

# A Functional Variant of IC53 Correlates with the Late Onset of Colorectal Cancer

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The *IC53* gene was reported to be upregulated in the colon adenocarcinoma cell line SW480. Here, we show that the expression level of *IC53* is positively correlated with the grade and depth of invasion in adenocarcinoma of the colon. Injection of *IC53* stably transfected HCT-116 cells into athymic nude mice promoted tumor growth. Furthermore, overexpression of *IC53* increased cell invasive growth, which could be dramatically prevented by knocking down *IC53* with siRNA. The effects of *IC53* on cell-invasive growth were mediated by upregulation of integrins, activation of phosphatidylinositol 3-kinase and phosphorylation of Akt. A single-nucleotide polymorphism rs2737 in the *IC53* gene created a potential microRNA379 target site, and microRNA379 expression inhibited *IC53* translation. Among 222 patients with colorectal cancer, the C/C rs2737 genotype was associated with late onset of colorectal cancer (median age 63.0 versus 55.3 years,  $P = 0.003$ ). The frequency of the C/C rs2737 genotype was much lower in patients who developed colorectal cancer below the age of 45 years than in individuals over age 45 years (10.8% versus 26.6%,  $P = 0.039$ ). These data indicated that *IC53* is a positive mediator for colon cancer progression, and *IC53*-rs2737 may serve as protection from the onset of colorectal cancer.

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## INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers in industrialized countries. In the United States, approximately 145,000 people are diagnosed with CRC annually, and the global figure is >875,000 (1). The genesis of CRC involves a series of steps, starting with environmental and/or endogenous carcinogens inducing or promoting cancer development via the activation of onco-

genes, such as *ras*, and the inactivation of tumor suppressor genes, such as *APC*, *Tp53* and *DCC*, and genes involved in DNA mismatch repair (2–5).

Genetic factors have been reported to play a key role in the predisposition to CRC as well as in the initiation and progression of the disease. High-penetrance mutations in several genes, such as *APC*; and DNA mismatch repair genes, *LKB1* and *SMAD4*, confer predispositions to fa-

miliar cases of CRC (that is, familial adenomatous polyposis, Lynch syndromes and hamartomatous polyposis), which account for <5% of all CRC cases (6). However, low-penetrance variants of these and other genes, such as common alleles at single nucleotide polymorphisms (6,7), account for much of the predisposition, resulting in sporadic cases of CRC and are likely responsible for much of the uncharacterized influence of inherited genetic changes on the development of CRC.

In the field of medical genetics, it has become increasingly apparent that few, if any, human diseases are homogeneous and solely the result of one mutation in a single gene. Even when a Mendelian disorder has one obvious predisposing genetic cause, the phenotype may still be subject to wide variation. The lack of a clear observed genotype/phenotype association, even in such single-gene disorders, suggests that additional modifier factors, including both environmental

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and genetic components, influence clinical phenotypes (8). For example, the phenotypes of FAP (familial adenomatous polyposis) and HNPCC (hereditary non-polyposis colorectal cancer), with regard to colonic disease, vary considerably, not only between families, but also within families (9,10). The considerable variation in disease expression (age of onset and tumor site) in these disorders cannot entirely be explained by the type and position of the mutation in the respective genes (11). Several reports have shown that genetic polymorphisms may be contributing factors to disease in HNPCC and sporadic cases of CRC (12–17).

MicroRNAs (miRNAs) are endogenously expressed RNAs 18–24 nucleotides in length that regulate gene expression through translational repression by binding to a target mRNA. Accumulating evidence suggests that miRNAs play a role in the pathogenesis of various human cancers (18–20). Recently, Chen and Rajewsky (21) reported that the variant rs2737 created a potential miRNA379 target site in the *IC53* gene, which was highly expressed in eight tumor cell lines, including the colon adenocarcinoma cell line SW480, compared with negligible expression in normal colon tissue (22). *IC53* was also overexpressed in tumor tissues of lung adenocarcinoma (23). In contrast, *IC53* was reported as a tumor suppressor in HeLa, H1299, HT1080 and U2OS cell lines (24–26). To our knowledge, the association between *IC53* and the development of CRC has not been established. These data led us to hypothesize that *IC53* could regulate colon cancer progression and the rs2737 in the *IC53* gene could modify the incidence of colon cancer as well as the timing of colon cancer onset.

