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• BRIEF REPORTS •

Detection of frameshift mutations of *RIZ* in gastric cancers with microsatellite instability

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Abstract

AIM: To study the frameshift mutations of the retinoblastoma protein-interacting zinc finger gene *RIZ* in gastric cancer with microsatellite instability, and to identify two coding polyadenosine tracts of *RIZ*.

METHODS: Frameshift mutations at $(A)_8$ and $(A)_9$ tracts of *RIZ* were detected in 70 human gastric cancer (HGC) specimens by DHPLC and DNA sequencing. Microsatellite instability (MSI) status was assessed by two mononucleotide markers, BAT26 and BAT25, by means of denaturing high-performance liquid chromatography (DHPLC).

RESULTS: In 70 HGC samples, 8 (11.4%) were found positive for instabilities at BAT26 and BAT25. In 7 of the 8 cases with instabilities at both BAT26 and BAT25 (MSI-H), 1 was unstable at BAT26 but stable at BAT25. Frameshift mutations were identified in 4 (57.1%) of the 7 samples with MSI-H in the $(A)_9$ tract of *RIZ* without mutations in the $(A)_8$ tract. In contrast, frameshift mutations were found in neither of the polyadenosine tracts in 63 samples of MSI-L or MSI stable tumors. Pro704 LOH detection in 4 cases with frameshift mutations did not find LOH in these cases.

CONCLUSION: Frameshift mutations of *RIZ* may play an important role in gastric cancers with MSI.

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INTRODUCTION

Two major pathways of genomic instabilities have been recently recognized, namely the chromosomal instability pathway (CIN) and the microsatellite instability (MSI) pathway (MIN), the former is characterized by the loss of heterozygosity (LOH) whereas the latter by MSI[1]. Some genes contain repetitive regions in their coding sequences that are often targets of MSI. Gastrointestinal tumors with DNA mismatch repair (MMR) defects often present MSI and harbor frameshift mutations in coding mononucleotide repeats of cancer-related genes. MSI (+) tumors arise from the defects in the MMR system through the mechanism presumably involving frameshift mutations of the microsatellite repeats within the coding regions of the affected target genes, whose function loss is believed to contribute to tumorigenesis[2-5].

The retinoblastoma protein-interacting zinc finger gene *RIZ* is a candidate tumor suppressor gene locus on 1p36, a region that commonly harbors alterations in many types of human cancers[6]. The interaction of *RIZ* with the retinoblastoma protein (Rb) suggests its involvement in the control of proliferation by an alternative mechanism. *RIZ* gene encodes two protein products, RIZ1 and RIZ2, which differ for a motif present in the N-terminal domain, defined as the PR domain that was previously identified as a common motif in several transcription factors. RIZ1 contains a PR domain indicative of tumor suppressor function, whereas RIZ2 does not contain this motif[7,8]. RIZ1 has the capacity to induce cell cycle arrest at G_2/M phase and cell apoptosis, and suppresses tumorigenicity in nude mice[9-11]. A role for *RIZ* has been recently proposed in cell cycle arrest and cell apoptosis through a transcriptional repression mechanism[7,12].

In MSI(+) tumors, *RIZ* was found to be affected by frequent frameshift mutations of one or two coding poly(A) tract, an $(A)_8$ tract at the coding nucleotide sequence 4273-4280 and an $(A)_9$ tract at 4462-4471 in exon 8. These mutations generate truncated RIZ1 proteins lacking the COOH-terminal PR-binding motif and are expected to have serious deleterious effects on the PR domain-specific function of RIZ1. *RIZ* plays an important role in hereditary tumors of the MIN pathway as suggested by the frequent frameshift mutations in HNPCC tumors[11]. The role of *RIZ* in gastric MSI(+) tumors remains to be investigated. In this study, we used denaturing high-performance liquid chromatography (DHPLC), a highly productive method, to rapidly detect frameshift mutations of $(A)_8$ and $(A)_9$ tracts and LOH of pro704 in gastric cancer specimens and to explore the role of *RIZ* gene in gastric carcinogenesis.

