1, The percentage of methylation in a sample was calculated by using a formula: Methylated reaction divided by unmethylated reaction plus methylated reaction×100%. FAL for each tumor was defined as the number of markers with loss of heterozygosity divided by the number of informative markers. The markers used were D4S1545, D4S2920, D8S264, D8S1752, D16S498, D16S514, and p53, LINE-1: Long interspersed nucleotide elements 1, FAL: Fractional allelic loss, HCCs: Hepatocellular carcinomas, HBV: Hepatitis B virus

DNA Methylation Analysis

It has been shown that a hypomethylation of the CpG site of LINE-1 at the promoter region, particularly at CpG site seven, which is the number 172 nucleotide of LINE-1 promoter (gene bank: X58075), is associated with the LINE-1 re-expression [27]. Therefore, the results of methylation specific polymerase chain reaction (PCR) (MSP), which detects specific methylation status at this CpG site, would indirectly indicate the expression status of the LINE-1 gene in a tested sample. For this experiment, genomic DNA was treated with bisulfite according to the procedure described by Herman *et al.* [28] and Du *et al.* [29]. We used the primer set LINE-1-7MF (5′- AGGAATAGTTTYGGTTTATAG-3′) and LINE-1-7MR (5′-ACAATACCTCRCCCTACTTCG-3′) for the amplification of the methylated DNA. For the amplification of unmethylated DNA, we used the primer set LINE-1-7UF (5′-AGGAATAGTTTYGGTTTATAG-3′) and LINE-1-7UR (5′-ACAATACCTCRCCCTACTTCA-3′). Real-time PCR of methylation level of CpG site seven was performed as described previously [27]. The percentage of methylation in a sample was

calculated using a formula: Methylated reaction divided by unmethylated reaction plus methylated reaction ×100% [27].

Analysis of loss of heterozygosity (LOH) using microsatellite markers and assessment of FAL.

FAL is one of the valuable parameters that indicate the extent of genomic instability in a tumor sample. To evaluate the actual value of FAL in each tumor, we selected the microsatellite markers that showed a high frequency of LOH in our previous studies [4,26,30,31]. We believed that a combination LOH at these loci, calculated as the FAL in a tumor sample, would represent a relevant index of the chromosome instability in each tumor. These microsatellite markers included D4S1545 (4q), D4S2920 (4q), D8S264 (8p), D8S1752 (8p), D16S498 (16q), D16S514 (16q), and p53 (17p). To evaluate the value LOH in p53 region, we used two markers reported previously (31). PCR reactions and LOH interpretation were carried out as described previously [32]. Briefly, a hot-start PCRs were performed in a mixture of 20 μl volume containing 1.5 mM magnesium chloride, 50 ng of sample DNA, PCR buffer and 1.25 unit Taq polymerase (GIBCO-BRL, Grand Island, NY). There is 20 pmol each oligonucleotide primer, 0.2 mM each dATP, dGTP, dTTP, 5 μM dCTP, and 1 μCi a-^{32} P-dCTP (3000 Ci/mmol; NEN DuPont, Boston, MA) in each PCR reaction. PCR was performed for 25 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and elongation at 72°C for 15 s. PCR products were separated by electrophoresis in the 6% urea-polyacrylamide gels, followed by autoradiography. LOH was scored when the band intensity of one allelic marker was significantly decreased (more than 50% reduction) by visual inspection in tumor DNA compared with normal DNA [33].

Statistical Analysis

The level of LINE-1 hypomethylation was assessed for associations with clinicopathological parameters, including HBV infection, cirrhosis, tumor size, tumor differentiation and FAL. All the analyzes were performed using the SPSS software Version 18.0 (Chicago, USA; SPSS Inc.), and significance of associations was determined using Fisher's exact test.

RESULTS

Methylation of the LINE-1 promoter in tumor tissues with PCR MSP.

A methylation specific PCR method has been previously developed to distinguish methylated and unmethylated DNA at LINE-1 promoter regions at CpG site 7 [27]. Hypomethylation at CpG site 7 of LINE-1 gene is correlated with the re-expression of this gene in our previous study, particularly a dramatically LINE-1 expression is observed when the methylation level of CpG site 7 < 68% [27]. Therefore, the detection of methylated PCR products by MSP is an indirect approach to assess the expression level of the LINE-1. We detected that 25 (89.3%) cases showed <68% of methylation at CpG site 7 among 28 HCCs. The mean methylation level at CpG site 7 of LINE-1 in 28 HCCs is 57.67 (range: 23.26-82.7). The hypomethylation level of each tumor at CpG site 7 of LINE-1 has shown in the Table 1. These observations are consistent with previous founding that there are frequent hypomethylation of LINE-1 in human HCCs compared with surrounding liver tissue [22].

FAL in HCCs

Together with our previous studies [3], an overall FAL in each of these tumors was calculated on the basis of a 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. The FAL value of each tumor has shown in Table 1. Representative results of the LOH analysis are shown in Figure 1.