## MATERIALS AND METHODS

### Materials

Protein kinase inhibitors (LY294002) and antibodies against Akt and phospho-Akt Ser<sup>473</sup> were obtained from Cell Signaling Biotechnology (Beverly, MA, USA). Antibodies against integrin  $\alpha_2$ ,  $\alpha_3$

and  $\beta_4$  and laminin  $\beta 1$  and  $\beta 2$  were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Wortmannin was obtained from Sigma Chemical (St. Louis, MO, USA). Nu/nu mice (BALB/c, 4- to 6-wk-old females) were purchased from the Laboratory Animal Center, Chinese Academy of Medical Sciences (Beijing, China). Colon cancer tissues and their corresponding normal mucosa were obtained from patients who underwent surgical resection of their tumors with informed consent. The human tissue collection protocol was approved by the Fuwai Hospital Ethics Committee. Informed written consent was obtained from patients themselves or their legal representatives. Animal experiments conformed to the guiding principles of China National Law for Animal Use in Medical Research and were approved by the Fuwai Hospital Committee for Animal Care and Use.

### Cell Lines

The colon cancer adenocarcinoma cell lines HCT-116, HT-29 and mouse embryonic fibroblast cell line NIH3T3 were obtained from the Institute of Cell Biology, Academic Sinica, and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Production and Purification of the *IC53* Monoclonal Antibody

The monoclonal antibody to *IC53* was produced in BALB/c mice against the keyhole-limpet hemocyanin (KLH)-conjugated synthetic peptide CQKQQ EALEEQAALEPKLD corresponding to amino acid residues 369–386 of human *IC53*. The first N-terminal cysteine residue was added to facilitate covalent KLH conjugation. BALB/c mice were immunized by intraperitoneal injection with the KLH-conjugated synthetic peptide. Murine antibodies were prepared by conventional hybridoma technology as previously described (27), and the re-

sulting hybridoma cells were screened for antibody production in an ELISA using bovine serum albumin (BSA)-conjugated synthetic peptide. Hybridoma cells producing anti-*IC53* monoclonal antibody were grown and subsequently injected into pristane-primed BALB/c mice. After 10 d, ascites fluid was collected. The IgG was extracted from the ascites fluid by using protein A-Sepharose CL-4B (Amersham Pharmacia Biotech, Amersham, UK).

### Tissue Microarray Analysis

The human tissue microarray of colon cancer tissue was obtained from Cybrdi (Xi'an, Shanxi, China). The array contained 182 dots in total and each dot represented one diseased tissue spot from one individual specimen that was selected and pathologically confirmed. The arrays were fixed with formalin, embedded in paraffin and immunostained with mouse monoclonal anti-*IC53* antibody (1:900 dilution) by using the avidin-biotin peroxidase complex method. The intensity of *IC53* staining was scored as weak (1+), moderate (2+) or strong (3+). To test the expression of miR-379, the locked nucleic acid (LNA)-modified probe U6, scramble and miR-379 were purchased from Exiqon (Vedbaek, Denmark). *In situ* hybridization was performed according to the manufacturer's protocol, and the intensity of miR-379 staining was scored as negative (0), weak (1+) or moderate (2+).

### Expression Plasmid Construction

The open reading frame of *IC53* was amplified by polymerase chain reaction (PCR) by using the EST clone (accession number AF110322) and the mammalian expression plasmid [pcDNA3.1/Myc-His(-) A-*IC53*], constructed as previously reported (22).

### Tumorigenicity

Tumorigenicity studies were performed as described previously (28). Briefly, cells from exponential cultures of HCT-116 transfectants and nontransfectants were resuspended in PBS and inoc-

ulated subcutaneously into 5-wk-old athymic nude mice ( $7 \times 10^6$ /mouse). Mice were maintained in a pathogen-free environment. Growth curves for xenografts were determined by externally measuring tumors in two dimensions. Volumes were determined by using the equation  $V = (L \times W^2) \times 0.5$ , where  $V$  = volume,  $L$  = length and  $W$  = width.

### Stable Transfection

Cells were placed in a six-well plate at a density of  $2 \times 10^4$  cells/well and grown for 16 h. The cells were then transfected with the empty plasmid or plasmids carrying the open reading frame of IC53 by using Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 24 h of transfection, fresh media were added containing G418 (200  $\mu\text{g}/\text{mL}$ ; Invitrogen). After 2 wks, stably transfected clones were pooled and propagated in DMEM containing G418 (200  $\mu\text{g}/\text{mL}$ ). The level of IC53 expression was determined by Western blot analysis.

### Stealth siRNA Treatment

IC53 stealth siRNA (number 111322F11) and the negative control were purchased from Invitrogen. HCT-116 cells were transfected by 20 pmol IC53 stealth siRNA or the negative control with Lipofectamine 2000 transfection reagent (Invitrogen) in 1 mL OptiMEM (Invitrogen) according to the manufacturer's instructions. The level of IC53 expression was determined by Western blot analysis.

### MTT Assay

The MTT assay (Sigma Chemical) was performed according to the manufacturer's instructions, with some modifications. Briefly, the cells (5,000 cells/well) were cultured in 96-well plates with 100  $\mu\text{L}$  media/well. MTT (20  $\mu\text{L}$ , 5 mg/mL) solution was added to each well at 72, 120 and 144 h after plating and incubated at 37°C for an additional 4 h in a CO<sub>2</sub> incubator. The absorbance at 570 nm was recorded with a microtiter plate reader (Bio-Rad, Hercules, CA, USA).

