**RIL, a LIM Gene on 5q31, Is Silenced by Methylation in Cancer and Sensitizes Cancer Cells to Apoptosis**

Yanis A. Boumer,1,2 Yutaka Kondo,1 Xuqi Chen,1 Lanlan Shen,1 Vazganush Gharibyan,1 Kazuo Konishi,1 Elihu Estey,1 Hagop Kantarjian,1 Guillermo Garcia-Manero,1 and Jean-Pierre J. Issa1,2

1Department of Leukemia, The University of Texas M. D. Anderson Cancer Center; 2Program in Cancer Biology, The University of Texas Graduate School of Biomedical Sciences, Houston, Texas

**Abstract**

Gene silencing associated with promoter methylation can inactivate tumor suppressor genes (TSG) in cancer. We identified **RIL**, a LIM domain gene mapping to 5q31, a region frequently deleted in acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS), as methylated in 55 of 79 (70%) of cancer cell lines tested. In a variety of primary tumors, we found RIL methylation in 55 of 92 (60%) cases, with highest methylation in AML and colon cancer, and in 30 of 83 (36%) MDS samples, whereas normal tissues showed either absence or substantially lower levels of methylation, which correlates with age. RIL is ubiquitously expressed but silenced in methylated cancers and could be reactivated by the hypomethylating agent 5-aza-2’-deoxycytidine. Restoring RIL expression in colon cancer cells by stable transfection resulted in reduced cell growth and clonogenicity and an ~2.0-fold increase in apoptosis following UV exposure. In MDS, RIL methylation is a marker of adverse prognosis independent of chromosome 5 and 7 deletions. Our data suggest that **RIL** is a good candidate TSG silenced by hypermethylation in cancer. [Cancer Res 2007;67(5):1997–2005]

**Introduction**

Tumor suppressor genes (TSG) can be inactivated in tumors by several mechanisms, including deletion [measured by loss of heterozygosity (LOH)] and mutation in the genomic sequence, or by epigenetic mechanisms, such as promoter DNA methylation. Most of the important TSG are lost by a combination of these mechanisms, with one or another mechanism prevailing depending on the type of tumor (1, 2). About 30% of all human genes have CpG islands. Methylation in these islands causes recruitment of CpG-binding proteins, histone deacetylases, histone methyltransferases, and associated repressor and DNA-binding proteins, which then lead to loss of expression and permanent gene silencing (3, 4). These changes may reinforce each other and thus result in a silencing loop (5, 6). In cancer, DNA methylation is a powerful way of inactivating tumor suppressor and DNA repair genes as shown for p16, RB, VHL, hMLH1, MGMT, etc. (7). This has led to the development of several techniques to clone novel TSGs based on aberrant DNA methylation, including arbitrarily primed PCR, restriction landmark genomic scanning, and methylated CpG island amplification-representational difference analysis (MCA/RDA; refs. 8–10). MCA/RDA is a powerful tool that allows PCR amplification and cloning novel CpG islands of genes that are hypermethylated in cancer (10).

LOH is commonly used to identify TSGs. In myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML), LOH or cytogenetic abnormalities involving 5q are common and carry a poor prognosis (11–13). Loss of chromosome 5 or deletion of the long arm [del(5q)] is a common abnormality in myeloid diseases, observed in ~10% of patients with MDS and de novo AML and in 40% of patients with therapy-related MDS or AML (14, 15). Common deletions in these malignancies implicate the presence of a possible TSG that might be important in these diseases. Several candidate TSGs were proposed in this region, including *IRF-1* (16). However, these genes are infrequently inactivated biallelically in leukemia.

Here, we report the MCA/RDA-based identification of **RIL**, a LIM domain gene mapping to 5q31.1, as a candidate tumor suppressor in this region. We show that **RIL** is frequently methylated in cancer, that methylation is associated with loss of expression, and that **RIL** reexpression leads to a suppression of cell growth and clonogenicity in soft agar and sensitizes cells to apoptosis. Finally, we found that **RIL** methylation is an adverse prognostic factor in MDS. Our data suggest that **RIL** is a strong candidate TSG for the 5q31 region.

**Materials and Methods**

**Cell lines and culture conditions.** HCT116 and RKO cells were grown in high glucose DMEM (Life Technologies, Gaithersburg, MD) plus 10% fetal bovine serum (FBS; InterGen, Purchase, NY), whereas HL-60 and ML-1 cells were grown in RPMI 1640 plus 10% FBS in plastic tissue culture plates in a humidified atmosphere containing 5% CO2 at 37°C.