Association between the level of the LINE-1 hypomethylation and clinicopathological parameters.

To evaluate the biological significance of hypomethylation of the LINE-1 in the hepatocarcinogenesis, we assessed the associations between the level of LINE-1 hypomethylation and clinicopathological parameters. All tumors were divided into two groups according to the mean value of the 7th CpG site methylation level: A high level of hypomethylation group (<57.67) and a low level of hyormethylation group (>57.67). The results showed that the LINE-1 promoter hypomethylated both in the advanced tumors (7/18, 39%) and in the early tumors (6/10, 60%). Moreover, the DNA hypomethylation of the LINE-1 promoter was found not only in the poorly differentiated tumors (8/13, 61.5%), but also in the well differentiated tumors (5/15, 33.3%) [Table 2]. To evaluate the association between the level of hypomethylation status and FAL, we calculated

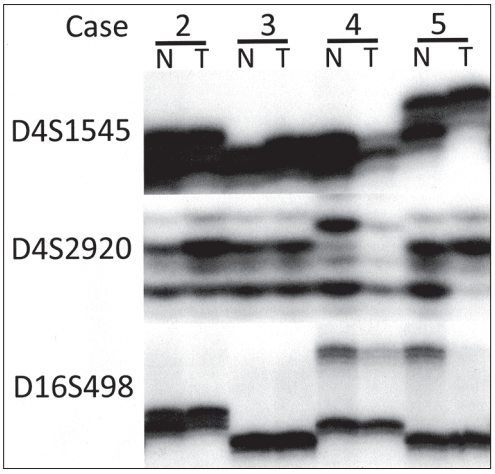

Figure 1: Representative autoradiographs of loss of heterozygosity (LOH). The tumor (T) and corresponding non-tumorous tissue (N) are shown with microsatellite markers indicated at the left. Case 5 is the positive control for LOH: Loss of the lower allele with markers D4S1545 and D4S2920; loss of upper allele with marker D16S498. Case 2: loss of lower allele with markers D4S1545, D4S2920, and D16S498. Case 3: retention of both allele with markers D4S1545 and D4S2920; not informative with marker D16S498. Case 4: loss of upper allele with markers D4S1545, D4S2920 and D16S498

Table 2: Clinicopathological characteristics of 28 HCCs with the level of LINE1 hypomethylation (n=28)

Hypomethylation of LINE-1	Tumor size (cm)*		Differentiation[a]**		FAL[b]***	
	<3	>3	Well	Poor	<0.535	>0.535
High level	6	7	5	8	7	6
Low level	4	11	10	5	10	5

[a]Differentiation: Tumor differentiation, Well: Grade I-II, Poor: Grade III-IV according to the Edmondson and Steiner grading system (Edmondson and Steiner, 1954), LINE-1: Long interspersed nucleotide elements 1, HCCs: Hepatocellular carcinomas, [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 *P=0.249, **P=0.133, ***P=0.380 (Fisher's exact test)

the mean of FAL in 28 HCCs to be 0.535. All cases were then divided into two groups: Low FAL (FAL < 0.535) and high FAL (FAL > 0.535). When the cases were divided into these two groups according to the FAL score, there were 41% (7/17) tumors with the high level of hypomethylation in the low FAL group and 54.5% (6/11) tumors with the high level of hypomethylation in the high FAL group [Table 2]. The results suggested that the high-level hypomethylation of LINE-1 occurred not only in the progression of HCC, but also in the early stage of HCC tumorigenesis. No associations between the level of LINE-1 hypomethylation and HBV infection, age, sex, and cirrhosis were found.

DISCUSSION

It has been demonstrated that CpG island methylation induces inhibition of LINE-1 promoter activity *in vivo* and *in vitro* [34]. It has also been reported that there is an inverse correlation between the amount of LINE-1 ORF1 product and methylation of the 5' end of LINE-1 [35]. Moreover, the inhibition of LINE-1 is thought to be strictly dependent on the position of methylation but not on the number of methylated CpG [34]. Couples of critical CpG sites for re-expression of LINE-1 transcription by DNA hypomethylation have been identified recently [34]. Our previous results indicated that the hypomethylation of at the 172 nucleotide (cytosine) on the LINE-1 promoter is associated with the re-expression of this gene [27]. Particularly, a significant re-expression of LINE-1 is observed when the methylation level of this CpG site is less than 68% in L02 cell line [27], which strongly indicates that hypermethylation of this specific CpG site is associated with down regulation of the LINE-1. This specific CpG site is very useful in designing primers for the MSP assay of the LINE-1. In another words, the detection of methylated PCR products by MSP is an indirect approach to assess the re-expression of the LINE-1 when the methylation level of this CpG site is <68% in a given DNA samples. Using this approach, we found 89.3% of HCCs showed <68% methylation at CpG site 7 in the LINE-1 promoter, which indicates that these tumors re-expressed the LINE-1 gene through an epigenetic pathway. This result is consistent with the previous findings that there is frequent hypomethylation of LINE-1 in human HCCs [22,23]. However, it is not clear how a hypomethylation of LINE-1 is involved in the hepatocarcinogenesis.

