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Original Article

Methylation-silencing *RCC1* expression is associated with tumorigenesis and depth of invasion in gastric cancer

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Abstract: Introduction: Regulator of chromosome condensation 1 (RCC1) is a critical cell cycle regulator. We firstly identified RCC1 gene hypermethylation in gastric tumor tissues using the differential methylation hybridization (DMH) microarray, but the role of RCC1 in the pathogenesis of gastric carcinoma is largely unknown. Methods: Three gastric cancer cell lines (AGS, MKN45, and TSGH9201) were used to analyze RCC1 gene methylation, mRNA and protein expressions. Furthermore, 85 pairs of matched human gastric carcinoma samples in a tissue microarray were used to analyze RCC1 expression by immunohistochemistry staining. Results: A differential methylation pattern was found in TSGH9201 (100%), MKN45 (87%), and AGS (62%) cell lines at the 9th CpG site of RCC1 exon 1. RCC1 mRNA and protein expressions in AGS cells were significantly higher than in TSGH9201 and MKN45 cell lines ($P < 0.05$). Tissue array data showed that RCC1 expression was detected in 21% (18/85) of gastric carcinoma tissues and in 80% (76/95) of adjacent non-tumor tissues. The expression of RCC1 in gastric carcinoma tissues was significantly lower than in adjacent non-tumor tissues ($P < 0.001$). Furthermore, an association between RCC1 expression and clinicopathological features showed that RCC1 expression was closely correlated with tumor differentiation and depth of invasion ($P < 0.05$). Conclusions: Our data indicate that RCC1 expression is frequently lost in poorly differentiated gastric cell lines and gastric carcinoma tissues. Loss of RCC1 expression is correlated with tumor differentiation and depth of invasion. These findings suggest that RCC1 may play a tumor suppressor role in gastric carcinoma.

Keywords: DMH microarray, gastric carcinoma, RCC1, DNA methylation, tumor tissue array, immunohistochemistry, invasion

Introduction

Despite a steady decline in gastric cancer incidence, gastric cancer still represents a major health problem [1]. It remains one of the most common malignancies, particularly in Eastern Asian countries, including Taiwan [2, 3]. Most gastric cancer patients present with advanced-stage disease [4]. The clinical outcome of gastric cancer has gradually improved, but the overall 5-year survival rate of gastric cancer patients remains approximately 20% [5].

The majority of gastric cancers are sporadic, and only a small percentage has a familial component, which is characterized by an autosomal pattern of inheritance [6]. Gastric cancer is a multi-factorial disease that involves both genetic and environmental factors. In sporadic gastric cancers, environmental factors seem to be predominant. Conversely, genetic factors play a major role in familial cancers. Approximately 90% of gastric cancers are classified as adenocarcinomas, while the remaining 10% are represented by squamous cell carcinomas, leiomyo-

sarcomas, non-Hodgkin lymphomas, and undifferentiated carcinomas [6, 7].

Gastric cancer has marked heterogeneity in its morphology and biology. Based on morphological characteristics of gland formation and histogenetic background, gastric adenocarcinomas can be divided into intestinal and diffuse types using the Lauren classification system [8] or as differentiated and undifferentiated using the Nakamura classification system [9]. Intestinal-type adenocarcinoma is considered to be equivalent to differentiated adenocarcinoma, and diffuse-type adenocarcinoma is considered to be equivalent to undifferentiated adenocarcinoma. These two gastric cancer types also display different biological and etiological characteristics. Tumor cells of the intestinal type form glandular-like structures, a feature that is lacking in the diffuse type. On the contrary, the diffuse type of gastric cancer is characterized by an infiltration and thickening of the gastric wall by tumor cells. The two histological subtypes are the result of distinct pathogenic pathways [10, 11]. Previous reports have also found that *Helicobacter pylori* infection leads to the development of chronic atrophic gastritis and intestinal metaplasia [12, 13].

Usually, the intestinal type of gastric cancer develops on a background of intestinal metaplasia; by contrast, the diffuse type develops on a background of gastric mucosa without intestinal metaplasia. The diffuse type of gastric cancer is characterized by reduced or abnormal E-cadherin expression [10]. The diffuse type has a worse prognosis and tends to develop in young patients; it can have any type of gastric localization, but it frequently develops at the level of the cardia. Although alterations in a large number of oncogenic and tumor-suppressive genes are reportedly implicated in gastric cancer [14-17], the molecular mechanisms underlying the development of gastric cancer are still poorly understood. Identification of its tumorigenesis mechanism is necessary for the development of targeted clinical therapy.

Regulator of chromosome condensation 1 (RCC1) has been identified as a critical cell cycle regulator [18, 19]. RCC1 is a guanine nucleotide releasing factor that promotes the exchange of Ran-bound GDP by GTP [20]. RCC1 plays a key role in nucleo-cytoplasmic transport, mitosis and nuclear envelope assembly and is involved in regulating the onset of chromosome condensation during S phase [18, 21]. RCC1 can bind both to nucleosomes and to double-stranded DNA, and the RCC1-Ran complex (together with other proteins) is a component in a signal transmission pathway that detects unreplicated DNA [21, 22]. This has led to the proposal that RCC1 is a signaling molecule, detecting unreplicated DNA and producing an inhibitory signal. However, the association between RCC1 expression and gastric carcinoma has not been fully studied.