**Human samples.** Samples of primary solid tumors and hematologic tumors were obtained from established tissue banks at M. D. Anderson Cancer Center and Johns Hopkins University. All samples were collected from consenting patients according to institutional guidelines.

**DNA, RNA extraction, and reverse transcription-PCR.** DNA was extracted using the phenol-chloroform method. Total cellular RNA was extracted with Trizol (Life Technologies) according to the manufacturer’s protocol and resuspended in diethylpyrocarbonate-treated water. Reverse transcription reactions were done using Moloney murine leukemia virus reverse transcriptase (Roche, Indianapolis, IN) on 2 μg of total RNA per reaction according to the manufacturer’s protocol. **RIL** and gyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR were done using the oligonucleotide primers shown in Supplementary Table S1. We initially confirmed
that PCR amplifications were done in the linear range and all reactions included negative controls where reverse transcriptase was omitted.

Methylated CpG island amplification-representational difference analysis. MCA/RDA was done as described (10). Briefly, 5 μg DNA was digested with SmaI followed by XmnI (New England Biolabs, Ipswich, MA). The restriction fragments were ligated to adapters and amplified by PCR. The reaction mixture was incubated at 72°C for 5 min and at 95°C for 3 min and then subjected to 25 cycles of 1 min at 95°C and 3 min at 77°C followed by a final extension of 10 min at 77°C. The MCA amplicon from the chronic myelogenous leukemia (CML) cell line K562 was used as the tester for RDA, and a MCA amplicon generated from a mixture of DNA from normal blood was used as the driver. RDA was done on these MCA amplicons using different adapters, JMCA and NMCA. After the third round of competitive hybridization and selective amplification, the RDA products were cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced.

Bisulfite modification of DNA. Bisulfite induces deamination of unmethylated cytosines, converting unmethylated CpG sites to UpG without modifying methylated sites. Bisulfite treatment of genomic DNA was done as described (17). DNA (2 μg) was used for bisulfite treatment. DNA was denatured in 0.2 N NaOH at 37°C for 10 min and incubated with 3 mol/L sodium bisulfite at 50°C for 16 h. Bisulfite-converted DNA was purified using the Wizard Clean-Up System (Promega, Madison, WI) and desulfonylated with 0.3 N NaOH at 25°C for 5 min. DNA was then precipitated with ammonium acetate and ethanol, dried, and resuspended in H2O.

Combined bisulfite restriction assay analysis and bisulfite sequencing. PCRs were carried out in 50 μL reactions using the oligonucleotide primers shown in Supplementary Table S1; combined bisulfite restriction assay (COBRA) primers are complementary to the RIL antisense strand. In each reaction, 2 μL of bisulfite-treated DNA, as well as 1.25 mmol/L deoxynucleotide triphosphate, 67 mmol/L Tris-HCl (pH 8.8), 16 mmol/L ammonium sulfate, 10 mmol/L β-mercaptoethanol, 0.1 mg/mL bovine serum albumin, 10 pmol of primers, and 1 unit of Taq polymerase, were used. All PCRs were done using a hot start at 95°C for 5 min. After amplification, PCR products were digested with the HpyCH4IV restriction enzyme (New England Biolabs), which digests alleles that were methylated before bisulfite treatment. The digested DNA was separated on non-denaturing polyacrylamide gels and stained with ethidium bromide. The proportion of methylated versus unmethylated product (digested versus undigested) was quantitated by densitometric analysis, done using a Bio-Rad (Hercules, CA) Geldoc: 2000 digital analyzer equipped with theQuantity One version 4.0.3 software. In case of bisulfite sequencing, the restriction enzyme digestion step was omitted; PCR products were directly cloned into a TOPO TA vector and individual clones were sequenced.

