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Epigenetic Activation of Tumor Suppressor miRNAs in Gastric Carcinoma

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Abstract

Background: Epigenetic silencing of tumor-related genes by CpG island methylation is an important mechanism for the development of many tumors, including gastric carcinoma. We examined the presence of DNA methylation-associated silencing of miRNAs in gastric carcinoma and observed that aberrant methylation of these miRNAs is associated with expression of target gene products.

Methods: The extent of promoter methylation of the miRNA-137 gene was assessed using methylation-specific polymerase chain reaction in 100 gastric carcinoma tissues and corresponding non-tumor tissues. The potential target gene products of miRNAs were evaluated by immunohistochemistry, and the relationship between the methylation profile of the miRNA promoters and clinicopathological parameters was analyzed.

Results: Methylation of the has-miR-137 CpG Island was observed frequently in tumor tissues (86%) and non-tumor tissues in 100 gastric carcinoma patients (78%), but not in normal gastric tissues (55%). Expression of the Cdc42 protein, which is a potential target of has-miR-137, was inversely correlated with the methylation level of miRNAs. The miRNA methylation status was closely associated with tumor size (p=0.03). However, there was no significant correlation between aberrant methylation of miRNA-137 and other clinicopathological factors. We found that has-miR-137 promoter methylation was higher in tumor tissues than non-tumor and normal tissues.

Conclusion: These results suggest that specific miRNA methylation in gastric carcinoma could be an important molecular mechanism causing loss of target regulation. It may be correlated with early-stage gastric carcinogenesis and could be used as an efficient diagnostic biomarker.

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Introduction

Epigenetic silencing of tumor-related genes by CpG island methylation has been reported recently as a genetic alteration in gastric carcinoma (GC) [1]. CpG islands are 0.5- to 2-kb regions rich in cytosine-guanine dinucleotides and are present in the 5' promoter region of approximately 40-50% of human genes [2]. DNA methylation has an important role in the transcriptional repression of imprinted genes and genes on inactivated X chromosomes, maintaining the integrity of chromosomes or acting as a defense against highly repeated mobile elements [3-6]. Aberrant methylation of CpG islands, which are normally protected from DNA methylation, is associated with DNA structural changes and consequent gene inactivation. Aberrant methylation of promoter CpG islands is an important mechanism for gene inactivation as an alternative to gene mutation or deletion in tumorigenesis [7,8].

GC has been shown to exhibit a high frequency of DNA hypermethylation [9]. Genes that are inactivated by CpG island hypermethylation, including those encoding tumor suppressors, cell-cycle regulators, tissue invasion-related proteins, and DNA mismatch repair proteins, have been reported in GC [10.11].

MicroRNAs (miRNAs) are non–protein-coding small RNAs ranging in size from 19 to 25 nucleotides that are cleaved from 70- to 100-nucleotide hairpin pre-miRNA precursors [12]. miRNAs bind to complementary sequences in the 3' untranslated regions (UTRs) of their target mRNAs and induce mRNA degradation or translational repression [13]. miRNAs play important roles in several cellular processes, such as proliferation, differentiation, apoptosis, and development, by simultaneously controlling the expression levels of hundreds of genes [14,15]. In recent years, a number of studies have provided evidence that dysregulation of miRNA expression contributes to the initiation and progression of human cancers. Tumor suppressor miRNAs usually repress growth-promoting genes and are downregulated in cancers. Conversely, oncogenic miRNAs target cell growth-inhibiting genes, and their expression is often upregulated in cancers [16]. However, the precise contribution of miRNAs to human metastasis and the mechanism underlying their dysregulation remain largely unexplored. Recently, many disease-associated miRNAs, such as *miR-9, miR-25, miR-34b, miR-124a, miR-127, miR-129, miR-137, miR-193a, miR-203a, and miR-342*, were reported to be silenced by aberrant DNA methylation of their promoter regions in cancer cells [17-23].

