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Induction of G1 Cell Cycle Arrest and P15^{INK4b} Expression by ECRG1 Through Interaction With Miz-1

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Abstract *ECRG1* is a novel candidate of tumor suppressor gene identified from human esophagus. To study the biological role of *ECRG1* gene, we performed a GAL4-based yeast two-hybrid screen of a human fetal liver cDNA library. Using the ECRG1 cDNA as bait, we identified two putative clones as associated proteins, Miz-1 and FLNA (Filamin A). The interaction of ECRG1 and Miz-1 was confirmed by glutathione-*S*-transferase (GST)-pull-down assays in vitro and co-immunoprecipitation experiments in vivo. ECRG1 was co-localized with Miz-1 in nucleus, as shown by confocal microscopy. Transfection of *ECRG1* gene into the esophageal cancer (EC) cells inhibited cell proliferation and induced G1 phase arrest of cell cycle. In the co-transfection of ECRG1 and Miz-1 assays, we found inhibition of cell proliferation and G1/S phase in EC cells, but the levels of cell proliferation inhibition and G1/S phase arrest were more strongly compared with the transfection of ECRG1 or Miz-1 alone. In addition, the interaction of ECRG1 and Miz-1 could induce expression of *P15^{INK4b}* gene in esophageal cancer 9706 (EC9706) cells. However, the transfection of ECRG1 or Miz-1 alone was not revealed the expressions of *P15^{INK4b}* gene. When antisense ECRG1 interdicted expression of endogenous ECRG1 in Balb/c-3T3 cells, Transfection of Miz-1 couldn't induce P15^{INK4b} expression. The results provide evidences that ECRG1 and Miz-1 in EC cells may be acting as a co-functional protein associated with regulation of cell cycle and induction of P15^{INK4b} expression. It suggests that ECRG1 may inhibit tumor cell growth by affecting cell cycle, and that expression of P15^{INK4b} may be likely to enhance G1 cell cycle arrest during the interaction of ECRG1 and Miz-1. The physical interaction of ECRG1 and Miz-1 may play an important role in carcinogenesis of EC. J. Cell. Biochem. 92: 65–76, 2004. © 2004 Wiley-Liss, Inc.

Key words: ECRG1; esophageal carcinoma; Miz-1; yeast two-hybrid; G1 cell cycle arrest; P15^{INK4b}

At present, the focus of biology studies is occurring a transition from the clone of new gene to function of the protein. During the era, identifying the function and interactions of disease-associated proteins produced by individual genes and their roles in specific disease may provide major opportunity to elucidate disease mechanisms and to identify new diagnostic markers and therapeutic targets [Blackstock and Weir, 1999; Emmert-Buck et al., 2000]. Esophageal cancer (EC) is one of the most common fatal cancers worldwide and occurs at very high frequency in certain areas of China [Lu, 2000]. Some of tumor suppressor genes and oncogenes are involved in the initiation and development of EC [Montesano et al., 1996]. However, no gene directly related to EC has been found. The *ECRG1* gene (GenBank accession no. AF071882.) was cloned by the effective technique of mRNA differential display by comparing the differential gene expression between normal esophageal epithelia and EC in our laboratory [Su et al., 1998]. Using the SMART^{TM} RACE technique, the full-length cDNA of *ECRG1* gene was generated [Liu

Abbreviations used: ECRG1, esophageal cancer related gene1 (accession no. AF071882); Miz-1, Myc interacted zinc finger protein; GST, glutathione-*S*-transferase; EC, esophageal cancer; AS-ODN, antisense phorithioate oligonucleotide; pB, pcDNA6-V5-HisB; PBS, phosphate-buffered saline.

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et al., 1990]. It contains 1,376 nucleotides, 1,173 nucleotides of the open reading frame that predicts 391 amino acids. Previous studies including RT-PCR and Northern Blot showed that *ECRG1* gene was expressed in normal esophagus, liver, colon, and lung tissues, but the expression was down-regulated in the adjacent and cancerous tissues, especially low frequency in EC [Liu et al., 1990]. The vitro and vivo assays indicated that *ECRG1* gene inhibited tumor cell proliferation [Wang et al., 1998]. It indicated that ECRG1 might be made an effect on tumor. But the mechanism of action remains unknown.

As most proteins perform their function by complex of proteins [Alberts, 1998; Lander et al., 2001]. It is necessary to identify the protein interaction, which not only promote to elucidate the function of the protein, but also to be sure conductor of specific signaling conduction gateway [Kumar and Michael, 2002; Gavin et al., 2002; Yuen et al., 2002]. To date, the yeast two-hybrid system that detects binary interaction through activation of reporter gene expression has been widely used for the identification of protein interaction [Fields and Song, 1989; Uetz et al., 2000; Ito et al., 2001]. Therefore, to examine the biological roles of ECRG1, we searched for associated proteins and protein–protein interactions by a GAL4-based yeast two-hybrid system. Using the full-length ECRG1 cDNA as bait to screen a human fetal liver cDNA library, we found that two positive clones could bind to the ECRG1 protein. Here we report one candidate clone, which encoded human Miz-1. The association of ECRG1 with Miz-1 was demonstrated in vitro by GST-pull-down assays and in esophageal cancer 9706 (EC9706) cells by co-immunoprecipitation experiments. Moreover, the fact that these two proteins co-localized in nucleus as shown by confocal microscopy also supported the interaction of ECRG1 and Miz-1. By considering the function of Miz-1 involved in the regulation of cell proliferation and G1 cell cycle arrest [Sakamuro and Prendergast, 1999], here we further present evidences of the functional interaction of the two proteins on cell proliferation and cell cycle in EC cells, and expression of $P15^{INK4b}$. It is noteworthy that Miz-1 is an interaction partner for ECRG1 correlating with suppression of cell proliferation and induction of G1 cell cycle arrest and $P15^{INK4b}$ expression.