To assess the role of RCC1 in gastric carcinoma, RCC1 expression was detected in different gastric cancer cell lines, gastric carcinomas and adjacent non-tumor tissues to evaluate the relationship between RCC1 expression and the clinicopathological characteristics of gastric carcinoma. The DNA methylation status of the *RCC1* gene in three different gastric cancer cell lines was also elucidated.

Materials and methods

Differential methylation hybridization (DMH) microarray assay

The protocol of differential methylation hybridization (DMH) on human CpG island microarray was based on our previously described [23, 24]. Briefly, the purified methylation amplicons (5 μg) from eight pairs of gastric tumor and non-tumor obtained from the tissue bank of Taichung Veterans General Hospital, Taiwan, were conducted using the BioPrime DNA labeling system (Invitrogen, CA, USA). Cyanine 5-ddUTP (Cy5-ddUTP) and Cyanine 3-ddUTP (Cy3-ddUTP) (Perkin-Elmer Life Sciences, NJ, USA) fluorescent dyes were coupled to tumor (T) and normal (NT) amplicons, respectively, and cohybridized to the microarray panel. The hybridization of DMH microarray was performed within a moistened hybridization chamber, GeneMachines HybChambers (Genomic Solutions, MI, USA), in a 65°C water bath from 12 to 16 h. The slides were scanned with the GenePix 4000B scanner (Axon, CA, USA) and the acquired images were analyzed with the software GenePix Pro 4.0 (Axon, CA, USA). The microarray data was analyzed as described previously [25].

Cell culture

Three human gastric cell lines, AGS, MKN45, and TSGH9201, were obtained from American

Type Culture Collection (ATCC; Rockville, MD, USA) and Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan). The AGS, MKN45 and TSGH9201 cells were kept in F-12K, DMEM, and RPMI 1640 media (Gibco, Carlsbad, CA, USA), respectively, which was supplemented with 10% fetal bovine serum (FBS) as well as 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). All of the cells were maintained at 37°C under an atmosphere of 5% CO_2.

Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from total RNA using a reverse-transcriptase system with the SuperScriptIII RT enzyme (Invitrogen, Carlsbad, CA, USA). The resulting cDNA was amplified using a QuantiFast Probe Assay kit (Qiagen, Foster, CA, USA) on an ABI Stepone™ Real-Time PCR system (Applied Biosystems, Foster, CA, USA). Relative quantification of target gene (*RCC1* and *GAPDH*) expression was evaluated using the comparative C_T method in which ΔC_T represents the normalization of C_T for a target gene relative to TBP. $\Delta\Delta C_T$ was calculated by comparing the mean ΔC_T values. Fold changes in gene expression were equivalent to $2^{-\Delta\Delta C}{}_T$ [26-28].

Western blot analysis

The gastric cancer cell lines were homogenized in lysis buffer containing protease inhibitor cocktail. Protein concentrations were measured by the Bradford method [29]. Before separation, the samples were re-suspended in loading buffer, denatured by boiling for 5 min and separated by 12% SDS-PAGE gel electrophoresis. The proteins were transferred to Trans-Blot polyvinylidene fluoride membranes, and the membranes were probed with a primary rabbit polyclonal antibody against human RCC1 (GeneTex Inc., Irvine, CA, USA) at a dilution of 1:12,000 for 3 h. The blots were washed in TBST and incubated with a goat anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody. The membranes were developed using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA) by exposure to x-ray film [30]. A housekeeping protein, β-actin, was used as an internal control for the quantification of protein expression levels as described previously [31].

Primer design and PCR amplification for gene methylation study

The primers that were designed to amplify *RCC1* covered the regions of the gene with the most CpG sites. Most of the selected amplicons were located in the promoter region and in exon 1, which spanned from -517 to 466 nucleotides (nt) relative to the transcription start site. The primers were designed using EpiDesigner software (http://www.epidesigner.com/) and are listed in Table S1. Genomic DNA was treated with sodium bisulfite as described previously [32, 33], and the bisulfite-treated genomic DNA was then amplified under the following conditions: initial denaturation, 94°C for 4 min, 45 cycles of 94°C for 20 sec, 56°C for 30 sec, and 72°C for 1 min, followed by 3 min at 72°C. The products were stored at 4°C for further analysis.