To explore the role of epigenetic mechanisms in the downregulation of miRNA-137, we examined DNA methylation-associated silencing of miRNAs in GC and observed that aberrant methylation of these miRNAs is associated with expression of their target gene products.

Material and Methods

Patients and samples

Gastric adenocarcinoma specimens were obtained from 100 patients who had undergone surgical resection at Chonnam National University Medical School in 2005. These cases were identified retrospectively from the surgical pathology files at the hospital.

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The panel of tumor specimens consisted of 50 early GCs and 50 advanced GCs. Early GC can be defined as a carcinoma limited to the mucosa or both the mucosa and submucosa, regardless of nodal status. A control group of 40 patients with GC corresponding non-tumor tissues and 20 patients with benign gastric pathology was also evaluated. To exclude the possibility of a cancer field defect, normal gastric mucosa tissues from a separate group of patients with benign gastric pathology were selected. Clinicopathologic data were available for the 100 GC patients. The average age of the patients was 60.9 years (range: 30-85 years), and there were 61 males and 39 females. The mean tumor horizontal diameter was 4.1 cm. Tumors were divided into two histological subgroups: the low-grade group, consisting of papillary and tubular adenocarcinomas that were well or moderately differentiated, and the high-grade group, consisting of poorly differentiated adenocarcinomas, signet ring carcinomas, and mucinous adenocarcinomas. Of the 100 tumors, 49 were high-grade, and 70 were intestinal type according to the Lauren classification. Tumor extent was in accordance with the criteria of the American Joint Committee on Cancer staging system [24]. Tumor metastasis to the lymph nodes and distant metastasis to other organs were observed in 46 and 15 of the 100 cases, respectively.

Microdissection and DNA extraction

Resected specimens were measured routinely, examined grossly, dissected from the representative tumor and non-tumor areas, fixed in 10% neutrally buffered formalin, embedded in paraffin, processed routinely, and stained with hematoxylin and eosin. Tissues were carefully dissected from carcinomas on H&E-stained slides as described previously [25]. Genomic DNA was extracted from microdissected tissue using a standard protocol. In brief, microdissected tissue was treated with 50 µl buffer containing 0.5% Tween 20 (Boehringer Mannheim, Germany), 20 µg proteinase K (Boehringer Mannheim, Germany), 50 mmol/L Trizma base at pH 8.9, and 2 mmol/L ethylene diaminetetraacetic acid and incubated at 56°C for 12-18 h. Proteinase K was inactivated by incubating the samples at 100°C for 10 min.

Bisulfite treatment of DNA and methylation-specific polymerase chain reaction

The methylation status of the has-miR-137 gene promoter was determined by bisulfite treatment of DNA followed by methylation-specific polymerase chain reaction (MSP), as described, with modification [26]. The primer sequences for all miRNA genes for the methylated and unmethylated reactions and the annealing temperature are described in Table 1. The bisulfate treatment was performed as follows. Briefly, 2 µg microdissected genomic DNA were denatured with 2 mol/L NaOH at 37°C for 10 min, followed by incubation with 3 mol/L sodium bisulfite (pH 5.0) at 50°C for 16 h in the dark. After treatment, DNA was purified using the DNA cleanup kit (Promega, Madison, WI, USA) as recommended by the manufacturer, incubated with 3 mol/L NaOH at room temperature for 5 min, precipitated with 10 mol/L ammonium acetate and 100% ethanol, washed with 70% ethanol, and finally resuspended in 30 µl distilled water. The methylation status of three gene promoters was determined using 2 µl bisulfite-treated DNA as a template for PCR using primers specific for the methylated and unmethylated alleles. Amplification was carried out in the GeneAmp PCR System 9700 thermocycler (Perkin Elmer, USA) for 40 cycles (30 s at 95°C, or 60 s at annealing temperature, then 60 s at 72°C, followed by a final 4-min extension at 72°C). The amplified PCR product (20 µl) was electrophoresed on 2% acrylamide gels and visualized by ethidium bromide staining. DNA from the RKO colon cancer cell line (American Type Culture Collection, Manassas, VA, USA) was used as a positive control for methylated DNA. A sample was considered positive for methylation if a band was seen for the DNA amplified by the methylated reaction primers.

