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Advances in Brief

Frequent Frameshift Mutations of *RIZ* in Sporadic Gastrointestinal and Endometrial Carcinomas with Microsatellite Instability[1]

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Abstract

Many lines of evidence suggest that the retinoblastoma protein interacting zinc finger gene *RIZ* is a strong candidate for the tumor suppressor locus on 1p36, a region commonly deleted in many human cancers with chromosomal instability. In addition, a role for *RIZ* in tumors of the microsatellite instability pathway is suggested by frequent frameshift mutations in hereditary non-polyposis colorectal carcinomas. Here we studied *RIZ* mutations in sporadic cancers with microsatellite instability. Frameshift mutations in the two coding polyadenosine tracks of *RIZ* were found in 19 (48%) of 40 gastric carcinomas, 6 (33%) of 18 endometrial carcinomas, 14 (26%) of 51 of colorectal carcinomas, and 7 (54%) of 13 cell lines. Eleven tumor tissues showed biallelic inactivation of *RIZ*. In contrast, no frameshift mutations were found in 70 microsatellite stable tumors. These results suggest an important role for *RIZ* in sporadic cancers with microsatellite instability.

Introduction

It is now widely accepted that cancer is the result of an accumulation of mutations in cellular cancer-causing genes. The mutations are thought to be driven by genetic instabilities, which commonly characterize tumor cells. Two major pathways of instabilities have been recognized, CIN[3] and MIN (1). The hallmarks of tumors of the CIN pathway are aneuploidy and LOH. In contrast, tumors of the MIN pathway are usually diploid and show massive instability in simple repeated sequences or microsatellites. In addition, epigenetic events can also facilitate genetic damage, as illustrated by the increased mutagenicity of 5-methylcytosine and the silencing of the MLH1 mismatch repair gene by DNA methylation in colorectal tumors (2).

MSI-positive (+) tumors are caused by defects in the mismatch repair system (3–5). MSI has been detected in HNPCC and HNPCC-associated tumors such as gastric carcinomas and endometrial carcinomas, as well as in many sporadic cases of these tumors (6). The mechanism of tumorigenesis of MSI(+) tumors is thought to involve frameshift mutations of microsatellite repeats within coding regions of affected target genes the inactivation of which directly contributes to tumor development (7).

The retinoblastoma protein-interacting zinc finger gene *RIZ* is a candidate tumor suppressor gene belonging to the PR or SET domain family of chromosomal regulators involved in chromatin-mediated gene activation and silencing (8, 9). The PR domain of *RIZ* is a protein-binding interface and can interact with a motif present in the COOH region of *RIZ* (9). The PR/SET domain family plays an important role in human cancers as evidenced by genetic mutations of several family members (10). *RIZ* gene normally produces two protein products, RIZ1 and RIZ2, that differ at the NH_2-region by the presence or absence of the PR domain (11). The RIZ1 (PR^+) product is considered a strong candidate for the tumor suppressor(s) on 1p36, a region commonly deleted in more than a dozen different types of human cancers (12). RIZ1 gene expression but not RIZ2 is commonly silenced in all of the human cancers examined including those of breast, liver, colon, and neuroendocrine tissues (13, 14). Consistently, RIZ1 has the capacity to induce G_2-M cell cycle arrest, apoptosis, or both, and tumor suppression in mice (13, 14). These observations suggest a role for *RIZ* in many tumors of the CIN pathway.

RIZ also appears to play an important role in hereditary tumors of the MIN pathway as suggested by the frequent frameshift mutations in HNPCC tumors (15). The mutations are located at two polyadenosine tracks within the coding region of *RIZ:* one $(A)_8$ track at coding nucleotide position 4273–4280 and one $(A)_9$ track at 4462–4471 in exon 8. These mutations generate truncated RIZ1/2 proteins lacking the COOH-terminal PR-binding motif and are expected to have serious deleterious effects on the PR domain-specific function of RIZ1.

Hereditary and sporadic MSI(+) colorectal carcinomas are caused by different tumorigenic events. Genetic defects in MSH2 and MSH3 causes HNPCC, and epigenetic silencing of MLH1 is associated with MSI(+) sporadic colorectal carcinomas (2). The role of *RIZ* in sporadic MSI(+) tumors remains to be investigated. Here we describe frequent frameshift mutations of *RIZ* in sporadic gastrointestinal and endometrial cancers. Importantly, many of these were biallelic or homozygous/hemizygous mutations, which suggests that *RIZ* inactivation is highly selected during the clonal evolution of these tumors.