Pyrosequencing. To study age-related methylation in normal colon samples, we used the pyrosequencing method (18). For PCR, we used 2 μL of bisulfite-treated DNA, 1.25 mmol/L deoxynucleotide triphosphate, 1 unit of Taq polymerase and the PCR buffer mentioned above, 10 pmol forward primer, 1 pmol reverse universal primer, and 9 pmol universal biotinylated primer (assays D, E, F, and G). In assay A, we used 1 pmol forward universal primer, 10 pmol reverse primer, and 9 pmol universal biotinylated primer. In assays B and C, the reverse primer was directly biotinylated. In a two-step PCR (assay D), the first step used forward and reverse universal primers only. All primer sequences and conditions are shown in Supplementary Table S1. The final biotin-labeled PCR product was captured by Streptavidin-Sepharose HP (Amersham Biosciences, Uppsala, Sweden). PCR products bound on the bead were purified and made single stranded using a Pyrosequencing Vacuum Prep Tool (Biotage, Sweden). The sequencing primers (0.3 μmol/L; Supplementary Table S1) were annealed to the single-stranded PCR product, and pyrosequencing was done using the Pyrosequencing System (Biotage). Quantiﬁcation of cytosine methylation was done using the provided software (PSQ HS 96A 1.2).

Plasmids and transfections. The open reading frame encoding RIL, the open reading frame encoding RIL, was PCR ampliﬁed from expressed sequence tag clone 169666 (ResGen-Invitrogen, Carlsbad, CA) using the CDS1 and CDS2 primers (Supplementary Table S1). The PCR products were directly cloned into pcDNA3.1/V5-HisTOPO TA vector (Invitrogen). Epitope-tagged expression plasmids were constructed by PCR using the CDS1 and CDS3 primers with eliminated stop codon in CDS3 for generation of V5/His-tagged protein. Plasmids were veriﬁed by sequencing and transferred using Fugene 6 reagent (Roche) according to the manufacturer’s protocol.

Mutation screening (single-strand conformational polymorphism, direct sequencing). The single-strand conformational polymorphism (SSCP) technique has been described previously (19, 20). Electrophoresis was carried out under different conditions on a SSCP mutation detection apparatus (Bio-Rad). The gels were stained with Silver Stain Plus kit (Bio-Rad), and the abnormally migrating bands were cut out, heated with double-distilled water, and puriﬁed using DNA absorbing resin (Promega). The puriﬁed products were reampliﬁed by PCR and sequenced at the M. D. Anderson Cancer Center sequencing facility. In some cases, PCR products from each exon were also directly sequenced. Primers and conditions are available on request.

In vitro cell growth and colony formation assay. For growth curves, 5 × 103 cells were added to six-well plates in triplicates and counted in a hemocytometer, with trypan blue exclusion, at 48-, 72-, and 144-h time points. For colony formation assay, 1 × 103 cells were mixed with 0.3% agarose and solidiﬁed on an underlying 0.6% agarose layer in six-well plates, in triplicates. Three weeks later, colonies were stained with p-ionotroten-trazolium violet and counted under the light microscope.

5-Aza-2′-deoxycytidine, trichostatin A, UV, and staurosporine treatment of cells. Cells were split 12 to 24 h before treatment and then treated with the following: (a) 5-aza-2′-deoxycytidine (5-aza-dC; 5 μmol/L; Sigma, St. Louis, MO) or PBS for 72 h (media containing 5-aza-dC or PBS were changed every 24 h); (b) trichostatin A (TSA; 300 nmol/L; ICN Biomedicals, Costa Mesa, CA) or an identical volume of ethanol added for 24 h; (c) 5-aza-dC/TSA [5-aza-dC (5 μmol/L) was used for 48 h followed by TSA (300 nmol/L) for an additional 24 h]; (d) UVC (254 nm, 30 J/m2); cells were collected at 1, 2, or 3 h (RKO cell line) or at 20, 24, or 30 h past treatment (HCT116 cell line); (e) staurosporine (4 μmol/L Sigma) or PBS for 24 or 48 h (media containing staurosporine or PBS were changed every 24 h).

Western blot analysis. Cellular protein (30–50 μg) was resolved by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in TBS [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl] containing 0.1% Tween 20 and then incubated with poly(ADP-ribose) polymerase (PARP; BD Pharmingen), anti-V5 (Invitrogen, San Diego, CA), or β-actin (Sigma) monoclonal antibodies. The primary antibody complex was then stained with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham Pharmacia Biotech, United Kingdom). Protein bands were visualized by enhanced chemiluminescence (ECL, Western Blotting Detection System, Amersham Pharmacia Biotech) using X-ray imaging film (Kodak, Rochester, NY).