Bisulfite treatment of DNA and methylation-specific polymerase chain reaction

Immunohistochemical staining for the potential target gene products of miRNAs was performed on paraffin-embedded tissue sections. Tissue sections (4 µm) of formalin-fixed, paraffin-embedded blocks were deparaffinized, rehydrated, rinsed with distilled water, and washed with Tris-buffered saline. Antigen retrieval was performed using a heat-induced epitope retrieval method. Avidin-biotin peroxidase complex staining with diaminobenzidine (Sigma) as the chromogen was performed using the streptavidin-horseradish peroxidase detection system (Ventana; Biotek Solutions, Tucson, AZ, USA). The primary antibody used for the immunohistochemical analysis was anti-cdc42 (1:200, Novus Biologicals, Littleton, CO, USA). The specificity of this antibody was confirmed previously by the manufacturer. Immunohistochemical staining was performed twice for samples with disagreement. A high level of concordance was achieved after this procedure. For a qualitative description of the relative intensity of immunoreactivity, the staining intensity was scored as follows: 0, no staining; 1, weak staining, scarcely above background level; 2, moderate staining; 3, strong staining. Immunoreactivity was considered abnormal if the intensity score was 0 or 1 and normal if the score was 2 or 3. Staining of tumor cytoplasm was evaluated on coded slides without knowledge of the molecular analysis results.

Statistical Analysis

All statistical analyses were conducted using SPSS for Windows

Gene	Type of primer	Primer SEQ.	Size (bp)	AT (°C)
hsa-miR-137① -M	Sense	5′-TTAGTTTATTTTTAGGTAGGGGCG-3′	124	60
	Antisense	5′-AAACCAAACTACCGCTACCG-3′		
hsa-miR-137① -U	Sense	5′-AGTTTAGTTTATTTTTAGGTAGGGGTG-3′	128	
	Antisense	5′-AAAAACCAAACTACCACTACCACT-3′		
hsa-miR-137② -M	Sense	5′-GTAGGGGGGTAGCGGTC-3′	157	60
	Antisense	5′-CAATCCTAATCACCAAAAACGCAACG-3		
hsa-miR-137③-U	Sense	5′-TGGTAGGGGGGTAGTGGTTG-3′	166	
	Antisense	5′-CATTTTCCAATCCTAATCACCAAAAACACAACA-3′		

Table 1: Primer sequence used in methylation-specific polymerase chain reaction.
AT; annealing temperature

(version 15.0; SPSS, Chicago, IL, USA). The methylation status of miRNA genes with respect to the progression of GC and the associations of miRNA gene methylation status with clinicopathologic variables were analyzed by Pearson's χ^2 test or two-tailed Fisher exact probability test, where appropriate. Agreement between the methylation status and immunohistochemical expression was estimated by calculating the kappa value. Comparison of Kaplan-Meier product limit survival curves was performed by application of the log-rank test. All reported p values were two-sided, and the level of significance was set at $p<0.05$.

Results

Frequency of miRNA-137 gene promoter methylation in gastric carcinoma and normal gastric tissues by MSP

Our analysis showed that the miRNA-137 gene and its 5'-UTR are located within CpG islands (Figure 1). Thus, we speculated that methylation of CpG islands leads to silencing of miRNA gene expression. To evaluate miRNA-137 gene promoter methylation in primary GC, 100 tumor samples were analyzed by MSP. Forty patients with corresponding non-tumor tissues and 20 patients with benign gastric pathology were evaluated as a control group.