### Cell Migration Assay

Cell migration assays were performed by using 24-well transwell migration chambers (Corning Costar, New York, NY, USA) with an 8- $\mu\text{m}$  pore size polycarbonate filter. Cells were starved in media containing 0.5% FBS for 12 h and then transferred to transwell chambers from the culture flasks by trypsin/EDTA digestion. Briefly, the transwell units were precoated with type I collagen (20  $\mu\text{g}/\text{mL}$ ), isolated from rat tails overnight at 4°C, washed with PBS and blocked with 0.1% BSA in PBS for 1 h at 37°C. The lower wells of the chamber were filled with 600  $\mu\text{L}$  0.5% FBS in DMEM. Cells were placed in the top chamber at  $1 \times 10^5$  cells/mL in 0.1 mL DMEM containing 0.1% BSA and allowed to migrate for 4 h at 37°C in a humidified CO<sub>2</sub> incubator. For antibody blocking experiments, the cells were preincubated with media containing antibodies against integrin  $\alpha_2$  (10  $\mu\text{g}/\text{mL}$ ),  $\alpha_3$  (10  $\mu\text{g}/\text{mL}$ ), and  $\beta_4$  (20  $\mu\text{g}/\text{mL}$ ) and the phosphatidylinositol 3-kinase (PI-3K) inhibitor wortmannin (100 nmol/L) or LY294002 (25  $\mu\text{mol}/\text{L}$ ), respectively, for 30 min at room temperature. After removing the cells from the upper surface of the membrane with a swap, cell numbers on the underside were determined by using the colorimetric crystal violet assay. Six independent fields per filter were counted, and the mean of six counts was used as the migrated cell number.

### Adhesion Assay

Cell adhesion was performed in 24-well plates (Corning Costar, New York, NY, USA) precoated with matrigel (5  $\mu\text{g}/\text{mL}$  in cold DMEM; BD Biosciences, Bedford, MA, USA) overnight at 4°C. Cells were serum-starved in media containing 0.5% FBS for 12 h, washed with PBS and blocked with PBS containing 2% BSA for 30 min at 37°C. The cells were then plated on coated plates at a density of  $2 \times 10^5$  cells/mL in 0.1 mL DMEM containing 0.1% BSA and incubated for 1 h. For antibody blocking experiments, cells were pretreated with or without antibodies against integrin  $\alpha_2$ ,  $\alpha_3$  or  $\beta_4$  for

30 min as described previously. After removing the media, along with the nonattached cells, 0.2% crystal violet was added, and the cells were incubated for 10 min. The plate was gently washed with tap water and then air-dried for 24 h. SDS (5%, 0.1 mL) containing 50% ethanol was added for 20 min, and the plate was read at 570 nm.

### Microarray Analysis

RNA was isolated from cultured HCT-116 cells either stably transfected with plasmids carrying IC53 or empty plasmids, and mRNA was isolated from the total RNA (200  $\mu\text{g}$ ) by using the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany). The mRNA was then used for microarray analysis. Briefly, cDNA was synthesized by *in vitro* transcription and labeled as a probe according to the manufacturer's manual (Clontech, Palo Alto, CA, USA). Hybridization of the cDNA probes to the Atlas human cancer array (Clontech; category number 7742-1) was performed by using a Hybridization Oven Robbin 1000 (Robbin's Scientific, Sunnyvale, CA, USA), and resultant spots were scanned with Phosphoimage (BAS-MS 2340; Fujifilm, Nakanuma, Japan). Data were analyzed by using ArrayGauge, version 1.0 (Fuji Photo Film, Tokyo, Japan). The data were then sorted to obtain genes differentially expressed  $\geq 2$ -fold.

### Immunocytochemistry

HCT-116 cells transfected with IC53 and control untransfected cells were plated onto glass coverslips in six-well plates and grown to 75% confluence. The cells were then serum-starved in media containing 0.5% FBS for 12 h, washed twice with PBS and fixed with 3.7% formalin for 20 min, and then rinsed twice with PBS. The cells were immunostained with polyclonal antibodies and detected by using the horseradish peroxidase staining method. Endogenous horseradish peroxidase was inhibited by incubating the cells in 3% H<sub>2</sub>O<sub>2</sub> solution for 10 min at room temperature and removed by washing twice with PBS. The cells

were incubated in media containing primary antibodies (anti-integrin  $\alpha_2$ ,  $\alpha_3$ , and  $\beta_4$  and anti-laminin  $\beta_1$  and  $\beta_2$ ) with a dilution of 1:100–1:400 for 30 min at 37°C, washed 3x with PBS and incubated with the peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 10 min at room temperature. Positive staining was visualized by applying the diaminobenzidine substrate (DAKO, Carpinteria, CA, USA) and then counterstaining with hematoxylin.