MATERIALS AND METHODS

Tissue specimen and DNA extraction

Gastric cancer samples and matched adjacent normal gastric tissues were obtained from 41 male and 29 female patients during surgical resection of the tumors with informed consent from the patients at Beijing Institute for Cancer Research, Beijing Cancer Hospital. The fresh samples were collected at the time of surgery and frozen at -80 °C. The sections from each specimen were examined by a pathologist. There were 39 intestinal-type tumors and 31 diffuse-type tumors. High-molecular weight genomic DNA was extracted by standard proteinase K digestion and phenol/ chloroform extraction[13].

Primers and PCR

Primers used for $(A)_8$ tract and $(A)_9$ tract in the *RIZ* gene amplification

were as follows: *RIZ* A8-F 5'-GAGCTCAGCAAAATGTCGTC-3', *RIZ* A8-R 5'-CAAGTCGGCCTTCTGCTTTG-3'; *RIZ* A9-F 5'-TCTCACATCTGCCCTTACTG-3', *RIZ* A9-R 5'-GTGATGAGT GTCCACCTTTC-3'. The *RIZ* Pro704 deletion polymorphism was assayed by PCR followed by DHPLC. The PCR primers were: RP145 5'-CCCAAGATAAACTAACTCCT-3', RP105 5'-ACTCCATGCTGGTGAGTC-3'.

The samples used for mutation screening and sequencing were amplified in 25 μL reaction solution containing 50 ng genomic DNA, 0.4 μmol/L sense and antisense primers for each tract, 200 μmol/L dNTPs (Perkin-Elmer, Foster City, CA, USA), 0.2 μL Taq polymerase (Ampli Taq Gold: Perkin-Elmer), and 2.0 mmol/L $MgCl_2$. After an initial activation of the enzyme by denaturation at 95 °C for 9 min, PCR amplification was performed for 35 cycles in the following sequence: at 94 °C for 30 s, at optimized annealing temperature for 45 s, and at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The annealing temperature for various primer sets was: 58 °C for *RIZ* A_8 tract, 60 °C for *RIZ* A_9 tract, and 55 °C for pro704.

Mutation analysis

For examining the heteroduplex content, 50-100 ng of the PCR products were subjected to DHPLC (WAVE™ system, Transgenomic, USA) under partial denaturation condition. The mobile phase consisted of a mixture of 0.1 mol/L triethylamine acetate (TEAA, pH 7.0) with or without 25% acetonitrile. The flow rate used in this study was 0.9 mL/min. The column temperatures for the PCR products were 57 °C for *RIZ* $(A)_8$ tract and 56 °C for *RIZ* $(A)_9$ tract. The PCR products were heated to 95 °C for 3 min followed by cooling to 25 °C over 45 min. Homozygous mutant DNA must be combined with the wild type at the ratio of approximately 1:1 prior to hybridization.

LOH analysis

The pro704 PCR products were directly used without a denaturation and reannealing process under non-denaturing conditions on the WAVE™ system. The gradient of buffer B from 1 to 7 min was 49-55%. The column temperature was 50 °C and flow rate was 0.75 mL/min.

DNA sequencing

The PCR product was treated with exonuclease and shrimp alkaline phosphatase based on the protocol provided by the United States Biochemical and sequenced by the Mayo Clinic DNA sequencing facility. Sequencing reactions were performed in the GeneAmp PCR System 9600 with fluorescent terminations, and the products were analyzed on an ABI 377 sequencer (Perkin-Elmer, Foster City, CA, USA). All sequence alterations were confirmed by bidirectional sequencing of the PCR products generated by at least two independent reactions.

MSI analysis by DHPLC

MSI analysis by DHPLC was performed as described previously[14]. Briefly, the PCR products were examined by DHPLC under fully denaturing conditions. The flow rate used in this study was 0.9 mL/min, with the column temperature of 80 °C. The gradient of buffer B from 0.1 to 7.1 min was 30-51%.

RESULTS

A total of 70 HGC samples and their matched normal tissues were analyzed for MSI status by two mononucleotide markers,