Representative results are shown in Figures 2-3. The degree of methylation of *the has-miR-137* CpG island was 86% in tumor tissues, 78% in non-tumor tissues, and 55% in normal gastric tissues (Table 2). Methylation of the *has-miR-137* CpG Island was frequently observed in tumor and non-tumor tissues of GC patients, but not in normal gastric tissues. However, there were no significant correlations between aberrant methylation of miRNA-137 and other clinicopathological factors.

		has-miR-137	
Group	Total	M	U
Normal	20	11 (55%)	9 (45%)
Margins	40	31 (77.5%)	9 (22.5%)
EGC	50	43 (86%)	7 (14%)
AGC	50	43 (86%)	7 (14%)
P value		0.27	

Table 2: Proportion of cases with methylation status in normal mucosa, margins, and gastric carcinoma by methylation-specific polymerase chain reaction. EGC; early gastric carcinoma, AGC advanced gastric carcinoma U; unmethylated, M; methylated

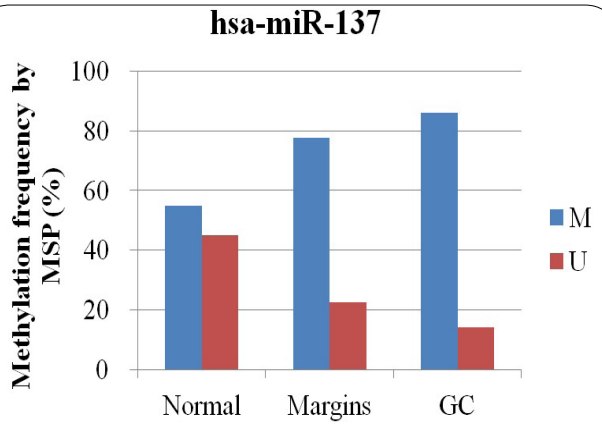

Figure 2: Methylation-specific polymerase chain reaction analysis of has-miR-137 gene methylation in normal mucosa, tumor margin, and gastric carcinoma tissues. Methylation frequency was significantly increased in gastric carcinoma.

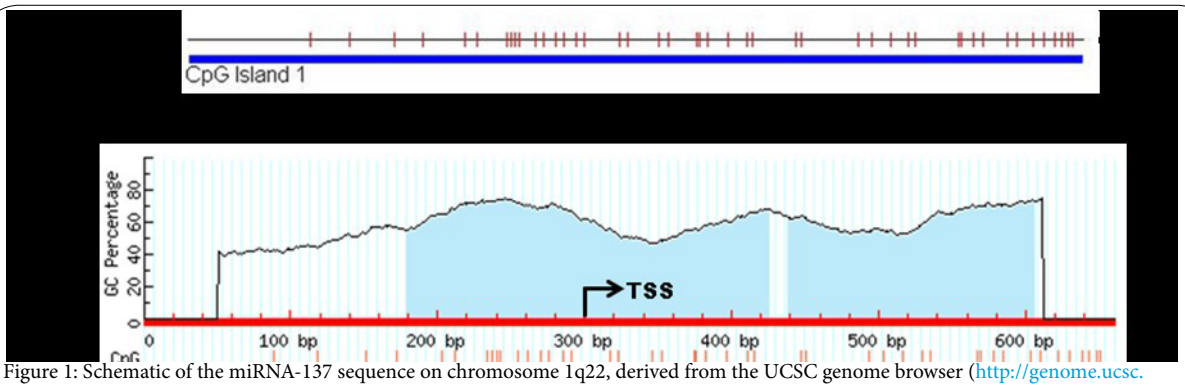

Figure 1: Schematic of the miRNA-137 sequence on chromosome 1q22, derived from the UCSC genome browser (http://genome.ucsc.edu). The regions of CpG dinucleotides in exon1 selected for methylation-specific polymerase chain reaction analysis are indicated below the CpG map. TSS: transcription start site.