MATERIALS AND METHODS

Bacterials and Yeast Strains

The Matchmaker GAL4 two-hybrid system 3 was obtained from Clontech (Palo Alto, CA). Yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura 3-52, his3-200, gal4Δ, gal80Δ, LYS2:-$GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-GAL2-TATA-ADE2, URA3:MEL1UAS-$MEL1_{TATA}$-LacZ) was use d for library screening and assay for protein and protein interactions. Strain AH109, which virtually eliminates false positives by using three reporters: ADE2, HIS3, and MEL1 (or LacZ)—under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes. These promoters yield strong and specific responses to GAL4. The ADE2 reporter alone provides strong nutrition selection, the HIS3 selection reduces the incidence of false positives, and the MEL1 or LacZ that encode α-galactosidase and β-galactosidase can be assayed directly on X-α-gal indicator plates. The *Escherichia coli* strains DH5α and BL21 were used to construct the plasmids and to express the fusion proteins, respectively.

Plasmid Constructs

The pGBkT7 vector, which expresses proteins fused to amino acids 1–147 of the GAL4 DNA binding domain (DNA-BD), pGADT7 vector, which expresses proteins fused to amino acids 768–881 of the GAL4 activation domain (AD), and the control plasmids: pGBKT7-53, pGBKT7-lam, pGADT7-T, and pCL1 were from Clontech. Plasmid pGBKT7-EC RG1 which encodes the full-length (391amino acids) *ECRG1* gene fused in frame to the GAL4 DNA-BD, was constructed by inserting the PCR-generated fragment into the EcoRI and the BamHI site of pGBKT7. Plasmid pGADT7-ECRG1 which encodes the full-length ECRG1 cDNA fused in frame to the GAL4-AD was constructed by inserting the PCR-generated fragment into the EcoRI and the BamHI site of pGADT7. The human fetal liver cDNA library, which was fused with the GAL4-AD in the pACT2 vector, was obtained from Clontech. Plasmid pGEX-4T-1-Miz-1, used to express the recombinant protein GST–Miz-1 containing a GST tag, was constructed by sub-cloning the Miz-1 PCR-generated fragment into the EcoRI site and the SalI site of pGEX-4T-1. For in vitro transcription and translation, ECRG1 protein-coding region was cloned into pB-HA. For

expression of Miz-1 and ECRG1 in human EC9706 cells, the PCR products of Miz-1 were inserted into pB-FLAG, while ECRG1 was inserted into plasmid pcDNA3.1(+).

Yeast Two-Hybrid Library Screening

Plasmid pGBkT7-ECRG1 was used as bait in two-hybrid screens of human fetal liver cDNA libraries by the Matchmaker two-hybrid system 3 protocol (Clontech). The yeast strain AH109 was transformed with the pGBKT7-ECRG1 by the lithium acetate method and expression of the bait was confirmed by Western blotting. A human fetal liver cDNA library in the pACT2 vector (Clontech) was transformed into the yeast strain expressing the prey protein. Transformants expressing both the bait and interactive prey proteins were selected on medium lacking tryptophan, leucine, histidine, and adenine (Sigma–Aldrich, St.Louis, MO). Plates were incubated at 30°C for 5–7 days, and tested for β-galactosidase activity using the filter lift assay. Approximately 3×10^6 colonies were screened and 23 positive clones were identified. The cDNA inserts of the positive clones were amplified by PCR using primers complementary to the pACT2 vector (5′-T ACC ACT ACA ATG GAT-3′ and 5′-GTG AAC TTG CGG GGT TTT TCA GTA TCT ACG A-3′). Subsequently, the pACT2-cDNA constructs were isolated from positive yeast colonies, as recommended by the supplier, transformed into super-competent *E. coli* DH5α by electroporation, grown under selection, re-isolated, and were analyzed by restriction digests. The unique purified constructs were then re-tested against the original pGBKT7-ECRG1 bait construct. To ensure that the interactions were specific, the positive clones were also tested against an irrelevant bait proteins lamin C and grown on selective plates lacking tryptophan, leucine, histidine, and adenine to test the specificity of interactions. The positive inserts were sequenced and analyzed in comparison with the GenBank sequence data bank.

Preparation of Recombinant Proteins and In Vitro Transcription and Translation

For the GST–Miz-1 binding experiments, the *E. coli* strain BL21 was transformed with pGEX-4T-1 and pGEX-4T-1-Miz-1, respectively, and grown in LB medium containing ampicillin (100 μg/ml). Expression of GST–Miz-1 fusion protein was induced for 2 h at 37°C in a 500 ml culture of *E. coli* BL21 by adding 1 mM isopropyl-β-D-thiogal actopyranoside (IPTG). The bacterial pellet was resuspended in 20 ml phosphate buffered saline (PBS) containing 0.5 mM dithiothreitol, sonicated thoroughly (10 × 30 s), and Triton X-100 was added to a final concentration of 1%. After gentle mixing for 30 min at 4°C, the lysate was spun at 12,000*g* for 10 min at 4°C. The induced GST fusion protein in the supernatant was bound to 250 μ l rewashed glutathione-sepharose beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) for 60 min at 4°C. The beads were washed extensively with PBS to remove non-specifically bound bacterial proteins and the quantity and purify of the GST-fusion protein bound to the glutathione-sepharose beads were analyzed by SDS–PAGE. In vitro translation reaction of pB-HA-ECRG1 was carried out using the TNT® Quick Coupled Transcription/Translation Systems (Promega Life Science, Madison, WI) according to manufacturer's protocol.

GST-Pull-Down Assay

GST–Miz-1 or GST was attached to glutathione-sepharose beads and incubated with in vitro translated pB-HA-ECRG1 in binding buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% NP-40) on ice for 2 h. The beads were recovered by centrifugation and were washed four-times with the same fresh buffer at room temperature, resuspended in 20 μl SDS sample buffer and boiled at 1,007°C for 3 min. The proteins were then separated on a 12% gel by SDS–PAGE, followed by Western blotting using anti-HA monoclonal antibody (Roche Diagnostics Indianapolis, IN), peroxidase-confugated goat anti-mouse IgG secondary antibody and the Western blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