Flow cytometry analysis (propidium iodide staining). To determine changes in cell cycle and apoptosis, 1 × 106 cells were seeded in 100-mm plates with or without treatment. At certain time points [2 and 3 h past UV treatment at 30 J/m2 dose (RKO), 20 and 30 h past UV treatment at 30 J/m2 dose (HCT116), and 24 and 48 h after treatment with staurosporine for both cell lines], both floating and attached cells were trypsinized [viability was determined by trypan blue exclusion (as described above)], fixed in 70% ethanol, and stored in ethanol before analysis. Cell cycle distribution and the number of apoptotic cells (detected as a sub-G1 fraction) were determined after propidium iodide staining and analysis using a Becton Dickinson FACSscan (San Diego, CA).

Annexin V staining. To measure and compare changes in apoptosis, 1 × 106 cells were seeded in 100-mm dishes and treated as described above, immediately trypsinized, counted for trypan blue exclusion, washed in PBS, and reconstituted in 100 μL binding buffer containing Annexin V (Annexin V-Fluos kit, Roche). Cells were analyzed by flow cytometry after the addition of propidium iodide. Annexin V binds to the cells that express phosphatidylserine on the outer layer of the membrane, and propidium iodide stains the cellular DNA in cells with compromised, permeable membrane. This allows one to discriminate between live (unstained), apoptotic (Annexin V positive), and necrotic cells (stained with both Annexin V and propidium iodide).
Statistics. To analyze methylation levels across the CpG island, we calculated average ± SEM for each region studied within the same group of patients (N = 10) using Microsoft Excel. To estimate correlation between methylation density and age for each region in 18 patient samples, nonparametric two-tail Spearman test (95% confidence interval) was used [GraphPad (San Diego, CA) Prism 4 software]. To calculate correlation between age and methylation in regions A to G in these samples (N = 18), Z-score analysis was used to normalize the data and allow the derivation of a mean methylation score. Z-score of methylation for each region was calculated as follows: Z-score = (methylation density of each sample – mean value of methylation density) / SD of methylation density. Then, a sum of the Z-scores was used to derive a mean methylation score and correlated with patient's age using Spearman test (GraphPad Prism 4 software). All cell growth and colony formation assays on cells were done three separate times, and the results were expressed as average ± SEM calculated using Microsoft Excel. Statistical differences of bisulfite sequencing data were calculated using Fisher's exact test.

Results

Identification of RIL by MCA/RDA. The strategy of MCA/RDA was reported previously (10). Briefly, we used MCA to enrich for hypermethylated CpG islands and RDA to select for those that are only methylated in cancer. DNA from the K562 CML cell line as well as a DNA mixture from five normal blood donors were digested with SmaI, which cuts unmethylated sites, only leaving "blunt ends," followed by XmaI (SmaI isoschizomer) digestions, the latter cutting methylated sites with "sticky ends." Adaptors were ligated to these sticky ends, and ligation products were subjected

to PCR using adaptor-specific primers. RDA was done on MCA amplicons to identify those that are methylated in K562 but not in normal blood. After two rounds of RDA, the PCR products were cloned, colonies containing inserts were identified by PCR and sequenced, and the results were analyzed by BLAST search. One of the recovered clones corresponded to the promoter-associated CpG island of RIL (Fig. 1). RIL was originally identified by subtraction cloning as a novel gene expressed in normal rat fibroblasts and down-regulated in H-ras–transformed derivatives (21). Human RIL maps to chromosome 5q31.1, a region frequently deleted in the malignant cells of patients with MDS and AML (22), and seemed to be a good candidate for the tumor suppressor gene that resides in this area.

**RIL methylation in cell lines and primary tumors by COBRA.** The RIL methylated sequence cloned by MCA/RDA was homologous to the 3’ part of the CpG island, in intron 1. To determine if the entire RIL CpG island is methylated, we used COBRA analysis. Bisulfite-converted DNA was amplified by PCR using primers designed to amplify the region around the transcription start site of the gene. The PCR products were then digested with HpyCH4IV restriction enzyme that distinguishes methylated from unmethylated DNA (Fig. 1A, region D). We initially studied normal tissues (WBCs, bone marrow, and colon) and found low levels of methylation (range, 0–34%; average, 13.7%), consistent with previous studies of methylation in normal aging cells (Fig. 1B; ref. 23). Using a methylation threshold of 30% (upper limit of normal) for calling a case methylated, we found that 55 of 79 (70%) cell lines from various tissues showed methylation. Using a different threshold did not change the results appreciably. As shown in Table 1, most tumor types studied showed evidence of RIL methylation, including nearly all colon cancers and hematologic malignancy cell lines. In primary tumors (Fig. 1B; Table 1), we found RIL methylation in 43 of 105 (41%) hematologic malignancies and in 53 of 85 (62%) solid tumors. To determine the extent of RIL methylation in cell lines and primary tumors, we also used bisulfite sequencing around the RIL transcription start. As shown in Fig. 1C, the COBRA results corresponded to dense methylation across the CpG island in both cell lines and primary tumors. Thus, RIL is a common target for methylation in multiple tissue types, with a specially high frequency and density of methylation in colon and leukemia/lymphoma cell lines as well as in primary colon cancer and leukemia (see Table 1).