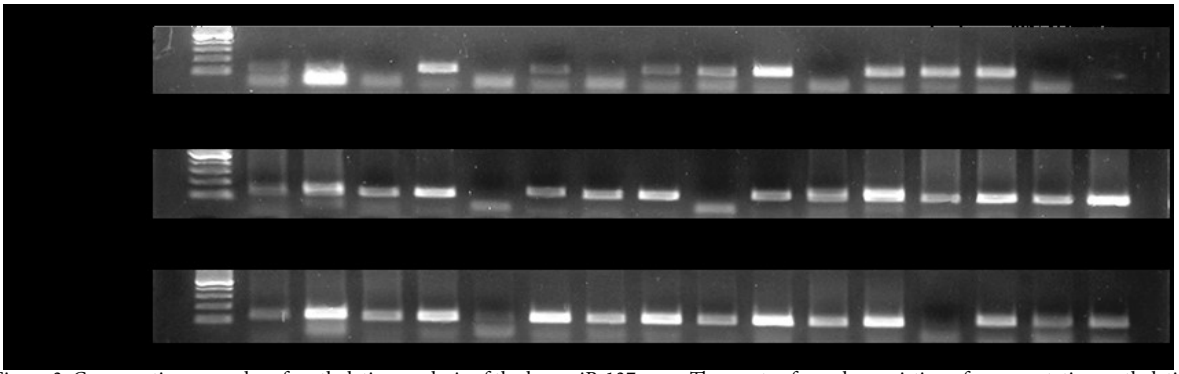

Figure 3: Comparative examples of methylation analysis of the has-miR-137 gene. Three sets of panels consisting of representative methylation-specific polymerase chain reaction electrophoretic products. Lane U, unmethylated; lane M, methylated; lane numbers, patient sample number.

Correlation between immunohistochemical expression of miRNA target genes and promoter methylation by MSP

As a target gene of *has-miR-137*, we focused on cdc42. The results of immunohistochemical analysis showed that expression of Cdc42 was preserved in normal gastric tissues and non-tumor tissues, whereas expression was lost in tumor tissues. These data suggest that DNA methylation-associated silencing of has-miR-137 prevents down regulation of the corresponding miRNA targets, such as oncoprotein Cdc42, and stimulates the progression and dissemination of the tumor.

Association between miRNA-137 gene promoter methylation and clinicopathological variables

The relationships between promoter methylation and clinicopathological variables of patients with GC are shown in Table 3. miRNA-137 promoter methylation by MSP was not associated with age, sex, histological type, Lauren classification, depth of invasion, or metastasis (p>0.05). However, the miRNA methylation status was closely associated with tumor size (p=0.03). Survival analysis was performed by log-rank analysis of Kaplan-Meier survival curves. In this study, the median follow-up after surgery was 63 months (range 49-68 months). However, the difference between the miRNA-137 methylated and unmethylated groups was not statistically significant.

			has-miR-137		
		Total	M	U	p value
Age (year)	<40	1	0	1	NS
	41-50	15	11	4	
	51-60	17	15	2	
	61-70	37	31	6	
	70<	30	30	0	
Sex	Male	61	51	10	NS
	Female	39	36	3	
Tumor size	≤4cm	67	56	11	<0.05
	>4cm	33	30	3	
Histologic type	Low grade	51	45	6	NS
	High grade	49	40	9	
Lauren classification	Intestinal	70	62	8	NS
	Mixed	14	13	1	
	Diffuse	16	12	4	
Depth of invasion	T1	51	44	7	NS
	T2	19	17	2	
	T3	27	23	4	
	T4	3	3	0	
LN metastasis	Absent	54	46	8	NS
	Present	46	41	5	
Distant metastasis	Absent	85	74	11	NS
	Present	15	13	2	

Table 3: Clinicopathological features of gastric carcinoma patients by promoter methylation status of miRNA-137 gene.
U; unmethylated, M; methylated, NS; not significant