Materials and Methods

Cell Lines and Tumor Samples. Fourteen cell lines were purchased from American Type Culture Collection. These were derived from colon (HCT116, SW-48, LOVO, LS441N, LS180, LS174T, DLD1, HCT15, HCT8), prostate (DU145), breast (Cal-51), and uterus (AN3CA, SK-UT-1B) cancers. MSI(−) colon cancer cell line SW620 was also included as controls. A total of 179 primary gastrointestinal and endometrial tumors from patients undergoing surgery were analyzed. Among them, 109 tumors were characterized as MSI-High including 40 gastric carcinomas, 18 endometrial cancers, and 51 colorectal cancers. The source of tumor samples has been described previously (16–18).

Analysis of MSI. MSI-High status in primary tumors was defined according to the criteria proposed by Boland *et al.* (19).

Mutation Analysis of *RIZ*. Frameshift mutations at $(A)_8$ and $(A)_9$ tracks in the *RIZ* were detected by PCR with Vent DNA polymerase and SSCP analysis, followed by sequencing. The $(A)_8$ track was amplified by PCR with primers RIZA8-F, 5′-GAGCTCAGCAAAATGTCGTC-3′, and RIZA9-R, 5′-CAAGTCGGCCTTCTGCTTTG-3′. The $(A)_9$ track was amplified by PCR with primers RIZA9-F, 5′-TCTCACATCTGCCCTTACTG-3′, and RIZA9-R, 5′-

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[3] The abbreviations used are: CIN, chromosomal instability; MIN, microsatellite instability; LOH, loss of heterozygosity; MSI, microsatellite instability; HNPCC, hereditary non-polyposis colorectal cancer; PR, PRDI-BF1-RIZ1 homology; SET, Suvar 3–9, Enhancer-of-zeste, Trithorax; SSCP, single-strand conformational polymorphism.

Table 1 *Frameshift mutations of RIZ in gastric carcinomas, endometrial carcinomas, and colorectal cancers and cell lines with MSI*

		Pro704		
Sample	Tissue type	Allele 1	Allele 2	*RIZ* mutation
KS02	Gastric	I[a]	I	$(A)_9\Delta A$
KS06	Gastric	NI	NI	$(A)_9\Delta A$
KS07	Gastric		LOH	$(A)_9\Delta A$
KS12	Gastric	NI	NI	$(A)_9\Delta A$
KS15	Gastric	NI	NI	$(A)_9\Delta A$ $(A)_9\Delta A$
KS18	Gastric	NI	NI	$(A)_9\Delta AA$
KS19	Gastric	NI	NI	$(A)_9\Delta A$ $(A)_8\Delta A$; $(A)_9\Delta A$
KS20	Gastric		LOH	$(A)_9\Delta A$
KS21	Gastric	I	I	$(A)_9\Delta A$
KS22	Gastric	ND	ND	$(A)_9\Delta A$
KS25	Gastric	I	I	$(A)_9\Delta A$
KS31	Gastric	NI	NI	$(A)_9\Delta AA$
KS33	Gastric	I	I	$(A)_9\Delta A$
KS34	Gastric	NI	NI	$(A)_9\Delta A$
KS36	Gastric	NI	NI	$(A)_9\Delta A$
E4	Endometrial	ND	ND	$(A)_9\Delta A$ $(A)_9\Delta AA$
E55	Endometrial	ND	ND	$(A)_9\Delta A$
E67	Endometrial	ND	ND	$(A)_9\Delta A$
E68	Endometrial	ND	ND	$(A)_9\Delta A$ $(A)_9\Delta AA$
E75	Endometrial	ND	ND	$(A)_9\Delta AA$ $(A)_8\Delta A$
E505	Endometrial	ND	ND	$(A)_9\Delta AA$ $(A)_9\Delta AA$
AC61	Colon	NI	NI	$(A)_9\Delta A$ $(A)_9\Delta A$
AC149	Colon	NI	NI	$(A)_9\Delta A$
AC334	Colon	ND		$(A)_8\Delta A$; $(A)_9\Delta A$ $(A)_9\Delta A$
AC353	Colon	ND		$(A)_9\Delta A$
AC410	Colon	I	I	$(A)_9\Delta AA$
AC411	Colon	NI	NI	$(A)_9\Delta A$
AC469	Colon	I	I	$(A)_9\Delta A$ $(A)_9\Delta A$
AC558	Colon	I	I	$(A)_9\Delta A$
AC584	Colon	I	I	$(A)_8\Delta A$
AC590	Colon	NI	NI	$(A)_9\Delta A$
IC31	Colon	ND	ND	$(A)_9\Delta A$
IC70	Colon	ND	ND	$(A)_9A$
IC824	Colon	ND	ND	$(A)_9\Delta AA$
IC2513	Colon	ND	ND	$(A)_9\Delta A$
AS95	Colon	I	I	$(A)_9\Delta A$
AS109	Colon	I	I	$(A)_9\Delta A$
AS111	Colon	NI	NI	$(A)_9\Delta A$
HCT116	Colon	ND	ND	$(A)_9\Delta A$ $(A)_9\Delta A$
LOVO	Colon	ND	ND	$(A)_9\Delta A$
LS411N	Colon	ND	ND	$(A)_9\Delta A$
LS180	Colon	ND	ND	$(A)_9\Delta A$
LS174T	Colon	ND	ND	$(A)_9\Delta A$
HCT-8	Colon	ND	ND	$(A)_9\Delta A$
AN3CA	Endometrium	ND	ND	$(A)_9\Delta A$ $(A)_9\Delta AA$