### Western Blot Assay

HCT-116 cells, HCT-116 cells transfected with empty plasmid, plasmids carrying the IC53 open reading frame, IC53 stealth siRNA or the negative control were grown to confluence in 75-mm dishes. For Akt and phospho-Akt Western blot analysis, HCT-116 cells transfected with empty plasmid or plasmids carrying the IC53 open reading frame were serum-deprived for 12 h and treated with platelet-derived growth factor BB (PDGF-BB) or LY294002 at a concentration of 30 ng/mL or 10  $\mu$ mol/L for 2 h, respectively. For analysis of the effects of micro379 on IC53 translation, HT-29 cells (with the T/C genotype) or LOVO cells (with the T/T genotype) were transfected with 20 pmol precursor miR-379 or pre-miR negative control.

Cells were harvested with trypsin, washed twice with PBS and directly lysed in ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.1% SDS, 0.02% sodium azide, 100  $\mu$ g/mL phenylmethylsulfonyl fluoride, 1  $\mu$ g/mL aprotinin, 1 mmol/L EDTA, 1 mmol/L EGTA and 1% NP-40. The cell lysate was incubated on ice for 10 min and centrifuged at 10,000g at 4°C for 5 min. Protein concentrations were quantified by using the Bradford colorimetric method (Bio-Rad). A total of 25  $\mu$ g lysate protein was boiled for 5 min in Laemmli sample buffer with 100  $\mu$ mol/L dithiothreitol and was then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to nitrocellulose membranes

(Amersham Pharmacia Biotech) and blocked with 5% nonfat milk. The membranes were then incubated with antibodies against Akt; phospho-Akt; integrins  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$ ; IC53 (1:1,000); or GAPDH (1:5,000) at 4°C for 16 h and then incubated with a horseradish peroxidase-conjugated anti-mouse IgG antibody at 25°C for 1 h. Protein bands were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology).

### Human IC53 3'UTR Luciferase Constructs

To construct IC53 3'UTR-luciferase reporter plasmids, a 75-bp sequence (Supplementary Table S1), carrying either the wild-type or the variant genotype of rs2737, was synthesized and cloned into the pMIR-REPORT vector (Ambion, Austin, TX, USA) by using restriction enzymes *Hind*III and *Spe*I. The reporter plasmid containing rs2737T was defined as pMIR-TT, and the reporter plasmid containing rs2737C was defined as pMIR-CC. The resulting constructs (pMIR-TT and pMIR-CC) were verified by sequencing.

### Luciferase Target Assay

HCT-116 cells ( $1 \times 10^5$  cells per well) were cotransfected with 0.8  $\mu$ g pMIR-CC or pMIR-TT plasmid, 50 ng Renilla and 20 pmol pre-miR miRNA precursor of miR-379 (Ambion) or pre-miR negative control (Ambion), all combined with Lipofectamine 2000 (Invitrogen). After 48 h, cells were washed and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA), and their luciferase activity was measured by using a luminometer (SIRIUS, Pforzheim, Germany). Firefly luciferase expression levels were adjusted on the basis of Renilla luciferase activity. Three independent experiments were performed for each reporter.

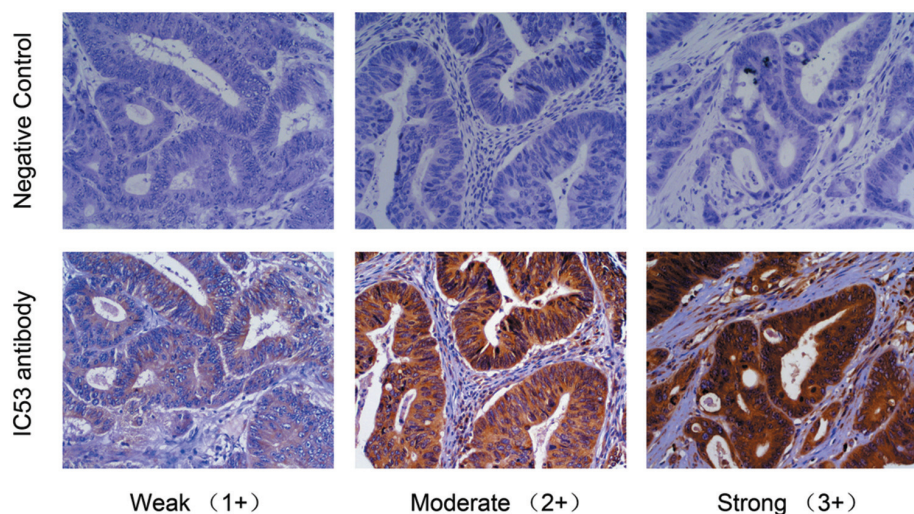
### Study Subjects

We consecutively selected 222 Chinese CRC patients with histologically confirmed colon or rectal adenocarcinoma between 2004 and 2006 in the Beijing Cancer Hospital. A total of 260 controls were se-