In vitro transcription and T-cleavage (RNase A digestion) assay

To neutralize unincorporated dinucleotide triphosphates (dNTPs) after amplification reactions, 0.3 U of shrimp alkaline phosphatase (SAP; Sequenom, San Diego, CA, USA) was used [34]. The components of the reaction included 1.7 μL of RNase-free ddH_2O and 0.3 μL of SAP, which were combined with the above PCR products. The mixture was incubated at 37°C for 20 min, 85°C for 5 min, and then at 4°C until use. Using T7 RNA polymerase, thymidine triphosphate was incorporated into the PCR product, finishing the transcription reaction. RNase A was added to the same reaction to cleave the transcripts (T-cleavage assay). The reaction mixture was incubated at 94°C for 30 sec, followed by 40 cycles of 94°C for 5 sec, 52°C for 5 sec, 5 cycles of 80°C for 5 sec and 72°C for 3 min, after which it was stored at 4°C. To remove the phosphate backbone, the T-cleavage/RNase A reaction products were diluted with RNase-free water and mixed with clean resin before performing MALDI-TOF mass spectrometry [34].

MALDI-TOF mass spectrometry for DNA methylation measurement

All of the RNase A and clean resin-treated products were robotically dispensed onto silicon matrix preloaded chips (SpectroCHIP;

Table 1. The hypermethylation gene list selected from gastric cancer samples in DMH microarray analysis[1]

Gene name	GenBank Accession No.	Locus of chromosome location	Description of gene function	Cy5/Cy3 ratio[2]
DXYS155E	BC028151	chrY:1677107-1677967	Mediates regulation of pre-mRNA splicing in a PKA-dependent manner	7.06
PLAG1	NM_002655	chr8:57285798-57286167	A zinc finger transcription factor that is consistently rearranged in pleomorphic adenomas of salivary gland	5.36
FLJ25791	NM_173559	chr6:109920727-109920873	Nucleoside phosphate kinase activity; ATP binding	4.33
KIAA1414	NM_019024	chr2:37157706-37157779	Unknown function	4.11
BC045801	BC045801	chr2:132729663-132730138	Sensory perception of light stimulus	3.76
ATP5A1	NM_001001937	chr18:41930633-41931167	Protein binding; ATPase activity; ATP binding	3.27
FAM90A1	NM_018088	chr12:8270997-8271748	Nucleic acid binding; zinc ion binding	3.03
C12orf32	NM_031465	chr12:2856916-2856990	Involved in mammary carcinogenesis	2.91
HOXC4	NM_014620	chr12:52696695-52697020	Plays an important role in morphogenesis in all multicellular organisms	2.51
DUSP10	NM_007207	chr1:219980942-219981545	Associated with cellular proliferation and differentiation	2.39
SPTBN4	AY004226	chr19:45765985-45766512	Determination of cell shape, arrangement of transmembrane proteins, and organization of organelles	2.28
ACSL3	NM_004457	chr2:223434579-223434768	Plays a key role in lipid biosynthesis and fatty acid degradation	2.08
ENSA	NM_207045	chr1:148868088-148869040	An endogenous regulator of KATP channels	2.06
PDE2A	AK131525	chr11:71978513-71979584	cGMP binding; cyclic-nucleotide phosphodiesterase activity	1.66
RCC1	NM_001269	chr1:28705597-28705835	Guanine nucleotide exchange factor for Ran GTPase	1.54

[1]DMH microarray: The differential methylation hybridization (DMH) microarray is a high-throughput microarray technique designed to identify changes in DNA methylation patterns as our previous reports [23-25, 32, 33]. 2Cy5/Cy3 ratio: Cyanine 5-ddUTP (Cy5) and Cyanine 3-ddUTP (Cy3) fluorescent dyes were coupled to gastric tumor (T) and non-tumor (NT) amplicons, respectively, and co-hybridized to the DMH microarray panel. The Cy5/Cy3 ratios (i.e., the hybridization intensity from the tumor amplicons to the hybridization intensity from the non-tumor amplicons) from each image are normalized guided by both the average global Cy5/Cy3 ratio from each image. The ratio of Cy5/Cy3 ≥ 1.5 was identified as the hypermenthylation gene.

A

Human gene	Primer design	Dye label	Detected transcripts (GenBank Acc. No.)
Rcc1	Hs_RCC1_QF_1 QuantiFast Probe Assay (QF00298438; Qiagen)	FAM	NM_001048194 (2633 bp) NM_001048195 (2591 bp) NM_001048199 (2715 bp) NM_001269 (2540 bp) NR_030725 (2858 bp) NR_030726 (2854 bp)
GADPH	Hs_GAPDH_QF_2 QuantiFast Probe Assay (QF00531132; Qiagen)	MAX	NM_002046 (1421 bp)