Co-Immunoprecipitation

For immunoprecipitation experiments, EC9706 cells were co-transfeced with pB-HA-ECRG1 (10 μg) and pB-FLAG-Miz-1 (3.3 μg) or with control vectors (pB-HA and pB-FLAG) in 100 mm dishes using Lipotectamine™ 2000 (Invitrogen Life Technologies, Carlsbad, CA). Forty-eight hours after transfection, cells were solubilized with 1 ml of lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% Nonidet p40, 0.5% sodium deoxycholate) (Roche Diagnostics)

on ice for 30 min. Insoluble material was removed by centrifugation for 20 s at 12,000*g* at 4°C. The supernatants were collected. Their protein concentrations were measured using Bradford method and adjusted to a final concentration of 1 mg/ml. The supernatants were rewashed with protein G (Roche) for 3 h at 4°C, 500 μl of lysates were then incubated with 3 μg anti-HA (Roche) or 6 μg anti-FLAG (Sigma) antibodies coupled to sepharose beads overnight at 4°C on a rocking platform. The samples were then washed with wash buffer (Wash 1: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet p40, 0.5% sodium deoxycholate; Wash 2: 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% Nonidet p40, 0.05% sodium deoxycholate; Wash 3: 10 mM Tris-HCl, pH 7.5, 0.1% Nonidet p40, 0.05% sodium deoxycholate). Proteins retained on the beads were eluted by SDS sample buffer and then separated by SDS–PAGE on a 12% gel, followed by immunoblot analysis with the anti-FLAG or anti-HA monoclonal antibody.

Confocal Immunofluorescence Microscopy

EC9706 cells seeded on glass coverslips in 6-cm plates were transfected with pB-FLAG-Miz-1 and pEGFP-C1-ECRG1. Two days later, the transfected cells were washed with PBS, fixed with 4% paraormaldehyde in PBS for 10 min at room temperature, and permeabilized for 4 min at 4°C in PBS containing 0.3% Triton X-100 and 10% goat serum. Subsequent incubations were performed at room temperature. After washing, cells were blocked for 60 min in PBS containing 3% bovine serum albumin and then incubated for 2 h with mouse anti-FLAG monoclonal antibody (Sigma) diluted 1:1,000 in blocking buffer. Cells were then incubated with Rhodamine (TRITC)-conjugated affinipure goat anti-mouse IgG (Amersham Pharmacia Biotech Inc.) (1:500) for 1 h. Microscopy was performed on a Bio-Rad Radiance 2000 MP confocal microscope.

Effect of *ECRG1* Gene and *Miz-1* Gene on Cell Proliferation of EC9706 Cells

EC9706 cells (human esophageal cancer cell line) transfected with pcDNA3.1(+)-ECRG1 or transfected with pcDNA3.1(+) vector using lipofectamine^{TM} 2000 were cultured in DMEM medium supplemented with 10% fetal bovine serum(FBS), 100 μ/ml of penicillin and 100 μg/ml streptomycin, 5% CO_2 at 37°C incubator. Geneticin (G418) at the concentration of 400 μg/ml was used to select for cells that neomycin-resistant, indicating that the vector was present in the cells. Cells were maintained in the media containing 400 μg/ml G418 and *ECRG1* gene re-expression was detected by Western blotting. The stable cell lines with characteristic of *ECRG1* gene expression and EC9706 cells were then transfected with pB-FLAG-Miz-1. The expression of Miz-1 was examined using anti-FLAG antibody (Sigma). To test the effect of ECRG1 and Miz-1 on EC9706 cell growth, EC9706 cells and above cells transfected or co-transfected with plasmids were adjusted to the desired concentration (1×10^6 cells, n = 12) and cultured for 6 days. Then were assayed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) every other day.

Effect of *ECRG1* Gene and *Miz-1* Gene on Cell Cycle of EC9706

Briefly, Cells were trypsinized and washed with ice-cold PBS once and then fixed with ice-cold 70% ethanol and stored at −20°C overnight. Fixed cells were then washed with PBS and stained with 500 μl of propidium iodide (10 μg/ml, Sigma) and 50 μg/ml RnaseA (Sigma) for 30 min at 37°C before analysis. The DNA content of the cells was then analyzed in a fluorescence cell sorter. Change of cell cycle was assayed by flow cytometory.

Effect of *ECRG1* and *Miz-1* Gene on Expression of P15^{INK4b}

To identify whether interaction of ECRG1 and Miz-1 induced P15 expression, we used ECRG1 and Miz-1 null EC9706 cells (identified by Northern blot) to be transfected or co-transfected with ECRG1 and Miz-1. Moreover, Balb/c-3T3 cells of endogenous ECRG1 expression (identified by Western blot) were transfected with Miz-1 or co-transfected with Miz-1 plus antisense phorithioate oligonucleotides (AS-ODNs: 5′-TAC TAA CGG CAA GAG TAA CAC-3′) for inhibition of ECRG1 expression specifically, then isolated RNA using TRIZOL reagent (Sigma). First strand synthesis of cDNA was carried out using 5 μg of total RNA as template, oligo(dT) first strand synthesis primers, and Superscript II reverse transcriptase according to manufacturer instruction (GIBICOBRL Life Technologies, Grand Island, NY). Primers P15^{INK4b} F: 5′-CAG GTC ATG ATG ATG GGC AGC-3′and P15^{INK4b} R: 5′-CCA GGC ATC GCG CAC GTC CAG-3′ were

designed basing on the reference [Muscarella et al., 2001] to generate 212 bp PCR fragment. PCR was performed to identify transcription levels of $P15^{INK4b}$ at first 95°C for 4 min, then 95°C for 60 s (denaturation), 58°C for 60 s (annealing), and 72°C for 90 s (elongation) for 35 cycles followed by 72°C incubation for 10 min. During the PCR experiment, β-actins as controls were also amplified. The protein extracts from the above groups was used to analyze expression of $P15^{INK4b}$ by Western blot using monoclonal anti-$P15^{INK4b}$ antibody (Santa Cruz).

RESULTS

A Yeast Two-Hybrid Screen of Cellular Proteins Interacting With ECRG1 Protein

To identify proteins that are interacted with the ECRG1 protein, a fusion construct of the GAL4 DNA-BD with the ECRG1 (open frame 82–1,257 bp) was generated and used as the bait for screening of a human fetal liver yeast two-hybrid cDNA library. Of 3×10^6 transformants screened, 23 clones grew in the absence of tryptophan, lencine, histidine, and adenine, and expressed β-galactosidase activity. The pACT2/cDNA plasmids were successfully isolated and retested for specificity of β-galactosidase expression. After re-transformation, two independent positive clones were identified and sequenced. The genes identified by the yeast two-hybrid approach were *Miz-1* and *FLNA* (Home sampiens Filamin A accession No.NM001456.1). Previous study had demonstrated that Miz-1 might be involved in cell cycle G1 phase arrest and inhibition of cell proliferation. In this article, we focus on the characterization of the interaction of ECRG1 with Miz-1.