**Methylation analysis across the RIL CpG island.** COBRA analysis of normal colon samples showed low levels of methylation (Fig. 1B). To know if it is uniform across the CpG island or more localized to certain CpG sites and whether in fact it represents age-related methylation, we designed seven pyrosequencing methylation assays, each covering several CpG sites (Fig. 1A, regions A–G; Supplementary Table S1) around the transcription start site as well as exon 1 and intron 1 of the gene. We found, in 18 normal colon samples, that the region around the transcription start site of the gene (~306 to +168; assays A–F on Fig. 1A) shows low degrees of methylation (average methylation density, 25.9 ± 1.9%), whereas the intron 1 region (+370 to +384; assay G on Fig. 1A) has very high degrees of methylation (average methylation density, 85.35 ± 0.8%; see Fig. 1D). Methylation by assays A to F was all generally similar, and each region showed increased methylation with age (R = 0.39, P = 0.101 for region A; R = 0.65, P = 0.003 for region B; R = 0.62, P = 0.048 for region C; R = 0.46, P = 0.074 for region D; R = 0.57, P = 0.028 for region E; R = 0.59, P = 0.016 for region F). A Z-score average methylation of all six regions showed a strong correlation with age (R = 0.62, P = 0.006). By contrast, region G had no correlation with age (R = −0.05, P = 0.844). In summary, methylation of the RIL promoter region is uniform and has a positive correlation with age in normal colon.

**RIL expression is ubiquitous in normal tissues, is lost in cancer cell lines, and can be restored by 5-aza-dC.** Using reverse transcription-PCR (RT-PCR), we examined the expression of RIL in normal tissues and cancer cell lines. We found that RIL is expressed in all 15 normal tissues tested (see Fig. 2A). However, we found that RIL expression is lost in 13 of 17 (82%) cancer cell lines tested. All highly methylated cell lines (Raji, Du145, Dupro, HT116, RKO, and SW48) have lost expression of RIL, whereas cell lines with low (U373) or partial methylation (K562) retained RIL expression (see Fig. 2B). Treatment of colorectal cancer cell lines HT116 and SW48 myeloid leukemia cell lines HL-60 and ML-1 with the hypomethylating agent 5-aza-dC led to reactivation of RIL expression, whereas treatment with the histone deacetylase inhibitor TSA alone had no effect (Fig. 2C). 5-aza-dC treatment resulted in hypomethylation of the RIL promoter in both HL-60 and ML-1 leukemia cells as measured by pyrosequencing (Fig. 2D). Finally, we analyzed RIL methylation status in CML patients before and shortly after 5-aza-dC treatment when WBC counts are still high and thus reduction of methylation is likely due to demethylation rather than elimination of leukemia. We found substantial reduction in methylation of RIL promoter as measured by pyrosequencing in two of six patients. Methylation analysis for one of them is shown in Fig. 2D. For the same patient, this was confirmed by