lected from patients admitted to orthopedics, general surgery or otorhinolaryngology wards, and patients with a prior history of malignant neoplasms were excluded. The age and sex distribution of the two groups was similar in both cases (mean  $\pm$  SD age, 59.0  $\pm$  13.9 years; 57.2% male) and control groups (mean  $\pm$  SD age, 59.6  $\pm$  9.3 years; 55.4% male). All subjects were of Han nationality, and all patients and controls provided written informed consent for the genetic studies, which were approved by the ethical committee of Beijing Cancer Hospital, China.

### Genotyping of rs2737

For IC53-rs2737 genotyping, to test association between rs2737 and risk of CRC, DNA was isolated from blood samples by using the RelaxGene Blood DNA System (TianGen, Beijing, China). To test correlation between expression of miR-379 and that of IC53 in subjects carrying a different genotype of rs2737, DNA was isolated from formalin-fixed paraffin-embedded tissue (Cybrdi) by using the MagneSil Genomic, Fixed Tissue Purification Module (Promega), according to the manufacturer's protocol. Variant rs2737 was genotyped by PCR–restriction fragment length polymorphism, analyzed by amplification of a 244-bp sequence with the following primers: forward 5'-CAAGA ACCCCACGAAAACAG-3', reverse 5'-AAGATGGAAAGCCACAGGAA-3'. The PCR assay was performed by using 50 ng genomic DNA, 5 pmol of each primer and EasyTag PCR SuperMix (TransGen, Beijing, China). The amplification protocol consisted of 35 cycles of 94°C for 30 s, followed by 59°C for 30 s and 72°C for 30 s. The resultant PCR products were digested with *Acc*I (New England Biolabs, Beverly, MA, USA), separated on a 4% agarose gel and stained with ethidium bromide. Two DNA fragments of 147 and 97 bp were expected for the C allele and only one band for the T allele (Supplementary Figure S1). In each plate (96 reactions), three positive and three blank controls were added; the positive control was from the sample confirmed by sequencing. Repro-



**Figure 1.** Immunohistochemical analysis of IC53 expression in the adenocarcinoma of colon samples. Representative IC53 immunohistochemical images of the tissue microarray are shown. The upper panel shows untreated tissue and the lower panel shows tissue treated with a monoclonal antibody against IC53 (1:900). The intensity of IC53 staining was scored as weak (1+), moderate (2+) or strong (3+) (200x magnification), and representative images from each of these scores are shown.

ducibility of genotyping was confirmed by sequencing in 50 randomly selected samples with 100% concordance.

**Statistical Analysis**

All experiments were repeated 3 or 4x, and each treatment was carried out

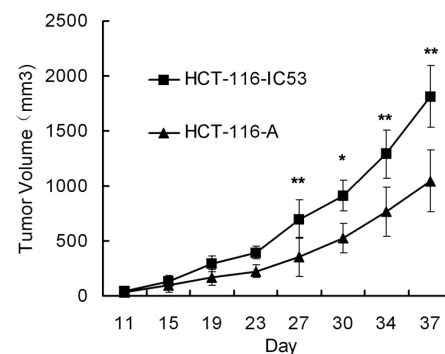
in triplicate, unless otherwise stated. Similar results were obtained in all cases. Each figure shows 1 of 3 representative experiments. Results were expressed as mean ± SD. The Student *t* test (two-sided) was used to compare the values of the test and control sam-

**Table 1.** IC53 expression level correlates with the grade and degree of invasion of adenocarcinoma of the colon.

Clinical pathological evaluation	Expression of IC53			<i>P</i>	Expression of IC53		<i>P</i>
	+	2+	3+		+	2+ and 3+	
<b>Adenocarcinoma of colon</b>							
Grade							
I	26	1	0	$1 \times 10^{-8}$	26	1	$1 \times 10^{-6}$
II	41	21	6		41	27	
III	10	15	10		10	25	
Depth of invasion							
T1/T2	15	3	0	0.018	15	3	0.025
T3/T4	62	34	16		62	50	
<b>Mucinous carcinoma of colon</b>							
Grade							
I	1	3	0	0.123	1	3	0.130
II	8	18	7		8	25	
III	8	7	0		8	7	
Depth of invasion							
T1/T2	1	5	1	0.371	1	6	0.269
T3/T4	16	23	6		16	29	

ples. A Spearman correlation analysis was used to calculate the correlation coefficients between IC53 expression levels and the grade of adenocarcinoma of the colon, degree of invasion or expression level of miR-379. An  $\chi^2$  test was used to test genotype frequencies of the single-nucleotide polymorphism rs2737; the associations between the variant and CRC were detected by using unconditional logistic regression models. The one-way analysis of variance (ANOVA) test was used to determine the statistical significance of the age at diagnosis of CRC between the groups with the T/T rs2737 genotype and the C/C rs2737 genotype. *P* < 0.05 was taken as significant.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).