ECRG1 and Miz-1 Interact In Vitro

To assess whether the yeast interaction data reflected direct association between ECRG1 and Miz-1, The GST–Miz-1 fusion protein or GST protein, which was expressed in *E. coli* strain BL21 (Fig. 1A,B) was loaded on glutathione-sepharose affinity column. In vitro-translated ECRG1 was tested for binding with glutathione-sepharose beads coupled with GST or GST–Miz-1 alone. Following extensive washing, bound proteins were eluted and analyzed by SDS–PAGE, followed by Western blotting. As shown in Figure 1C, ECRG1 was bound to GST–Miz-1 (lane3) but not to the GST control protein (lane2). These results are consistent with the interaction between ECRG1 and Miz-1.

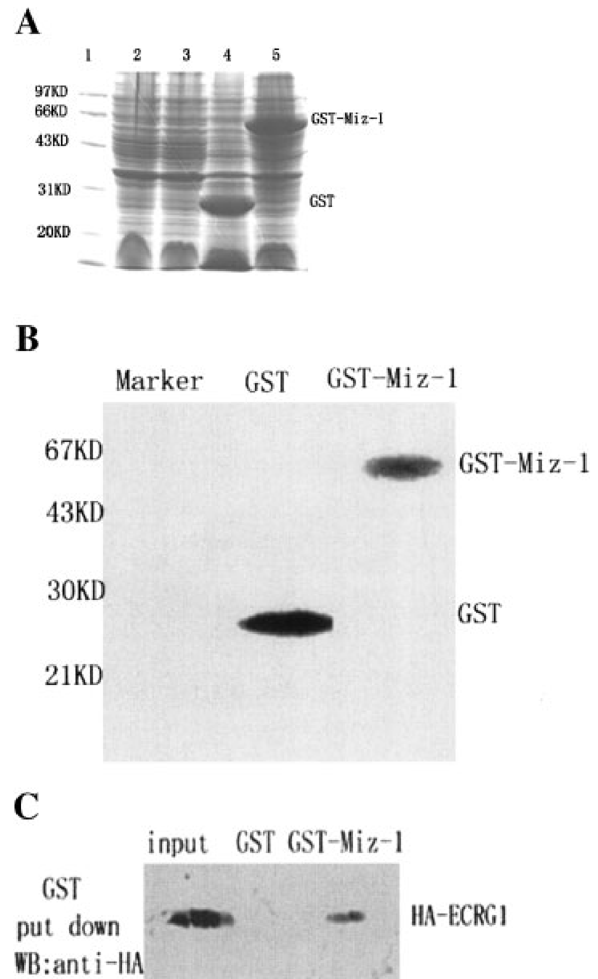

Fig. 1. **A**: SDS–PAGE analysis about expression of GST–Miz-1 fusion protein and GST protein. **Lane 1**, marker; **lane 2**, BL21; **lane 3**, BL21 transformed with pGEX-4T-1-Miz-1 but was not induced; **lane 4**, **5**, the supernatant of BL21 transformed with pGEX-4T-1 or pGEX-4T-1-Miz-1, respectively, and was induced by IPTG (1 mM). **B**: Identified affinity purified proteins of GST or GST–Miz-1 by Western blot. **C**: GST-pull-down assays. Input (**lane 1**) was the positive control. GST alone was used as a negative control. HA-tagged ECRG1 was visualized after Western blotting using the anti-HA monoclonal antibody when bound to GST–Miz-1 (**lane 3**) but not visualized when bound to GST (**lane 2**).

ECRG1 Interacts With Miz-1 In Vivo

To investigate whether the interaction between ECRG1 and Miz-1 occurred in vivo, we performed co-immunoprecipitation of the complex from EC9706 cells transiently expressing the two proteins. FLAG epitope tagged Miz-1 was transiently co-expressed in EC9706 cells together with pB-HA-ECRG1. As negative control, a pB-FLAG empty vector replaced pB-

FLAG-Miz-1. The cell lysate with a high protein expression of HA-ECRG1 (Fig. 2A, lane 1, 2) and FLAG-Miz-1 (Fig. 2B, lane 1, 2) detected in the immunoblots was then immunoprecipitated with anti-FLAG monoclonal antibody. Immunoprecipitated proteins were separated by SDS–PAGE and blotted with a monoclonal anti-HA antibody. HA-ECRG1 was present in immunoprecipitates from cells transfected with FLAG-Miz-1 and pB-HA-ECRG1 (Fig. 2A lane 4) but not in control immunoprecipitates (Fig. 2A lane 3). In the inverse experiment, a pB-HA empty vector replaced pB-HA-ECRG1 as negative control. The protein lysates were immunoprecipitat-ed with anti-HA antibody, then co-immunoprecipitated materials were immunoblotted with anti-FLAG antibody. This immunoblot also showed that immunoprecipitates contains FLAG-Miz-1 when co-transfected with pB-HA-ECRG1 and pB-FLAG-Miz-1 (Fig. 2B, lane 4) but, to the negative control, immunoprecipitates did not contain FLAG-Miz-1 (Fig. 2B lane 3). It showed that in vivo ECRG1 and Miz-1 occurred interaction. Altogether, the GST-pull-down assays and the co-immunoprecipitation studies support an interaction between ECRG1 and Miz-1.