B

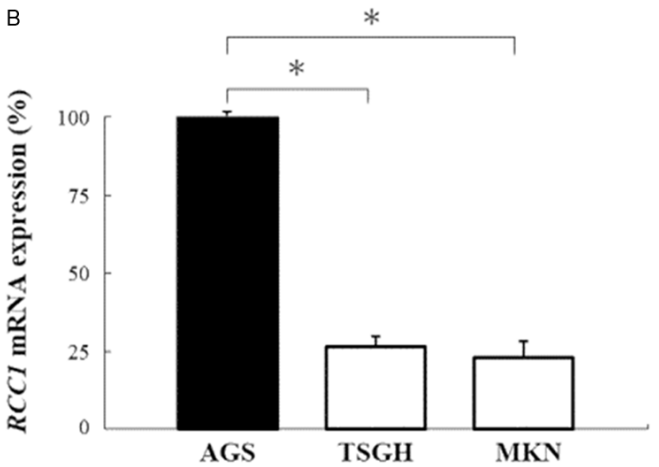

Figure 1. Validation of *RCC1* mRNA expression in gastric cancer cell lines by quantitative reverse transcription PCR (qRT-PCR). A. Primer and probe designs of *RCC1* and *GAPDH* genes for qRT-PCR analysis. B. Quantitative data of relative *RCC1* mRNA expression levels in three different differentiation states of gastric cancer cell lines. The results are shown as the mean ± SD of three independent experiments. Each bar represents the relative *RCC1* expression percentage normalized to the *GAPDH* housekeeping gene and compared to the AGS cell line. *P=0.002 (Kruskal Wallis test).

Sequenom, San Diego, CA, USA), and mass spectra were collected using a MassARRAY compact MALDI-TOF analyzer (Sequenom). Mass spectra methylation ratios were generated using EpiTYPER (ver. 4.0; Sequenom).

Immunohistochemical staining of tissue microarray

Gastric carcinoma and adjacent non-tumor tissue microarray (TMA) sections with stage and grade information were purchased from Biomax, Inc. (Rockville, MD, USA). We used the following tissue microarray sections, containing different numbers of cases and cores: ST1004 (55 cases with 100 cores) and ST801a (40 cases with 80 cores). The sections were in duplicate cores per case. The TMA sections were processed, stained, and analyzed essentially as described previously [35]. In this study, the total number of exam samples in the TMA sections included 85 pairs of human gastric cancer tissues with adjacent non-tumor tissue plus 10 samples of normal stomach tissue. According to the manufacturer's instructions, the specimens were stained with an anti-RCC1 antibody (GeneTex Inc., Irvine, CA, USA), and the nuclei were counterstained with hematoxylin [36]. Positive staining was detected as a brown color that was present in more than 25% of the cells.

Statistical analysis

All statistical analyses were carried out using SPSS software (IBM, standard version 10.0). Fisher's exact test, the Pearson chi-square test, and the Kruskal Wallis test were used to determine statistically significant differences. A P-value < 0.05 was considered statistically significant.

Results

Identification of hypermethylation genes from gastric cancer by DMH microarray

The DMH microarray hybridization results showed that hundreds of gene spots were obviously more hypermethylated in gastric tumor genomes than their adjacent non-tumor tissues. Yellow spots (Cy5:Cy3 = 1.0) represent equal amounts of bound DNA from each amplicon, an indication of no methylation differences

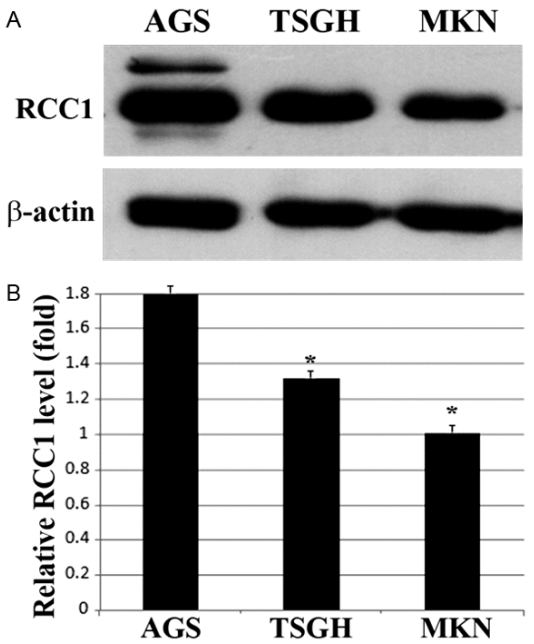

Figure 2. Protein expression levels of RCC1 analyzed in three different gastric cancer cell lines by western blotting. A. Protein expression levels of RCC1 and β-actin in three gastric cancer cell lines (AGS, TSGH9201, and MKN45) as determined by western blot analyses. B. Quantification of relative RCC1 protein levels in three different gastric cancer cell lines using image J. β-actin was used as an internal control. $^{*}P < 0.05$ compared to AGS cells.

between gastric tumor and non-tumor genomes. Selection of genes was based on the criteria described above. We conducted a confirmation study to determine whether the cutoff ratio (≥1.5) could accurately identify hypermethylation. These newly collected genes are consistent hypermethylation in all of the eight gastric cancer genomes as shown in **Table 1**. Upon further examination, we selected the *RCC1* gene to examine in greater details by quantitative RT-PCR, protein expression, MALDI-TOF mass spectrometry of methylation status analysis, and also the tumor tissue array assays.

mRNA expression of RCC1 in different human gastric cancer cell lines

To explore the role of RCC1 in gastric carcinoma, we first examined the mRNA expression levels of *RCC1* in various gastric cancer cell lines. The mRNA expression levels of *RCC1* were quantitatively analyzed in the three gastric cancer cell lines using real-time PCR (**Figure 1A**). As shown in **Figure 1B**, the AGS (100%) cell line expressed relatively higher levels of *RCC1* mRNA than did the TSGH9201 (25%) and MKN45 (20%) cell lines. The relative expression of *RCC1* mRNA in the AGS cell line was statistically significantly higher than that in the TSGH9201 and MKN45 cell lines ($P < 0.05$).