Discussion

miRNAs are 19- to 25-nucleotide-long RNAs that bind complementary sequences in the 3'UTR of several target mRNAs to induce their degradation or repress their translation. They play a crucial role in the initiation and progression of human cancers [12,14,15]. Epigenetic inactivation of miRNAs in human cancer constitutes an emerging mechanism involved in the progression of cancer. Studies have reported that miR-137 down regulation is common in various cancers, such as pancreatic, hepatocellular, and head and neck cancers. Epigenetic silencing of miR-137 is an early event in colorectal carcinogenesis [19]. Expression of miR-137 is restricted to the colonocytes in normal mucosa and inversely correlated with the level of methylation. Promoter methylation of miR-137 was associated with female gender and inversely associated with body mass index in squamous cell carcinoma of the head and neck (SCCHN). Promoter methylation of miR-137 appears to be a relatively frequently detected event in the oral rinse of SCCHN patients and may have future utility as a biomarker in DNA methylation panels [10].

In this study, we confirmed that miR-137 was more frequently downregulated in GC tissues than in the corresponding non-cancerous tissues. We also evaluated whether downregulation of miR-137 is mediated by epigenetic mechanisms in GC. We found that the miR-137 promoter was methylated in 21 of 30 (70%) cancer tissues and 2 of 30 (6.7%) adjacent non-cancerous tissues. The expression of miR-137 was further determined in a panel of five human GC cell lines (AGS, SGC-7901, MKN28, MKN45, and BCG823). We examined a further link between miR-137 CpG island hypermethylation and its gene silencing by the treatment of these cancer cell lines with decitabine (DAC). After treatment with DAC, the expression of miR-137 was restored. From a functional standpoint, we next examined whether epigenetic inactivation of miR-137 inhibits growth suppression in GC cells. By restoring miR-137 expression in GC cells, we indeed showed that miR-137 suppressed cell growth, induced apoptosis, and inhibited the cell cycle in GC cells, suggesting a tumor-suppressive role of miR-137.

Three independent studies screening for important oncogenes have found that Cdc42 is commonly altered by retroviral insertions. Cdc42 was over expressed in colorectal cancer samples, and this expression was associated with silencing of ID4 with statistical significance [13,19]. The up-regulated Cdc42 activity may impair c-Cbl-mediated EGFR degradation, contribute to EGFR hyperactivity, and induce proteasomal degradation of p21CIP1, leading to an increase in cell proliferation and migration. These functional outcomes may occur through regulation of the PAK1, GSK3b, MLC, ERK1/2, and JNK pathways. In addition, down regulation of Cdc42 signals can inhibit anchorage-independent growth and induce apoptosis via the PI (3)K-Akt and Erk signaling cascades and the p53 tumor suppressor [16,17]. Cdc42 was found to be a direct target of miR-137 in colorectal cancer cells, and ectopic expression of miR-137 reduced Cdc42 expression [8]. Similarly, we found that exogenous miR-137 suppressed the expression of Cdc42, resulting in decreased phosphorylation of ERK1/2. Similar results were also obtained after transfection of siRNAs targeting Cdc42. Cdc42 was shown to be a direct target of miR-137 by a luciferase reporter assay. Additionally, we found that inactivation of Cdc42 by siRNA and the exogenous expression of miR-137 induced apoptosis in AGS cells. Apoptosis in GC cells induced by siRNAs targeting Cdc42 was similar to the results obtained from ectopic expression of miR-137. It was suggested that inactivation of the Cdc42/ERK pathway is involved in miR-137-induced apoptosis in GC.

Conclusion

These results suggest that specific miRNA methylation in GC could be an important molecular mechanism causing loss of target regulation. It may be correlated with early-stage gastric carcinogenesis and could be used as an efficient diagnostic biomarker.

Competing Interests

The authors have no competing interests with the work presented in this manuscript.

Author Contributions

All the authors substantially contributed to the study conception and design as well as the acquisition and interpretation of data and drafting the manuscript.

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