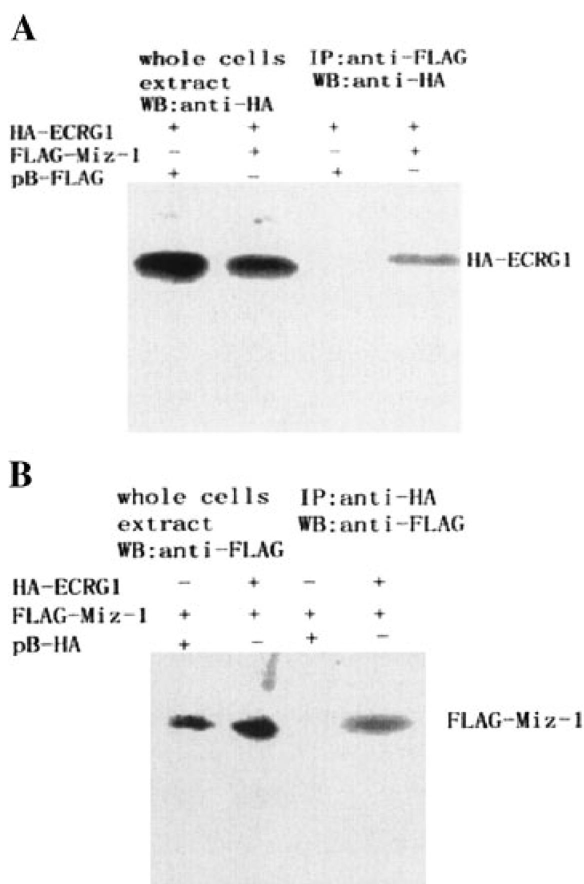

Fig. 2. Co-immunoprecipitation (co-IP) of ECRG1 and Miz-1. **A**: Esophageal cancer 9706 (EC9706) cells transiently transfected with HA-ECRG1 and FLAG-Miz-1, were immunoprecipitated by anti-FLAG antibody and were detected by anti-HA monoclonal antibody. As negative control, a pB-FLAG empty vector replaced pB-FLAG-Miz-1. The HA-ECRG1 proteins were visualized after Western blotting using anti-HA monoclonal antibody as shown in **lane 1**, **2**, and **4**. **B**: EC9706 cells transiently transfected with HA-ECRG1 and FLAG-Miz-1 were immunoprecipitated by anti-HA antibody and detected by anti-FLAG antibody. As negative control, a pB-HA empty vector replaced pB-HA-ECRG1. The FLAG-Miz-1 proteins were visualized after Western blotting using anti-FLAG as shown in **lane 1**, **2**, and **4**.

ECRG1 and Miz-1 Proteins Co-Localize Mostly to Nuclei and Slightly to Cytoplasm

We next examined whether the interaction between ECRG1 and Miz-1 resulted in co-localization of the two proteins in vivo using immunofluorescence staining and auto-fluorescence in the EC9706 cells. For this purpose, ECRG1-null EC9706 cells were transiently co-transfected with GFP-ECRG1 and FLAG-Miz-1. Immunofluorescence and confocal laser microscopy showed that these two proteins were all localized to nuclei and slightly to cytoplasm (Fig. 3D). Taken together, our in vitro and in vivo interaction data indicate that ECRG1 is physiologically associated with Miz-1.

Effect of *ECRG1* Gene and *Miz-1* Gene on Cell Proliferation in EC9706 Cells

In initial experiments, the expression of ECRG1 and Miz-1 in EC9706 cell line was examined. The results showed the ECRG1 and the Miz-1 were absent in EC9706 cells (identified by Northern blot, no data shown). So, to test their effect on cell proliferation, we used EC9706 cell line to be transfected with pcDNA3.1(+)-ECRG1 and FLAG-Miz-1, respectively, or co-transfected with pcDNA3.1(+)-ECRG1 plus pB-FLAG-Miz-1. EC9706 cells and EC9706 cells transfected with pcDNA3.1(+) vector were used as controls. The expression of ECRG1 (Fig. 4A lane 4, 5) or Miz-1 (Fig. 4B lane 3, 5) was identified by Western blotting, respectively, confirming that transfection of ECRG1 and Miz-1 is successful. MTT assay (Fig. 4C) showed that ECRG1 or Miz-1 could inhibit rate of cell proliferation ($P < 0.05$) compared with empty vector. When the two genes were present, the inhibition was markedly enhanced compared to the action of ECRG1 or Miz-1 alone

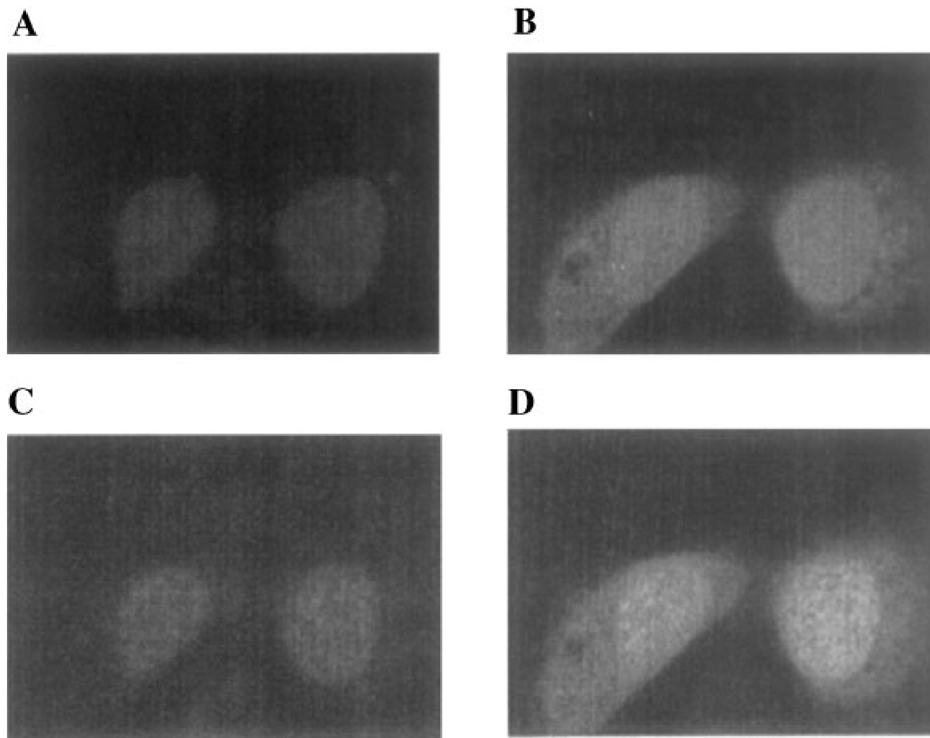

Fig. 3. Immunofluorescence confocal microscopy of EC9706 cells co-transfected with genes for fusion proteins GFP-C1-ECRG1 and FLAG-tagged Miz-1 (**A**) label nuclei by Hoechst 33342; (**B**) GFP-tagged ECRG1excited by only Ar488; (**C**) Rhodamine red staining of FLAG-tagged Miz-1 excited by only Kr543; (**D**) co-localization of GFP-tagged ECRG1, Rhodamine red staining of FLAG-tagged Miz-1, and Hoechst blue staining of nuclei. When transfected with these two plasmids, they were all localized mostly to nuclei and slightly to cytoplasm.