Protein expression levels of RCC1 in different gastric cancer cell lines

In western blot analysis, RCC1 expression was observed in all of the analyzed cell lines (**Figure 2A**). After being normalized to a β-actin internal control, the AGS cell line (1.8-fold) expressed more RCC1 protein than did the TSGH9201 (1.3-fold) and MKN45 (1.0-fold) cell lines, which was statistically significant ($P < 0.05$) and is shown in **Figure 2B**. These protein expression results are in concordance with the measured RNA expression, meaning that RCC1 mRNA and protein expression was higher in the AGS cell line than in the TSGH9201 and MKN45 cell lines. These gastric cancer cell lines exhibited different types of cell differentiation: the AGS cell line was established from a well-differentiated gastric epithelial cancer [37]; the TSGH9201 cell line was established from a human signet ring gastric carcinoma with a moderately differentiated cell type [38], and the MKN45 cell line was established from a poorly differentiated liver metastasis of a gastric carcinoma patient [39]. These results suggested that RCC1 expression levels may be correlated with the differentiation status of gastric cancer cells.

MALDI-TOF mass spectrometry analysis of CpG site methylation in the RCC1 gene in different human gastric cancer cell lines

Epigenetic mechanisms, especially DNA methylation, are associated with gastric carcinoma development and progression [40, 41]. We therefore analyzed the methylation patterns of the *RCC1* gene in the three gastric cancer cell lines. The locations of CpG islands in the human *RCC1* gene were predicted by EpiDesigner software, and our data showed that a typical CpG island was found within the *RCC1* gene promoter that extended to the exon 1 region, between -49 to 466 nucleotides (**Figure 3A**). A total of 24 potential CpG sites (**Figure 3B**) were analyzed by MALDI-TOF mass spectrometry, and 20 CpG sites could be detected (**Figure 3C**). The results showed that significantly different