[a] I, informative; NI, not informative; ND, not determined; ΔA, deletion of one A.

GTGATGAGTGTCCACCTTTC-3′. PCR was carried out with Vent DNA polymerase as described previously (17). PCR was performed with primers RIZA8-F and RIZA-9R for SSCP analysis as described (17). The mutated bands in SSCP gel were sequenced using the Big Dye terminator cycle sequencing kit (Perkin-Elmer Corp.).

Assessment of LOH. The *RIZ* Pro704 deletion polymorphism, which has been discovered during sequence analysis of RIZ, was assayed by PCR, then followed by electrophoresis on denaturing gel as described previously (20). The PCR primers were RP145 (5′-CCC AAG ATA AAC TAA CTC CT-3′) and RP105 (5′-ACT CCA TGC TGG TGA GTC-3′).

Results and Discussion

We detected *RIZ* mutations in 19 (48%) of 40 MSI(+) gastric carcinomas, 6 (33%) of 18 endometrial cancers, 14 (26%) of 51 colorectal carcinomas, and 7 (54%) of 13 MSI(+) cell lines (Fig. 1 and Table 1). Some of the cell lines have also been studied by Chadwick *et al.* (15) and served as positive control for our studies here. The mutations found in tumor tissues were somatic because the corresponding normal counterparts were wild type. With the exception of a mutation in the $(A)_8$ track in KS19, E75, AC334, and AC590, all of the mutations targeted the $(A)_9$ track. No mutations in the $(A)_8$ or $(A)_9$ track were found in 70 MSI(−) gastric carcinomas, which indicated that these mutations are specific for MSI(+) tumors (46/122 *versus* 0/70; $P = 0.000000$ by Fisher's exact test).

Among the 46 cases with *RIZ* mutations (7 cell lines, 19 gastric carcinomas, 6 endometrial carcinomas, and 14 colorectal carcinomas), 11 cases (KS15, KS19, E4, E68, E75, E505, AC91, AC334, AC469, HCT-116, AN3CA) were biallelic mutations. KS15, E68, E505, AC91, AC469, and HCT-116 showed homozygous/hemizygous mutations (Fig. 1 and Table 1). KS19 and AC334 had a 1-bp deletion at both $(A)_9$ and $(A)_8$ tracks in one allele and a 1-bp deletion at $(A)_9$ track in the other allele, whereas E4 and AN3CA showed a 1-bp deletion at $(A)_9$ track in one allele and a 2-bp deletion at $(A)_9$ track in the other allele. E75 had a 1-bp deletion at $(A)_8$ track in one allele and 2-bp deletion at $(A)_9$ track in the other allele. To determine whether *RIZ* is also affected by chromosomal deletions in MSI(+) cancers, LOH studies were performed on 25 cases with frameshift mutations for which the matched normal DNAs were available. The RIZpro704 deletion polymorphism, a three-nucleotide deletion at codon Pro704 in exon 8 (20), allowed us to detect LOH in 2 of 12 informative tumors, KS07 and KS20, which had frameshift mutations in one allele (Table 1). Therefore, 11 cancers (KS07, KS15, KS19, KS20, E4, E68, E75, E505, AC91, AC334, AC469) and 2 cell lines (HCT-116, AN3CA) had evidence of biallelic inactivation of *RIZ*. The high frequency of mutation and biallelic inactivation suggest that frameshift mutation of *RIZ* is clonally selected during tumorigenesis.