($P < 0.001$). It suggests that the ECRG1 protein co-operate with Miz-1 to affect cell proliferation.

Effect of *ECRG1* Gene and *Miz-1* Gene on the Alteration of Cell Cycle in EC9706 Cells

The above five groups were then analyzed for cell cycle by flow cytometry. The results showed that the evident increase of cell cycle G1/S phase in EC9706 cells transfected with ECRG1 (65.7) or co-transfected with ECRG1 and Miz-1 (69.5) was observed, compared with the control groups (57.5, 57.8). Miz-1 results in slight increase in cell cycle G1/S phase (61.6). In comparison with transfection of ECRG1 or Miz-1 alone, we also found that the interaction of the two genes increase G1cell cycle arrest (Table I). It indicated that the two proteins were positively associated with G1 cell cycle arrest.

Effect of *ECRG1* and *Miz-1* Gene on Expression of P15^{INK4b}

Above results indicated that ECRG1 and Miz-1 involved in same physiological process. As Miz-1 may induce G1 cell cycle arrest by upregulating P15^{INK4b}, we hypothesized that ECRG1 might be involved in the process to induce G1 cell cycle arrest. So we used ECRG1and Miz-1 null EC9706 cells (determined by Northern blot no data shown) to be transfected or co-transfected with ECRG1 and Miz-1, and Balb/c-3T3 cells of endogenous ECRG1 expression (identified by Western blot no data shown) to be transfected with Miz-1 or Miz-1 plus AS-ODNs of ECRG1, respectively. Then, P15^{INK4b} expression was analyzed by RT-PCR and Western blot. The results showed that ECRG1 or Miz-1 did not induce expression of P15^{INK4b} in EC9706 cells. When co-transfected with both ECRG1 and Miz-1 in EC9706 cells, *P15^{INK4b}* gene was expressed (Figs. 5A lane 6 and 5B lane 5). To Balb/c-3T3 cells of endogenous ECRG1expression, transfection of Miz-1 up-regulated P15^{INK4b} expression (Figs. 5C lane 3 and 5D lane 3), which was consistent with previous studies about Miz-1 [Staller et al., 2001]. When AS-ODNs of ECRG1 interdicted endogenous ECRG1 protein of Balb/c-3T3 cells, transfection

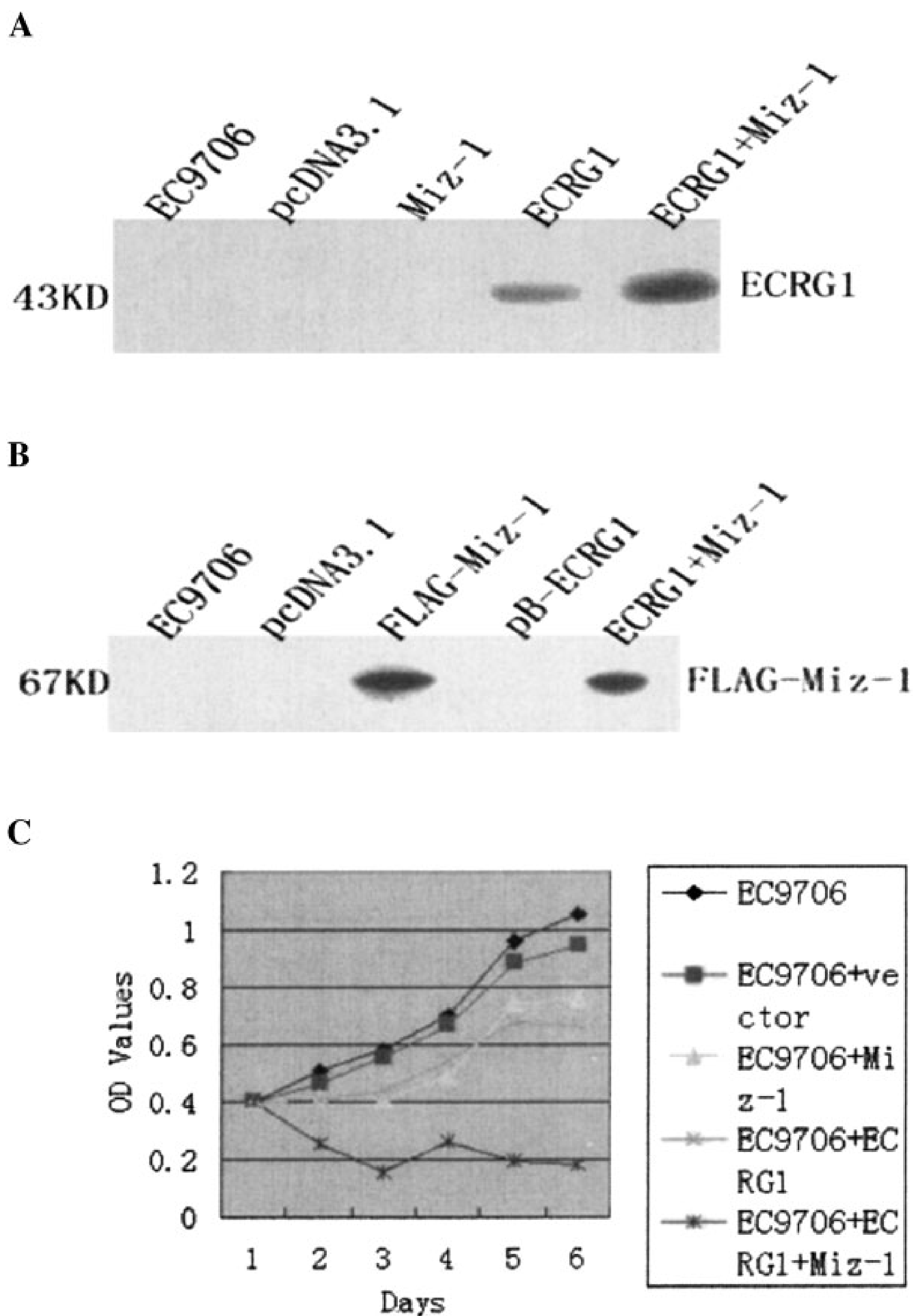

Fig. 4. **A**: Western blot results using anti-ECRG1 antibody to detect expression of ECRG1 after transfection. **B**: Western blots results using anti-FLAG antibody to detect exprssion of Miz-1 after transfection. **C**: Cell growth curve determined by MTT assay. The cell numbers decreased in groups of transfected ECRG1 or Miz-1 alone. Specially, the co-transfection of ECRG1 and Miz-1 inhibited significantly cell proliferation than transfection of empty vector, Miz-1, or ECRG1 alone respectively ($P < 0.001$).

of Miz-1 couldn't induce $P15^{INK4b}$ expression (Figs. 5C lane 2 and 5D lane 2). It suggested that the interaction between ECRG1 and Miz-1might induce expression of $P15^{INK4b}$.