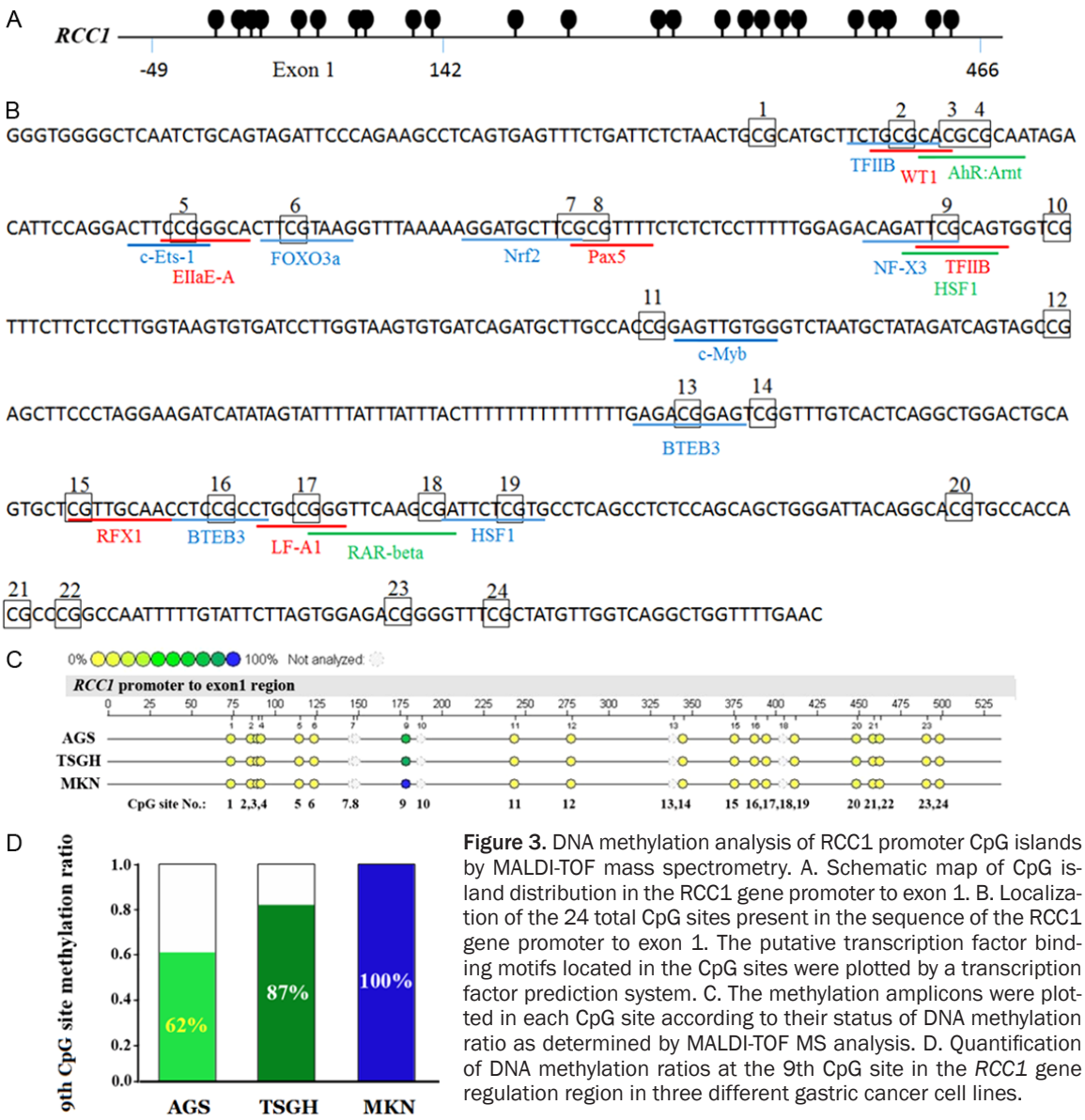

Figure 3. DNA methylation analysis of RCC1 promoter CpG islands by MALDI-TOF mass spectrometry. A. Schematic map of CpG island distribution in the RCC1 gene promoter to exon 1. B. Localization of the 24 total CpG sites present in the sequence of the RCC1 gene promoter to exon 1. The putative transcription factor binding motifs located in the CpG sites were plotted by a transcription factor prediction system. C. The methylation amplicons were plotted in each CpG site according to their status of DNA methylation ratio as determined by MALDI-TOF MS analysis. D. Quantification of DNA methylation ratios at the 9th CpG site in the *RCC1* gene regulation region in three different gastric cancer cell lines.

methylation levels of the *RCC1* gene were found in these three gastric cell lines at a specific location in the 9th CpG site ($P < 0.05$).

The expression of *RCC1* gene was higher in the AGS cell, which corresponded to a lower percentage (62%) of methylation at the 9th CpG site. However, the *RCC1* gene exhibited higher methylation levels (87% and 100%) at the 9th CpG site in the TSGH9201 and MKN45 cell lines, which corresponded to lower RCC1 protein expression in both (**Figure 3D**). Using a transcription factor prediction system, we further found that there were three specific transcription factor binding motifs, called heat shock factor 1 (HSF1), transcription factor IIB (TFIIB), and nuclear factor-X3 (NF-X3), located around the 9th CpG site. DNA methylation at the 9th CpG site of the *RCC1* gene may lead to interference in *RCC1* gene regulation and reduce its expression in gastric cancer cells.

Loss of RCC1 expression is frequently found in gastric carcinomas

To understand the role of RCC1 in gastric cancer development and cell differentiation, immunohistochemical (IHC) analysis was performed to observe the expression levels of RCC1 in the gastric carcinoma tissue microarray (TMA) containing matched non-tumor tissues (**Figure 4**). RCC1 expression was evaluated in the nuclei