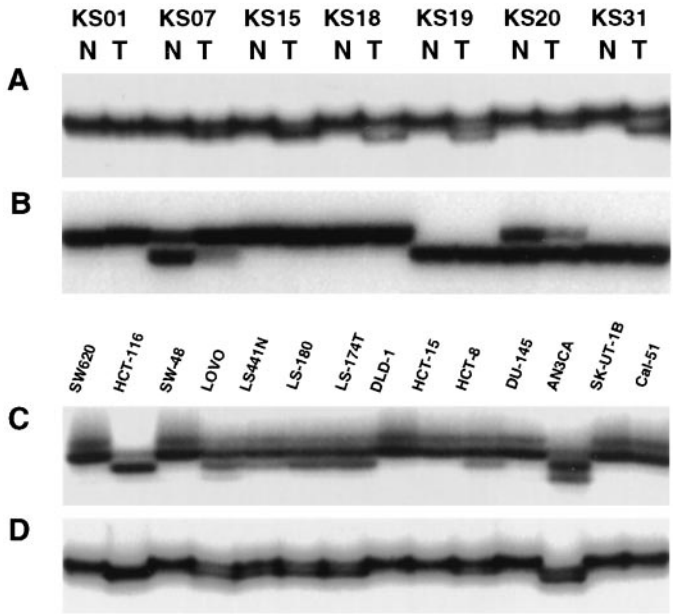

Fig. 1. Frameshift mutations of *RIZ* at coding (A_8) and (A_9) tracks in gastric carcinomas and cell lines with MSI. *A*, LOH analysis of gastric carcinomas using RIZpro704 deletion polymorphism. Case numbers are shown *at the top* with matched normal (*N*) and tumor (*T*) DNA. Tumor KS07 lost lower allele, and tumor KS20 lost upper allele. *B*, SSCP analysis of PCR products amplified with primers RIZA8-F and RIZA9-R including both $(A)_8$ and $(A)_9$ tracks. Tumor KS07 and KS20 carried a 1-bp deletion at $(A)_9$ track. Tumor KS18 and KS31 carried a 2-bp deletion at $(A)_9$ track. Tumor KS19 had two mutation bands: *upper one* had a 1-bp deletion at $(A)_9$ track; *lower one* had a 1-bp deletion at both $(A)_8$ and $(A)_9$ tracks. Tumor KS15 had a homozygous or hemizygous 1-bp deletion at $(A)_9$ track. *C*, denaturing gel electrophoresis analysis of PCR products amplified with primers RIZA9-F and RIZA9-R using Vent DNA polymerase. The specific MSI and control cell lines are indicated *at the top*. One-bp deletion was seen in HCT-116, LS441N, LS-180, LS174T, DLD-1, and DU-145. HCT-116 was a homozygous mutation. AN3CA had two mutations: one was 1-bp deletion at $(A)_9$ track, and the other one was 2-bp deletion at $(A)_9$ track. *D*, SSCP analysis of PCR products with primers RIZA8-F and RIZA9-R including both $(A)_8$ and $(A)_9$ tracks. SSCP results were consistent with those of denaturing gel electrophoresis as shown in *C*.

All of the frameshift mutations here found in *RIZ* are predicted to lead to the production of the COOH-terminal domain truncated proteins. The deletion of one adenosine in the $(A)_8$ track at amino acid residue 1436 produces a stop codon two residues from the deletion and a mutant protein lacking COOH-terminal 293 amino acids. It has been demonstrated that the COOH-terminal domain (amino acid 1502–1668 of RIZ1) is a PR domain-binding motif, which may play a role in RIZ1 folding or binding to other proteins or RIZ1 itself (oligomerizaton; 9). Thus, deletion of this COOH-terminal protein-binding interface is likely to seriously affect RIZ1 functions.

Our results show that RIZ frameshift mutations are common in sporadic MSI(+) cancers, including gastric, endometrial, and colorectal carcinomas. Importantly, many of these mutations are biallelic or homozygous/hemizygous, which suggests that *RIZ* fits the Knudson's two-hit model of tumor suppressor genes (21). Given the characteristic low frequency of LOH in MSI(+) tumors, it is not surprising that LOH is not commonly found at the *RIZ* locus in these tumors. Future studies will be required to determine whether other genetic or epigenetic events may be responsible for the inactivation of the remaining wild-type allele. Biallelic inactivation of *RIZ* was not found in the recent study on HNPCC tumors (15). This may be related to the genetic differences between hereditary and sporadic cancers but more extensive studies on HNPCC will be required to determine whether heterozygous *RIZ* mutation is a characteristic of this tumor.

Together with the location on 1p36 and the silencing in many cancers, our finding here suggests that *RIZ* is a common target of both the CIN and MIN pathways of cancer. In view of the recent report of a role of RIZ in estrogen-receptor signaling (22), our finding of *RIZ* mutation in endometrial carcinomas is consistent with an important role in the hormone-dependent growth-control pathways in the endometrium.

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