DISCUSSION

A formidable challenge of postgenetic biology is to understand how genetic information

TABLE I. Cell Cycle Distribution of Cells by Expression of ECRG1 or Miz-1

Transfected EC9706 cells	G1 (%)	S (%)	G2 (%)
EC cells	57.5	31.0	11.4
EC/pcDNA3.1	57.8	33.3	8.9
EC/ECRG1	65.7	21.4	12.9
EC/Miz-1	61.6	26.1	12.3
EC/ECRG1 + Miz-1	69.5	24.2	6.3

results in the concerted action of gene products in time and space to generate function [Roses, 2000]. Therefore, the immense task will be exploration of the structure, function, and partner of the proteins [Akhilesh and Mattias, 2000; Chambers et al., 2000; David et al., 2000]. The *ECRG1* gene was a novel candidate tumor suppressor gene associated with esophageal carcinoma cloned in our laboratory (GenBank accession No. AF071882.). Identifying the function of *ECRG1* gene product may provide opportunities to elucidate the EC mechanisms and its role in tumor development and progression. As a key question about a protein, in addition to when and where it is expressed, is with which other proteins does it interact [Garbed and Erica, 2001], we searched for associated proteins with a yeast two-hybrid system using the ECRG1 cDNA fragment as bait. On screening a human fetal liver cDNA library, we identified two putative clones as associated proteins including Miz-1 (Myc interacted zinc finger protein) and FLNA (Home sampiens Filamin A accession no. NM001456.1). Since human *Miz-1* gene is closely linked with cancer [Simpkins, 2000], it is possible for us to understand the cellular functions of the ECRG1 protein through its linkage to Miz-1. As the yeast two-hybrid system provides only potential interaction that have to be confirmed by further biological experimentation [Ito et al., 2000], we performed GST-pull-down assays in vitro, co-immunoprecipitation experiments, and co-localization of the two proteins in vivo using immunofluorescence staining and auto-fluorescence to test the association of ECRG1 with Miz-1. Our results confirmed perfectly the interaction between ECRG1 and Miz-1.

Miz-1, a Myc interacting zinc finger protein [Peukert et al., 1997], arrested cell in G1/S phase, inhibited cyclinD-associated kinase activity and occurred loss of heterozygosity (LOH) frequently in tumor [Schwab, 1996; Staller et al., 2001]. Moreover, it down-regulated expression of cyclin-dependent kinase inhibitor P15^{INK4b} by interacting with Smad, Max, and Myc [Seoane et al., 2001; Staller et al., 2001]. Current studies indicated that Miz-1 might be linked with TGF signaling conduction gateway [Orian and Eisenman, 2001]. These findings showed that Miz-1 could play an important role in controlling expression of a gene or genes involved in cell cycle arrest and cell proliferation. So we reasoned that ECRG1 as a partner of Miz-1 might be co-regulate cell cycle and proliferation. In this article, we further examined the potential functional interaction of ECRG1 and Miz-1 in EC cells. In order to get a better understanding of the association of ECRG1 with Miz-1 on cell proliferation and cell cycle as well as various physiological processes, we used ECRG1 and Miz-1 null EC9706 cell lines (identification by Northern blot. no data shown) to be transfected with ECRG1 or Miz-1 alone, respectively, and co-transfected with the two genes. The results showed that ECRG1 or Miz-1 inhibited cell proliferation, and induced G1 cell cycle arrest. When co-transfected with the two genes, the inhibition of cell cycle G1/S phase and cell proliferation was more markedly enhanced than the ECRG1 or Miz-1 alone. It indicated that they might act as a co-functional protein associated with cell proliferation and cell cycle in EC cells.

It has been reported that Smad, Sp1, and Miz-1 complex up-regulated expression of P15^{INK4b} [Seoane et al., 2001]. Based our data, we hypothesize that ECRG1 may be involved in the complex to affect the expression of P15^{INK4b}. So we further investigate whether interaction between ECRG1 and Miz-1 up-regulate expression of P15^{INK4b}. To identify the idea, we analyzed expression of P15^{INK4b} in EC9706 cell and EC9706 cell transfected with ECRG1 or Miz-1 alone and co-transfected with both ECRG1 and Miz-1 by RT-PCR and Western blot. In addition, we transfected Balb/c-3T3 cells with Miz-1 or Miz-1 plus antisense sequence-specific phosphorothioate oligodeoxynucleotides (ODNs) of the ECRG1 to interdict endogenous ECRG1, then identified the change of P15^{INK4b} expression. The results showed that P15^{INK4b} was not induced by ECRG1 or Miz-1 alone but by both ECRG1 and Miz-1 in EC9706 cells. To Balb/c-3T3 cells of endogenous ECRG1