It has been proposed that the hypomethylation is present at different steps of carcinogenesis in different tumor types [23]. In ovarian tumors, it showed a stepwise increase in global hypomethylation among benign cystadenomas, low malignant potential tumors, and carcinomas [36]. On the other hand, tumors in the gastrointestinal tract showed a global hypomethylation at an earlier stage of carcinogenesis [20,21]. In HCC it has been suggested that global hypo-methylation may be promoting a disease progression via chromosomal instability, activation of protooncogenes, and reactivation of transposable elements [13,37]. In this study, a high level of LINE-1 promoter hypomethylation presented both in the advanced tumors and in the early tumors. Moreover, the high level of hypomethylation of the LINE-1 was found not only in the poorly differentiated tumors, but also in the well differentiated tumors. These data suggest that a hypomethylation of LINE-1 may be an early event in hepatocarcinogenesis and raise the possibility of utilizing LINE-1 methylation-based assays as potential biomarkers for the early detection of HCC, especially in high-risk populations, as has been proposed previously [22,38].

Chromosomal instability is the hall marker of the cancer cells and contributes to not only tumor initiation but also tumor progression and metastasis [32,39]. Many methods have been used to evaluate chromosomal instability in a tumor, including FAL and the genomic damage index [3,40]. In the allelotype studies, FAL has been widely used for the evaluation of chromosomal instability in different tumor types, including in HCC [32]. To evaluate FAL in a tumor, we used a set of microsatellite markers, which have shown a high frequency of LOH in our previous deletion mapping studies in HCC [26,30,31,41]. A combination of LOH results at these loci in a tumor represents a relevant value of chromosomal instability in each tumor in our collection. When HCCs were divided into two groups according to the mean FAL score, there were 41% tumors with a high level of hypomethylation in the low FAL group and 54.5% HCCs with a high level of hypomethylation within the high FAL group. Forty-one percent of HCCs with low FAL exhibited the high level of LINE-1 hypomethylation, indicating that the LINE-1 is re-expressed before the occurrence of an extensive chromosomal instability and suggesting that the re-expression of LINE-1 might play a role in the early stage of malignant transformation in the early hepatocarcinogenesis. On the other hand, 54.5% of high FAL tumors showed a high level of LINE-1 hypomethylation, suggesting that the LINE-1 remains in its re-expression state during the process of tumor progression. This interpretation is also strongly supported by the results that the high level of DNA hypomethylation of LINE-1 was found not only in the poorly differentiated tumors (61.5%), but also in the well differentiated tumors (33.3%). Furthermore, LINE-1 equally showed a high level of hypomethylated in both the advanced tumors (39%) and in the early tumors (60.0%). Our results are consistent with the observations in the colon and stomach cancers, in which LINE-1 hypomethylation was present at an earlier stage of carcinogenesis [20,21]; however, the results differ from the observations that LINE-1 hypomethylation is considered a poor prognostic marker involved in the tumor progression and aggressive tumor behavior in HCC [27,37,38] and other type of cancers [42-44]. This difference may be

in part due to the different type of markers of methylation, methodology, and criteria used in assessing hypomethylation of LINE-1.

Currently, serum alpha-fetoprotein (AFP) is the most widely used tumor marker for detecting patients with HCC in the United States. When AFP is used for screening of high-risk populations, a sensitivity of 39%, specificity of 76%, and a positive predictive value of 9% to 32% has been reported [45-49]. Moreover, AFP is not specific for HCC. Titers also rise in acute or chronic hepatitis, in pregnancy, and in the presence of germ cell tumors [50]. Therefore, it is imperative to find other markers with a higher sensitivity and specificity to early detect this disease. Previously it has been observed that when <68% of methylation at CpG site seven of LINE1 in a cell would lead to re-expression of LINE-1 [27]. Although there is only 60% of early HCC in the LINE-1 higher methylation group in our study, actually there are 25 out of 28 (89.3%) HCCs showed <68% of methylation at CpG site seven of LINE1, indicating that these tumors are expressing LINE-1 gene. In other words, the sensitive by using this test to detect HCC would be about 89.3%. A hypomethylation of LINE-1 is not specific for HCC tumorigenesis; however, patients with a long time history of hepatitis or cirrhosis, who are in the high risk populations for HCC, with hypomethylation of LINE1, may indicate underlying HCC. These patients need further clinical and radiological workup to rule out HCC. This approach is highly feasible due to the fact that LINE1 hypomethylation could be detected in the serum [24]. Our results are preliminary for the immediate clinical usage for this purpose. The early diagnostic significance of LINE-1 methylation observed in this study should be further validated in a prospective and large-scale clinical study in the future.

In summary, our results strongly suggest that a hypomethylation of LINE-1 plays a role in the hepatocarcinogenesis, particularly in the early stage of malignant transformation. This also raises the possibility that serum LINE-1 hypomethylation could be potentially used as an early diagnostic marker for HCC.

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