**Figure 2.** Effect of IC53 on tumorigenesis *in vivo*. The tumor sizes were twice as large in the animals injected with HCT-116 cells carrying IC53 than in the animals injected with HCT-116 cells carrying the control vector (*P* = 0.0003). HCT-116 cells were stably transfected with IC53 cDNA or their corresponding vector. The cells ( $0.5 \times 10^5$ ) were injected subcutaneously into 5-wk-old male nude mice. The animals were monitored for tumor formation every 4 d. Tumors were measured externally with a caliper in two dimensions on the indicated days. Tumor volumes were determined from the following equation:  $V = (L \times W^2) \times 0.5$ , where *L* is length and *W* is width. Each point on the graph represents the mean volume (± SE) of six xenografts. \**P* < 0.05; \*\**P* < 0.01.

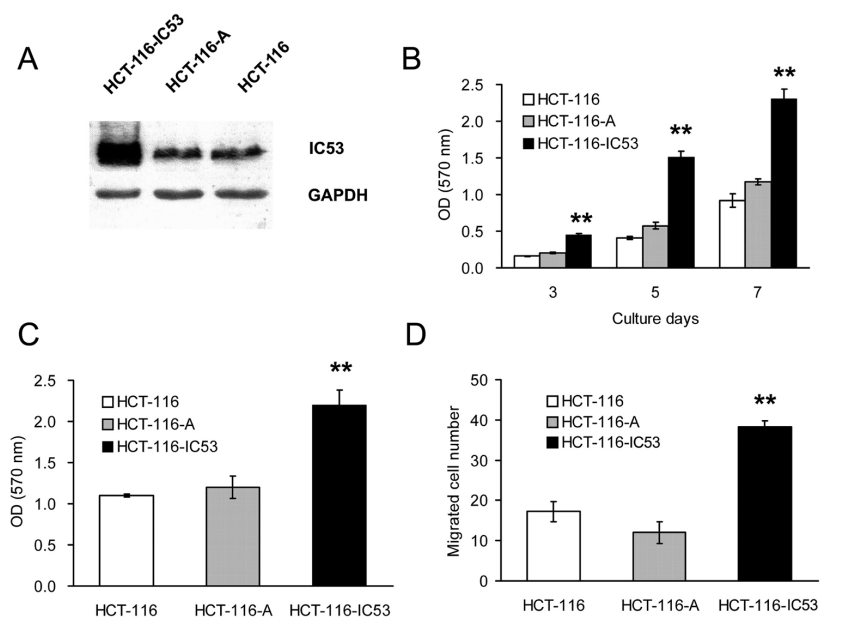
RESULTS

Expression Level of IC53 Was Positively Correlated with the Grade and Depth of Invasion in Adenocarcinoma of the Colon

To confirm whether IC53 is expressed in normal colon epithelial, we performed immunohistochemistry, showing that IC53 was low or weak when expressed in the normal colon epithelial (Supplementary Figure S2). To evaluate the expression of IC53 in colon cancer and investigate the association of IC53 expression levels with various clinical pathological parameters, we performed tissue microarray analysis. The intensity of IC53 staining was scored as weak (1+), moderate (2+) or strong (3+) (Figure 1). We found a strong correlation between the IC53 expression level and the grade of adenocarcinoma of the colon (correlation coefficient 0.47,  $P = 1 \times 10^{-7}$ , Table 1), a weak association between the IC53 expression level and the degree of invasion (correlation coefficient 0.21,  $P = 0.018$ , see Table 1) and no association between the IC53 expression level and the grade or the degree of mucinous carcinoma of the colon ( $P = 0.13$  and  $P = 0.27$ , see Table 1). These results indicated that IC53 may contribute to the development of colon cancer.

Overexpression of IC53 Promoted the Tumorigenicity of HCT-116 Cells

To determine whether IC53 has the ability to transform NIH3T3 cells, IC53 stable transfectants were generated and injected into athymic nude mice. After 37 d, no tumor formation was detected in the animals injected with NIH3T3 cells carrying exogenous IC53 or those carrying control vectors (data not shown). Whereas in the animals injected with HCT-116 cells transfected with IC53 expression plasmids, tumors formed that were twice as large as those in the animals injected with HCT-116 cells carrying the control vector (Figure 2). These results indicated that IC53 has the potential to promote cancer cell growth but is unable to transform cells.