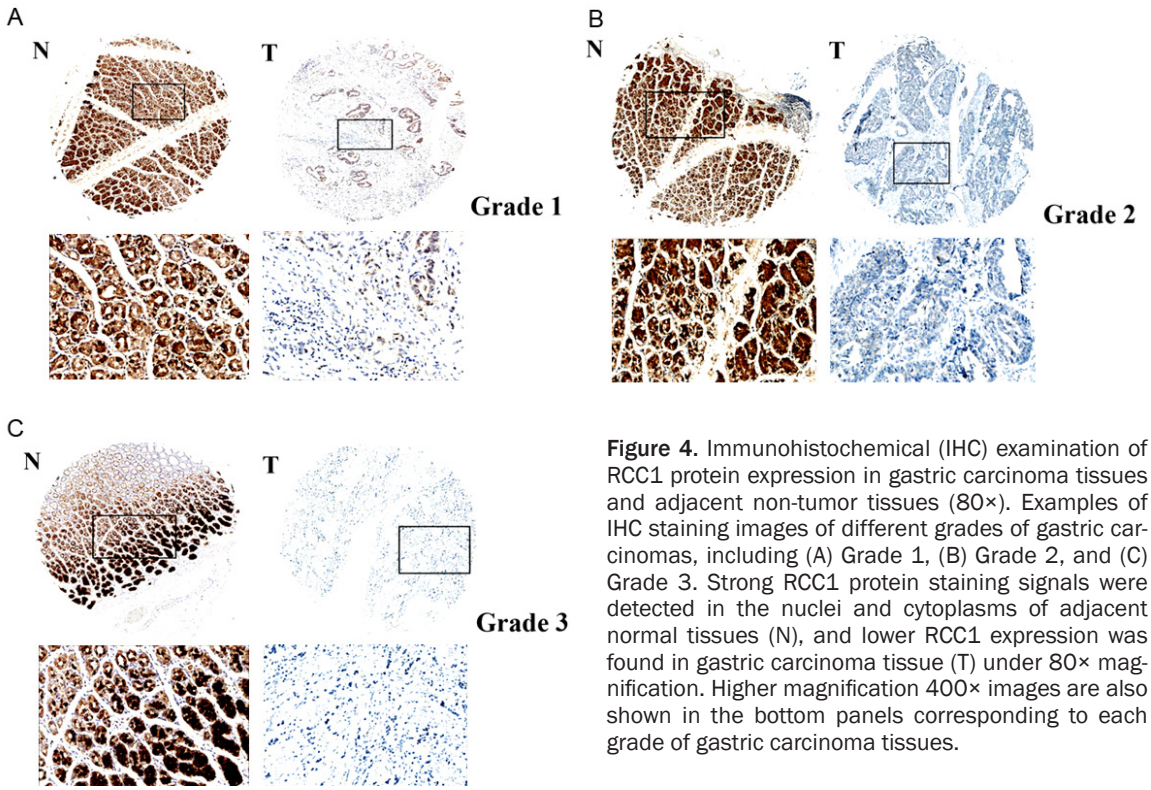

Figure 4. Immunohistochemical (IHC) examination of RCC1 protein expression in gastric carcinoma tissues and adjacent non-tumor tissues (80×). Examples of IHC staining images of different grades of gastric carcinomas, including (A) Grade 1, (B) Grade 2, and (C) Grade 3. Strong RCC1 protein staining signals were detected in the nuclei and cytoplasms of adjacent normal tissues (N), and lower RCC1 expression was found in gastric carcinoma tissue (T) under 80× magnification. Higher magnification 400× images are also shown in the bottom panels corresponding to each grade of gastric carcinoma tissues.

and cytoplasms of 85 pairs of gastric cancer tissues and adjacent non-tumor tissues plus 10 normal stomach tissues. If 20% of an image's total area showed positive staining for RCC1 in the nucleus and cytoplasm, it was scored as RCC1 positive (+). In adjacent non-tumor tissues, positive RCC1 staining was located in the regenerative compartment of the gastric epithelium, known as the gastric mucous neck region (**Figure 4A-C**; left panels). Moreover, lymphocytes within gastric mucosa always showed strong RCC1 staining. Positive RCC1 expression was detected in 21% (18/85) of gastric cancer tissues and in 80% (76/95) of adjacent non-tumor tissues (**Table 2**). The expression of RCC1 in gastric carcinoma tissues (**Figure 4A-C**; right panels) was significantly lower than in adjacent non-tumor tissues ($P < 0.001$).

Correlation analysis of RCC1 expression and clinicopathological characteristics of gastric carcinoma

We further analyzed the relationships between the clinical characteristics of gastric carcinoma and RCC1 expression using the Pearson chi-square and Fisher's exact tests as summarized in **Table 3**. Tumor differentiation ($P < 0.01$) and depth of invasion ($P < 0.05$) were significantly correlated to RCC1 expression. However, there were no significant correlations between RCC1 expression and other clinicopathologic features, including age, gender, stage, lymph node metastasis, and distant metastasis ($P > 0.05$).

Discussion

In this study, we demonstrated that RCC1 downregulation was common both in gastric cancer cell lines and in clinical gastric carcinomas. A loss of RCC1 expression was significantly correlated with tumorigenesis, cell differentiation, and depth of tumor invasion.

Gastric carcinoma is a heterogeneous disease that displays various biological and clinical characteristics. The control of this disease in patient is currently based on easily detectable clinical and pathological characteristics. Recently, many molecular markers have been proposed for the prediction of the prognoses of gastric carcinoma patients, but their roles in determining the risk level of an individual patient are quite limited. Gastric carcinoma may be considered the final step in a progres-

Table 2. Expression levels of RCC1 protein in gastric cancer tissue microarrays (ST801a and ST1004, Biomax US)

Tissue	Total No.	RCC1 expression		*p*-value
		Positive	Negative	
Adjacent tissue	95	76 (80%)	19 (20%)	<0.0001
Gastric cancer tissue	85	18 (21%)	67 (79%)	

P < 0.0001 (Pearson chi-square).

sive imbalance between mucosal cell proliferation and apoptosis due to the activation of oncogenes and the inactivation of tumor suppressor genes [42, 43].

RCC1 was first identified during premature chromosomal condensation in BHK cells and was found to affect mammalian cell cycle progression [44]. Cell lines with mutant forms for RCC1 display either cell cycle arrest or an immature chromatin structure phenotype at different cell cycle boundaries. RCC1 encodes a bulky nuclear protein that associates with chromosomes and belongs to a class of highly conserved proteins present in all eukaryotic species [45]. In recent years, functional studies of the RCC1 protein have shown that it is a guanine nucleotide exchange factor (GEF) that acts on the nuclear Ras-like G protein Ran [20]. Ras and Ran function as biological switches, and RCC1 may carry out its biological functions through Ran, which is essential for nuclear pore transport function [21]. In particular, this suggests the presence of two functional domains in RCC1: one to receive upstream signals and the other to transfer them into downstream events. A loss of RCC1 induces premature initiation of mitosis, resulting in G1 arrest with micronuclei possessing mitotic condensed chromosomes [46]. However, little is known about the relationship between RCC1 and carcinomas.