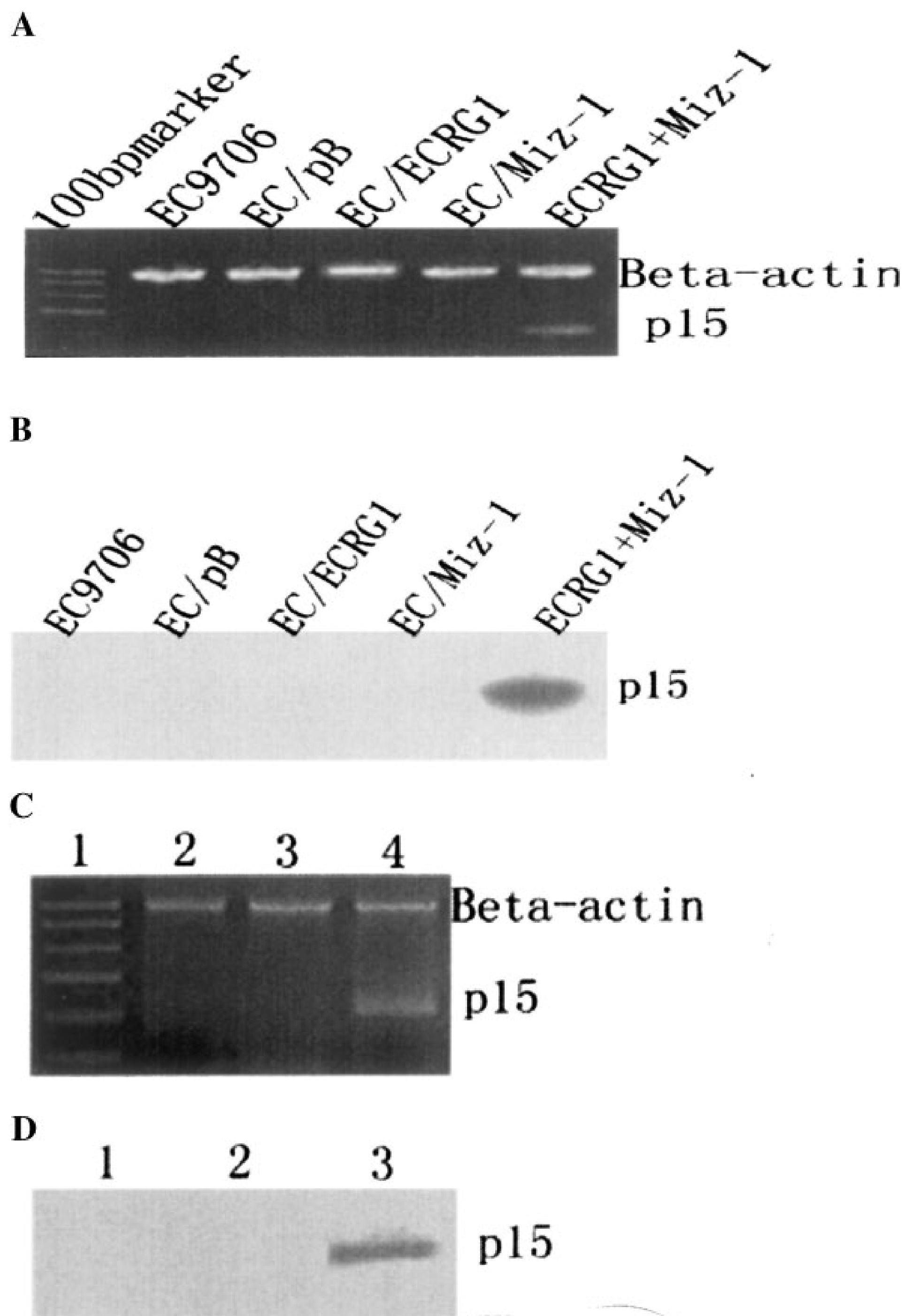

Fig. 5. **A**: Upregulating P15^{INK4b} mRNA through ECRG1 and Miz-1 by RT-PCR in EC9706 cells. **B**: Western blot showing expression of P15^{INK4b} in EC9706 cells (**C**) upregulating P15^{INK4b} mRNA through ECRG1 and Miz-1 by RT-PCR in Balb/c-3T3 cells. (1) 100 bp Marker; (2) Balb/c-3T3 cells; (3) Balb/c-3T3 cells transfected with pB-FLAG-Miz-1 and AS-ODNs of ECRG1; (4) Balb/c-3T3 cells transfected with pB-FLAG-Miz-1; (**D**) Western blot showing expression of P15^{INK4b} in Balb/c-3T3 cells (1) Balb/c-3T3 cells; (2) Balb/c-3T3 cells transfected with pB-FLAG-Miz-1 and AS-ODNs of ECRG1; and (3) Balb/c-3T3 cells transfected with pB-FLAG-Miz-1.

expression, transfection of *Miz-1* gene induced P15^{INK4b} expression, which accorded with previous studies [Staller et al., 2001]. When AS-ODN of ECRG1 interdicted the effect of endogenous ECRG1 in Balb/c-3T3 cells, transfection of Miz-1 could not induced P15^{INK4b} expression. It indicated that the interaction between ECRG1 and Miz-1 could induce P15^{INK4b}

expression, and ECRG1 might be a necessary component of activation complex of P15^{INK4b}. It is well known that P15^{INK4b}, a cyclin-dependent kinase inhibitor, can results in the G1 cell cycle arrest of cells by blocking the phosphorylation of Rb protein [Hannon and Beach, 1994; Reynisdottir et al., 1995; Sandhu et al., 1997; Warner et al., 1999]. So the reason of the increased cell cycle G1/S phase arrest by the interaction of ECRG1 and Miz-1 might be expression of P15^{INK4b} whereas ECRG1 or Miz-1 alone inducing G1 cell cycle arrest may be due to the change of other G1 phase regulatory gene. The expression of P15^{INK4b} was found to be dramatically induced by TGF beta, which suggested its role in the TGF beta induced growth inhibition. So, ECRG1 may also be involved in the TGF beta-signaling pathway to regulate cell cycle.

Studies of cell cycle have revealed that some oncogenes and tumor suppressor genes directly involved in regulatory process that ensured the orders of events. Once the genes mutated, it would occur deregulation of cell cycle including abnormality of cell cycle start, progression, and end. Then results in tumor development [Hunter and Pines, 1991; Hartwell and Kastan, 1994; Hunter, 1994; Marx, 1994; Steel, 1994; Clurman, 1995; Jacks and Weinberg, 1996; Weinberg, 1996]. So, alteration of cell cycle plays a major role in cacinogenesis. Here, our results suggested that in the absence of ECRG1, the genetic instability might contribute to uncontrolled proliferation. It is important to understand the mechanism on inhibition of cell proliferation and alteration of cell cycle. As our knowledge of the process increases, we will be able to use molecular and cellular assays to estimate the cell cycle missing in specific tumors.

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