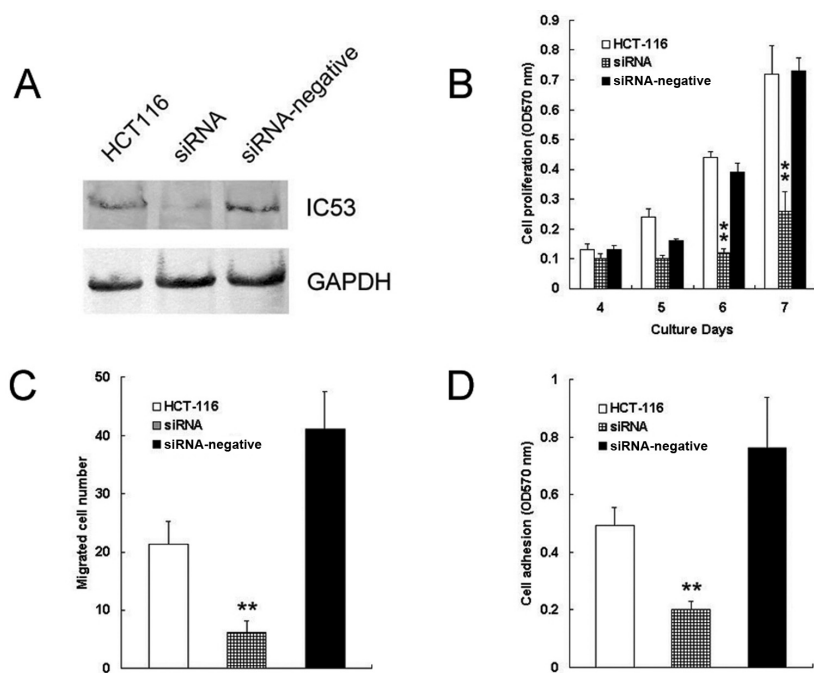


**Figure 3.** Overexpression of IC53 promoted proliferation, migration and adhesion of HCT-116 cells. (A) Stable transfection of IC53 plasmids increased IC53 expression. IC53 expression was analyzed in serum-starved HCT-116 cells by Western blot analysis with an anti-IC53 monoclonal antibody (1:1,000). The expression level of IC53 in the cells transfected with IC53 plasmids (HCT-116-IC53) was four-fold greater than that of the cells transfected with empty plasmid (HCT-116-A) or the untransfected control (HCT-116). An anti-GAPDH antibody was included in the analysis as a loading control. (B) Overexpression of IC53 induced HCT-116 cell proliferation. Cell proliferation was assayed by using the MTT assay. The cells were maintained in DMEM containing 10% FBS and 200 μg/mL G418 and cultured in 5% CO<sub>2</sub> at 37°C. The media were replaced every 48 h. Stable transfection of IC53 (HCT-116-IC53) markedly promoted HCT-116 cell proliferation 2.1-fold on day 3, 2.6-fold on day 5 and 1.97-fold on day 7 after plating, compared with either the untransfected (HCT-116) control or the empty vector control (HCT-116-A), as determined by the optical density (OD) measurements. (C) Overexpression of IC53 induced HCT-116 cell adhesion. HCT-116 cells were suspended in serum-free medium and seeded into collagen I (20 μg/mL)-coated 24-well plates. After incubation for 60 min at 37°C, the percentage of adhered cells was determined by using the colorimetric crystal violet assay. Cells stably transfected with the IC53 expression construct (HCT-116-IC53) dramatically promoted HCT-116 cell adhesion by 183% after 60 min compared with the untransfected control (HCT-116) or cells transfected with empty vector (HCT-116-A). (D) Overexpression of IC53 induced HCT-116 cell migration. Cell migration was analyzed by using the transwell assay. The cells were maintained in DMEM containing 10% FBS and 200 μg/mL G418 and cultured in 5% CO<sub>2</sub> at 37°C. Cells stably transfected with IC53 expression construct (HCT-116-IC53) migrated 180% or 300% more than their parental (HCT-116) or vector transfected cells (HCT-116-A), respectively. This figure represents 1 of 3 independent experiments, with quadruplicate samples in each experiment. The results represent the mean ± SD in triplicate. \*\* $P < 0.01$ .