### Table 1. RIL methylation in different cell types

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Methylation frequency, total (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemia/lymphoma</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>Colon</td>
<td>12/13 (92)</td>
</tr>
<tr>
<td>Prostate</td>
<td>3/4 (75)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>5/7 (71)</td>
</tr>
<tr>
<td>Renal</td>
<td>4/7 (57)</td>
</tr>
<tr>
<td>Breast</td>
<td>5/9 (56)</td>
</tr>
<tr>
<td>Lung</td>
<td>7/13 (54)</td>
</tr>
<tr>
<td>CNS</td>
<td>4/8 (50)</td>
</tr>
<tr>
<td>Ovarian</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>Patient samples</td>
<td></td>
</tr>
<tr>
<td>Hematopoietic malignancies</td>
<td></td>
</tr>
<tr>
<td>AML</td>
<td>6/7 (80)</td>
</tr>
<tr>
<td>ALL</td>
<td>7/15 (47)</td>
</tr>
<tr>
<td>MDS</td>
<td>30/83 (36)</td>
</tr>
<tr>
<td>Nonhematopoietic malignancies</td>
<td></td>
</tr>
<tr>
<td>Breast cancer</td>
<td>7/19 (37)</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>5/10 (50)</td>
</tr>
<tr>
<td>Colon adenoma</td>
<td>11/13 (85)</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>30/43 (70)</td>
</tr>
<tr>
<td>Normal samples</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>1/22 (5)</td>
</tr>
<tr>
<td>Blood</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>0/3 (0)</td>
</tr>
</tbody>
</table>

Abbreviations: CNS, central nervous system; ALL, acute lymphoblastic leukemia.
bisulfite sequencing and the difference was statistically significant \((P < 0.001)\). In this analysis, we observed that before treatment all sequenced alleles had some RIL methylation (mostly very dense), whereas after treatment 4 of 13 alleles are completely free of methylation (Fig. 2D).

**RIL suppresses tumor cell growth and colony formation.** To determine whether RIL expression has an effect on the growth of cancer cells, we reintroduced the gene into the colorectal cancer cell lines RKO and HCT116, both of which have endogenous RIL gene methylated and have lost RIL expression. The RIL-V5-His TOPO vector or antisense vector was transfected using Fugene 6. Individual G418-resistant colonies were expanded and RIL-expressing clones were verified by Western blotting (Fig. 3A). For each cell line, we compared three randomly selected RIL-expressing clones with three antisense clones and averaged the results. As shown in Fig. 3B, RIL-transfected RKO and HCT116 clones showed, on average, an \(~6\)-fold (range, 3–18) reduction in cell growth on plastic surfaces. We also compared the clonogenic efficiency of the RIL-transfected and antisense vector-transfected cells and found, on average, an \(~6\)-fold (range, 3–6) reduction of colony formation in soft agar for RKO cell line and \(~4\)-fold (range, 3–6) reduction of colonies for HCT116 (Fig. 3C). RIL RKO untagged clones showed similar results (data not shown); hence, here, we describe the data obtained with RIL RKO V5-tagged clones only. Thus, RIL has

---

**Figure 2.** RIL expression in normal tissues and cancer cell lines and reactivation by 5-aza-dC. **A,** RIL expression pattern in normal human tissues. RIL expression was determined by RT-PCR. All reactions included RT (-) controls, and amplification using GAPDH primers was used as a control to verify the integrity of RNA. **B,** RIL expression in hemimethylated cell lines (U373 and K562) and in highly methylated cell lines (RAJI, DU145, LNCAP, SW48, RKO, and HCT116); GAPDH was used as a control for the integrity of RNA. **C,** restoration of RIL expression by hypomethylation in four cell lines. **D,** top left, methylation levels in HL-60 and ML-1 cell lines before and after treatment with 5-aza-dC; bottom left, methylation levels in CML-1 patient at different time points (days 1–192) after 5-aza-dC treatment as measured by pyrosequencing. Cycle 1 of 5-aza-dC started on day 1, cycle 2 on day 44. Right, bisulfite sequencing of CML-1 patient at days 1 and 6 after treatment.
growth-suppressive properties and is able to suppress colony formation, which is consistent with our hypothesis of *RIL* functioning as a TSG.