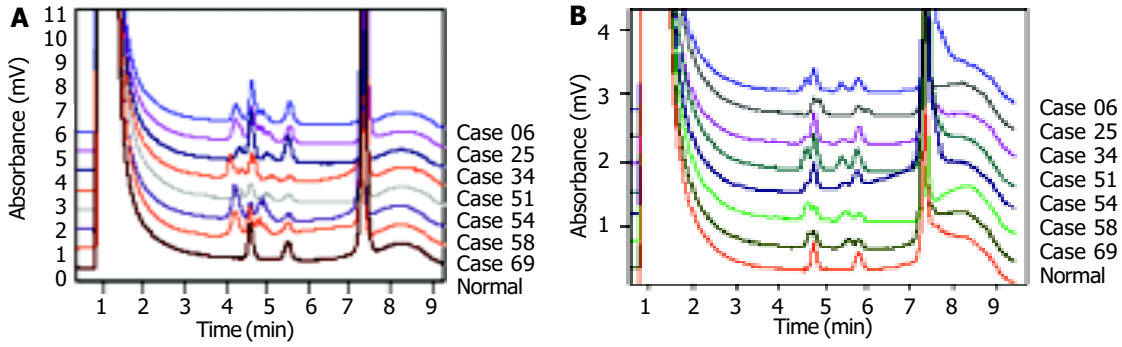

Figure 1 MSI analysis of 70 paired HGCs by DHPLC. Curves at the bottom in both Panels A and B represent normal DNA as indicated by the specification on the bottom right, and the rest curves represent tumor DNA in specified cases. Column: DNASep™; mobile phase: 0.1 mol/L TEAA (pH 7.0); linear gradient: 30-51% B in 7 min; flow rate: 0.9 mL/min; temperature: 80 °C; detection: UV at 260 nm. Panel A: DHPLC chromatograms of HGCs at BAT26; Panel B: DHPLC chromatograms of HGCs at BAT25.

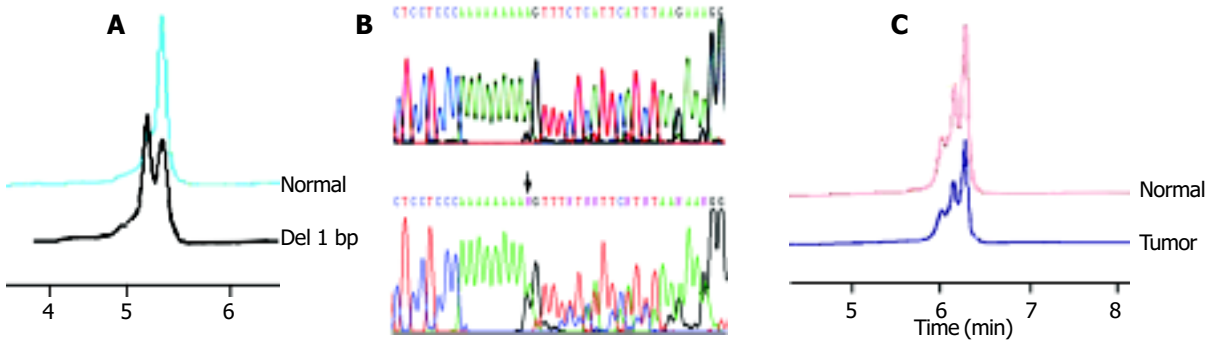

Figure 2 Typical elution profiles of DHPLC analysis and sequencing. Panel A: DHPLC elution profiles for *RIZ*$(A)_9$ tract, column temperature: 56 °C, flow rate: 0.9 mL/min, mobile phase: 0.1 mol/L TEAA (pH 7.0), linear gradient: 51-60% B in 4.5 min, detected with UV at 260 nm; Panel B: Sequence tracings for the same samples. The upper panel is a normal control, and the lower is a frameshift mutation (1-bp deletion). The arrow indicates the mutant nucleotide; Panel C: DHPLC elution profiles for pro704 LOH, column temperature: 50 °C, flow rate: 0.75 mL/min, mobile phase: 0.1 mol/L TEAA (pH 7.0), linear gradient: 49-55% B in 7 min, detected with UV at 260 nm.

BAT26 and BAT25, by means of DHPLC. In 70 HGC samples, 8 (11.4%) were found to contain sequence variation at BAT26 and BAT25 and 7 of them were shown to be unstable at both BAT26 and BAT25 (Figure 1), classified as MSI-H. One was unstable at BAT26 but stable at BAT25.

Frameshift mutations of *RIZ* $(A)_9$ and $(A)_8$ tract were identified. HCT-116 cell line had been studied previously and frameshift mutations were identified in the polyadenosine tracts[11], which served as the positive control in this study. In 7 samples with MSI-H, 4 (57.1%) were found to have mutations in the $(A)_9$ tract of *RIZ* by DHPLC and DNA sequencing, including 3 intestinal-type tumors and 1 diffuse-type tumor. All of them had a 1-bp deletion (Figure 2A, B). No mutations were detected in the $(A)_8$ tract. No mutations in the $(A)_9$ or $(A)_8$ tract were found in the 63 MSI-L or MSI stable samples.