This study is the first to identify a relationship between RCC1 and gastric carcinoma. We found that the transcription and translation of RCC1 were significantly different among three different types of differentiated gastric cancer cell lines (AGS, TSGH9201, and MKN45). The AGS cell line was established from a well-differentiated gastric epithelial cancer cell [37], and the TSGH9201 cell was established from a human signet ring gastric carcinoma with a moderately differentiated cell type [38]; however, the MKN45 cell line was established from a liver metastasis of a gastric carcinoma patient and had poorly differentiated cells [39]. RCC1 expression decreased in the moderately and poorly differentiated cell lines (TSGH9201 and MKN45, respectively). To further understand *RCC1* gene regulation by epigenetics, a total of 24 CpG sites present in the promoter to exon 1 region of *RCC1* were analyzed by MALDI-TOF mass spectrometry. The results showed that significantly different methylation levels of the RCC1 gene were found in these three gastric cell lines at a specific location in the 9th CpG site. This might explain how the hypermethylation of the *RCC1* gene at the 9th CpG site of the promoter to exon 1 region in both the TSGH9201 and MKN45 cell lines correlated to a downregulation of RCC1 expression.

When analyzing transcription factor (TF) binding motifs, we further found that there were three specific TF binding motifs, called HSF1, TFIIB, and NF-X3, located within the 9th CpG site of the *RCC1* promoter to exon 1 region. Promoter hypermethylation-mediated silencing of tumor suppressor genes (TSGs) is a hallmark of oncogenesis. In a previous study of nasopharyngeal carcinoma (NPC), a candidate TSG called oxidored-nitro domain-containing protein 1 (NOR1) was found, which was downregulated during tumorigenesis. Furthermore, a functional NOR1 promoter that is regulated by HSF1 and nuclear respiratory factor 1 (NRF1) was identified [47]. It is known that the transcription factor TFIIB plays a central role in preinitiation complex assembly, providing a bridge between promoter-bound TFIID and RNA Polymerase II [48]. TFIIB possesses sequence-specific DNA-binding ability and interacts with a TFIIB-recognition element (BRE) that can be disrupted by DNA methylation [49], which is present in many promoters, such as in the *RCC1* gene in this study.

The expression patterns of RCC1 were not only analyzed in three gastric cell lines but also in clinical gastric carcinoma and adjacent non-tumor tissue microarray (TMA) sections. The TMA chip displays a novel method for the serial analysis of gene expression in hundreds of tissue specimens at a time. Commonly, this method has proven to be highly effective for the analysis of molecular alterations in different

Table 3. Relationship between RCC1 and clinicopathological characteristics of patients with gastric carcinoma

Characteristic	*RCC1 (+) (n=18)*	*RCC1 (-) (n=67)*	*P*-value
Age			0.178[a]
<60	8	40	
≥60	10	27	
Gender			1.000[b]
Male	13	49	
Female	5	18	
Tumor differentiation			0.004[b]
Well	0	10	
Moderate	1	24	
Poor	14	30	
Unknown	3	3	
Stage			1.000[b]
I/II	15	55	
III	3	12	
Depth of invasion			0.044[a]
T1/T2	9	17	
T3/T4	9	50	
Lymph node metastasis			0.348[b]
No	12	53	
Yes	6	14	
Distant metastasis			1.000[b]
No	18	65	
Yes	0	2	

[a]Pearson's chi square; [b]Fisher's exact test.

stages of one particular tumor, such as in gastric, liver, kidney, or prostate cancers [50]. In this study, we examined the immunohistochemical staining of the RCC1 protein in 85 pairs of human gastric carcinoma tissues with adjacent non-tumor tissue plus 10 normal stomach tissues. In the gastric carcinoma tissues, we found a higher percentage of either the loss of or a reduction in RCC1 expression. In contrast, adjacent non-tumor tissues exhibited strong RCC1 expression in the majority of cases (**Table 2**). The significant relationship between the loss or weak expression of RCC1 and poorly differentiated carcinomas might reflect an increased selection pressure on and clonal expansion of the cells in which RCC1 expression has been suppressed, which therefore may exhibit a more aggressive phenotype, such as a greater depth of invasion in gastric cancer.

In conclusion, we demonstrated for the first time that the RCC1 protein is downregulated in gastric carcinoma tissues. This loss of RCC1 expression may be due to hypermethylation of CpG islands in the *RCC1* gene in a region spanning from its promoter region to exon 1, particularly at the 9th CpG site. In addition, we observed strong and significant associations between RCC1 expression and degree of differentiation and depth of invasion, indicating that moderate or poor differentiation is associated with reduced RCC1 expression in gastric cancer cell lines as well as in gastric carcinoma tissues. Therefore, our data suggest that RCC1 may play a tumor suppressor role in gastric carcinoma.

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Disclosure of conflict of interest

None.

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Table S1. MALDI-TOF MS analysis of amplicon products of *RCC1* gene

Human gene	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Amplicon size (bp)
RCC1	aggaagagagGGGTGGGGTTTAATTTG-TAGTAGAT	cagtaatacgactcactatagggagaaggc-tATTCAAAACCAACCTAACCAACATA	515