**RIL sensitizes cancer cells to apoptosis.** RIL has previously been shown to interact with TRIP-6 and PTP-BL, two proteins that function in apoptotic pathways (24, 25). To test the hypothesis that RIL suppresses growth through effects on apoptosis and cell survival, *RIL*-transfected RKO and HCT116 were exposed to UV radiation and apoptosis-inducing agents, such as staurosporine. *RIL*-transfected cells did not show a different degree of apoptosis at baseline as measured by propidium iodide staining. After treatment with UVC (254 nm) at 30 J/m², *RIL*-transfected clones exhibited higher cleavage of PARP at 2 and 3 h when compared with antisense vector clones (Fig. 4A). We also compared apoptosis 3 h following UVC by staining cells with propidium iodide and measuring cell cycle distribution and sub-G₁ fraction by fluorescence-activated cell sorting (FACS). In RKO cells, on average, *RIL* transfectants showed 1.5-fold higher degree of apoptosis 3 h after treatment when compared with antisense transfectants. In HCT116 cells, *RIL*-transfected cells showed 2.0-fold higher degree of apoptosis by propidium iodide staining at 30 h after treatment (Fig. 4B and C). Finally, we measured apoptosis in both cell lines at the same time points by staining cells with Annexin V and analyzing them using FACS and obtained similar results (1.7-fold compared with antisense transfectants; Fig. 4B and C). A similar increase in apoptosis was obtained in a series of experiments where we treated the cells with staurosporine (data not shown). In contrast to increased apoptosis, we found no difference in cell adhesion, migration, and invasion using Becton Dickinson Transwell chambers with and without Matrigel in *RIL*-transfected clones (data not shown).

**Mutation analysis.** Mutation analysis of *RIL* was undertaken using SSCP and direct sequencing. Briefly, PCR primers were designed to amplify each of the seven exons of *RIL* from genomic DNA. A panel of 38 cell lines and 13 tumors was used in the study. PCR products were analyzed by SSCP analysis or, alternatively, directly sequenced and then scrutinized for the presence of mutations. All bands that were abnormally migrating during SSCP analysis were excised from the gel, purified, reamplified with PCR, and sequenced. We found four polymorphisms in *RIL*: one polymorphism in nine samples in exon 3 (position 255, T→G), two polymorphisms in five samples in exon 3 (position 561, C→T; position 591, A→G), and one polymorphism in two samples in exon 6 (position 722, C→T). None of these changed the *RIL* coding sequence.

**RIL methylation is a poor prognostic factor in MDS.** 5q31 deletions are associated with a poor prognosis in MDS. To determine whether *RIL* contributes to this poor prognosis, we analyzed 83 MDS patient samples for the presence of *RIL* methylation and found methylation (>10%) in 30 of 83 (36%) cases. We calculated patient risks using the International Prognostic Scoring System score (26) and found that *RIL* methylation was positive in only 2 of 32 low-risk patients compared with 28 of 51 cases with intermediate-1 risk or higher (P < 0.001). Among the higher risk patients, the 28 patients with *RIL* methylation had a median survival of 55 weeks compared with 119 weeks for the patients without methylation (P = 0.008). This survival difference was preserved when considering those patients without deletions of 5q31 or the closely linked chromosome 7 deletions (median survival of 74 weeks versus 120 weeks for patients with/without methylation, respectively). Figure 5 shows Kaplan-Meier curves of survival by *RIL* methylation. These findings support the hypothesis that *RIL* is one of the TSGs on 5q31 targeted for inactivation in hematologic malignancies.
Discussion

Using MCA/RDA, we identified the promoter CpG island of RIL as methylated in cancer. In a panel of 79 cell lines tested derived from different cell types, we found methylation in 55 (70%). We have also observed age-related RIL methylation in normal colon and a high frequency of methylation in primary tumors. Methylation was associated with transcriptional silencing of this gene, and restoring expression by transfection suppressed growth and clonogenicity, in part by sensitizing cells to apoptosis. RIL maps to 5q31.1, a locus involved in neoplastic transformation to AML and which has been suspected to harbor a TSG for many years (11). It is located within a 1.5-Mb segment commonly deleted in AML and MDS (22). RIL is distal to interleukin-9 and just 250 kb telomeric to the IRF-1, a gene proposed as the 5q31.1 TSG (16) but not altered and expressed in majority of cases and thus unlikely involved. A large number of other candidate TSGs for 5q31 region have been proposed recently, including 5qVCA, ETF1, PURA, HSPA9, CDC23, TTID, and SMAD5, but none of them seem to be mutated or frequently down-regulated (27-34). It has previously been proposed that the gene involved in 5q31 is not a classic TSG where the remaining allele is inactivated due to mutations in coding sequence or small deletions, but rather, it is silenced by other mechanisms, perhaps by aberrant methylation (14). RIL is thus far the only gene described in the 5q31 region that is frequently inactivated biallelically due to a combination of LOH and methylation. Furthermore, RIL inactivation by methylation carries the same poor