To determine whether *RIZ* was also affected by chromosomal deletion in MSI(+) tumors, pro704 LOH studies were performed on 4 samples with frameshift mutations for which the matched normal DNAs were available. We observed that both the PCR products of the tumor DNA and matched normal DNA resulted in the same peak chromatogram on DHPLC under the condition used for DNA sizing at 50 °C. LOH was not found at the *RIZ* locus in these tumors (Figure 2C).

DISCUSSION

Approximately 10-15% of gastrointestinal tumors are caused by defective MMR[15], characterized by the presence of tumor MSI (MSI-H) and the absence of protein expression for any of the various genes involved in DNA MMR including hMLH1, hMSH2, hMSH6 or hPMS2[16]. Gastric cancer with an MSI-H phenotype often harbors somatic frameshift mutations in the coding mononucleotide repeats of cancer-related genes. Frameshift mutations in *TGFßRII*, *BAX*, *IGFIIR* and *E2F4* genes are often observed in cancers exhibiting a high frequency of MSI[17]. Recently, the new candidate tumor suppressor gene, *RIZ*, which may be targeted for deletion, was identified. RIZ is a protein with two alternative forms of RIZ1 and RIZ2, which differ for a PR domain present in the N-terminal domain. The PR domain is necessary for the negative regulatory function of *RIZ*. Frameshift mutations in either $(A)_8$ or $(A)_9$ tract are thought to lead to C-terminal domain loss of the RIZ protein involved in PR binding. In this study, we detected frameshift mutations in *RIZ* $(A)_9$ tract, whereas no mutation was found in $(A)_8$ tract. All of the frameshift mutations here found in *RIZ* are assumed to lead to the production of the COOH-terminal domain-truncated protein, which is likely to seriously affect RIZ1 functions.

Frameshift in short mononucleotide tracts is common in gastrointestinal tumors of the microsatellite mutator phenotype (MMP). MSI is considered a hallmark of the mutator phenotype, and determination of MSI is critical for understanding tumor biology. Separation of HGCs based on their mutator phenotypes is an effective first step to allow the distinction of these two different pathways of carcinogenesis. In the present study, we analyzed MSI status by two mononucleotide markers, and detected *RIZ* mutations in 4 (57.1%) of the 7 MSI-H tumors but in none of the 63 MSI-L or MSI stable gastric cancers, indicating that these mutations are specific for MSI-H tumors that exhibit a tendency to accumulate frameshift mutations in reiterated sequence of the coding regions of cancer-related genes known to facilitate cancer development and progression. These mutations may contribute to cancer progression either by inactivating their tumor suppressor functions or acting as secondary mutator mutations in MMP(+) gastric tumors[18]. Our study has shown that MSI-H gastric cancers accumulate frameshift mutations in the *RIZ* gene. On the basis of our findings, we suppose that *RIZ* is a candidate target gene in MSI tumorigenesis.

Two kinds of genetic instability, MIN and CIN, have been documented in colorectal cancers. To determine whether *RIZ* is also affected by chromosomal deletion in MSI-H cancers, we detected the pro704 LOH in the 4 cases with frameshift mutations but failed to find LOH at the *RIZ* locus in these tumors, suggesting that *RIZ* frameshift mutations are common in MSI(+) gastric cancers, whereas LOH is not. More extensive studies on gastric cancers are needed to clarify whether *RIZ* is affected by two different ways. In MSI(+) tumors (MIN pathway), frameshift mutations in the COOH-terminal interfere with the interactions between the C terminus of the protein and its PR domain. In MSI(-) tumors (CIN pathway), mutations or deletions of the PR domain of *RIZ1* may have similar effects.

In this study, we detected MSI status and LOH by DHPLC analysis. DHPLC has been described as a novel technology for the detection of gene mutations in inherited diseases or cancers and for the identification of single nucleotide polymorphisms (SNPs)[19]. The present study demonstrates the efficacy of DHPLC in analysis of the MSI status and LOH, which allows automated examination of MSI and LOH with considerable precision at relatively low cost, without any special labeling procedure.

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