Figure 4. RIL sensitizes to UV-induced apoptosis. A, PARP Western blot results for the RKO cell line. To study apoptosis, we treated three antisense vector and two RIL clones with 30 J/m² UVC at 254 nm and collected protein at indicated time points. Untreated antisense clone was used as control (C). B, FACS propidium iodide (PI) and Annexin V results for RKO and HCT116 cells. RKO cells were treated as described above and collected at the 3-h time point; HCT116 cells (three antisense vector and three RIL clones) were treated with 30 J/m² UVC at 254 nm and collected at the 30-h time point. For propidium iodide analysis, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry (left). X axis, DNA content; Y axis, cell number. Percentage of sub-G₀ cells undergoing apoptosis is indicated. For Annexin V analysis, cells were stained with propidium and Annexin V-FITC and analyzed by flow cytometry. X axis, Annexin V; Y axis, propidium iodide. Bottom right quadrant; cells binding Annexin V and retaining propidium iodide were apoptotic (% cells indicated). C, average for sub-G₀ apoptosis as measured by propidium iodide staining in antisense and RIL-expressing clones in RKO and HCT116 cells. Data are the summary of three independent experiments for each clone. Columns, average values for each cell line; bars, SEM. *, P < 0.05. In the average table for Annexin V experiments, data of one experiment for RKO and two experiments for HCT116 clones are shown. Both apoptotic and secondary necrotic cells were counted and shown in the table.
cytoskeletal proteins containing one NH$_2$-terminal PDZ domain and one COOH-terminal LIM domain (37). This group consists of RIL, CLP 36/HCLIM1, and actin-in-associated LIM protein (38, 39). All three members have been shown to associate with the cytoskeleton, and their functional role is largely unknown. Cytosolic LIM-only proteins lacking homeodomain, such as RIL, often serve as adaptor proteins (40). The RIL LIM domain can interact both with the PDZ domain of RIL itself and with the PDZ domain of the protein tyrosine phosphatase PTP-BL. This LIM domain also contains a consensus tyrosine phosphorylation site, which can be phosphorylated by an unknown kinase and dephosphorylated by PTP-BL (25). The human homologue of PTP-BL, known as PTP-BAS or FAS-associated phosphatase 1, has been previously found to bind the COOH-terminal domain of the FAS receptor and affect FAS-mediated apoptosis (41). In addition, the RIL PDZ domain can interact with the second LIM domain of another LIM protein, TRIP-6, which has been shown to act as a transcriptional coactivator of v-rel/nuclear factor-κB (NF-κB; refs. 24, 42). Thus, through protein-protein interactions, it is possible that RIL modulates PTP-BL and/or TRIP-6 activities and thus regulates apoptosis through effects on the FAS/NF-kB pathways. Our data on increased apoptosis in RIL-transfected cells support this concept and provide a potential mechanism for its tumor-suppressive properties. Interestingly, other predominantly cytosolic LIM domain proteins lacking a homeobox, such as DALP, DRAL, and Hic-5, have also been recently shown to induce apoptosis in other cellular systems (43, 44).

In summary, we present evidence that RIL, a gene located in a frequently deleted segment on 5q31.1, is a suppressor of cellular proliferation and clonogenic growth, sensitizes cancer cells to apoptosis, and is epigenetically inactivated in multiple cancer types. Because RIL exhibits proapoptotic functions, loss of RIL expression in tumors might alter normal response to DNA damage and chemotherapy and provide a growth advantage to tumor cells. Deciphering signal transduction pathways and proteins interacting with RIL that might be involved in regulation of proliferation, transformation, and apoptosis would now be of interest to understand further the role this gene plays in carcinogenesis.

Acknowledgments

Received 8/22/2006; revised 11/28/2006; accepted 12/29/2006.

Grant support: NIH grants CA100632 and CA108631 and Uehara Memorial Foundation of Japan (Y. Kondo).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Jill Villaythong, Saira Ahmed, and Yi Guo for excellent technical assistance.

References


RIL, a LIM Gene on 5q31, Is Silenced by Methylation in Cancer and Sensitizes Cancer Cells to Apoptosis

Yanis A. Boumber, Yutaka Kondo, Xuqi Chen, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/67/5/1997

Supplementary Material Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2007/02/28/67.5.1997.DC1

Cited articles This article cites 43 articles, 18 of which you can access for free at: http://cancerres.aacrjournals.org/content/67/5/1997.full.html#ref-list-1

Citing articles This article has been cited by 15 HighWire-hosted articles. Access the articles at: /content/67/5/1997.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.