Overexpression of IC53 Promoted Proliferation Migration and Adhesion of the Human Colon Cancer Cell Line

The process of cancer development is closely related to the unrestricted invasive growth of cancer cells. To test whether the IC53 gene encodes a colon cancer progression regulator, we first studied the effect

of overexpression of the IC53 gene on cell proliferation in the established colon cancer cell line HCT-116. The cells were transfected *in vitro* by IC53 plasmids and empty plasmids as controls. The expression level of IC53 in the cells transfected with IC53 plasmids was increased four-fold compared with cells transfected with



**Figure 4.** Knockdown of IC53 blocked cell proliferation, migration and adhesion. (A) Stealth siRNA against IC53 eliminated IC53 protein expression. IC53 expression was analyzed in serum-starved HCT-116 cells. The cells were transfected with stealth siRNA against IC53 (siRNA) or a negative control (siRNA negative). The IC53 proteins were detected by using Western blot analysis with an anti-IC53 monoclonal antibody (1:1,000). An anti-GAPDH antibody was included in the analysis as a loading control. (B) Knockdown of IC53 inhibited HCT-116 cell proliferation. IC53-mediated cell proliferation was analyzed by using the MTT assay. The cells were maintained in DMEM containing 10% FBS and cultured in an incubator with 5% CO<sub>2</sub> at 37°C. The culture media were replaced every 48 h. HCT-116 cells transfected with stealth siRNA against IC53 showed a 64% decrease in proliferation compared with cells transfected with the negative control. (C) Knockdown of IC53 inhibited HCT-116 cell adhesion. HCT-116 cells were suspended in serum-free medium and seeded into collagen I (20 µg/mL)-coated 24-well plates. After incubating for 60 min at 37°C, the percentage of adhered cells was determined by using the colorimetric crystal violet assay. HCT-116 cells transfection with stealth siRNA against IC53 showed a 74% decrease in cell adhesion compared with cells transfected with the negative control. (D) Knockdown of IC53 inhibited HCT-116 cell migration. IC53-mediated cell migration was analyzed by the transwell assay. The cells were maintained in DMEM containing 10% FBS at 37°C. HCT-116 cells transfected with stealth siRNA against IC53 showed an 85% decrease in migration compared with cells transfected with the negative control. This figure represents 1 of 3 independent experiments, with quadruplicate samples in each experiment. The results represent the means ± SD in triplicate. \*\**P* < 0.01.

empty plasmids (Figure 3A). Overexpression of IC53 markedly promoted HCT-116 cell proliferation 2.1-fold on day 3, 2.6-fold on day 5 and 1.97-fold on day 7, in IC53 plasmid-transfected cells compared with their untransfected or empty vector-transfected counterparts ( $P = 9 \times 10^{-9}$ ). These results indicated that overexpression of IC53 promoted human cancer cell line proliferation (Figure 3B).

Next, we tested whether IC53 could stimulate cancer cell adhesion to an extracellular matrix. We found that overexpression of IC53 dramatically promoted HCT-116 cell adhesion by 183% after 60 min compared with untransfected controls or with cells carrying the empty vector ( $P = 9 \times 10^{-6}$ , Figure 3C).

We then examined the effects of IC53 on HCT-116 cell motility by using trans-

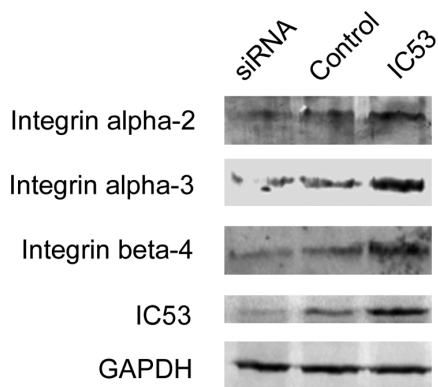
well migration chambers. Cells stably transfected with the IC53 expression construct showed increased motility of 300% or 180% compared with the parental or empty vector transfected cells, respectively ( $P = 2 \times 10^{-7}$ , Figure 3D).

#### Knockdown of IC53 Blocked Cell Proliferation, Migration and Adhesion

To further examine the effect of endogenous expression of IC53 on cell proliferation, migration and adhesion, the expression of IC53 in HCT-116 cells was suppressed by its siRNA. The expression level of IC53 in the cells transfected with IC53 siRNA was <10% of that in the control cells (Figure 4A). Additionally, cell proliferation was 36% ( $P = 5 \times 10^{-6}$ , Figure 4B), migration was 15%, ( $P = 2 \times 10^{-7}$ , Figure 4C) and adhesion was 26% ( $P = 5 \times 10^{-6}$ , Figure 4D) of that in the controls cells.

#### IC53 Upregulated Expression of Integrins

Because IC53 is upregulated in colon cancer and promotes colon cancer cell proliferation, migration and adhesion, we next investigated the target genes that are important in the mediation of IC53-regulated cell invasive growth. Atlas human cancer array from Clontech was used to identify gene expression profile in response to overexpression of IC53 in HCT-116 cells. Total RNA was isolated from HCT-116 cells that were stably transfected with IC53 or control vector; mRNA was isolated from 200 µg total RNA and used to generate <sup>33</sup>P-labeled cDNA probes for microarray analysis. We found that overexpression of IC53 upregulated the expression of genes encoding various integrins, macrophage stimulating 1 and laminins, which have long been linked to cancer progression (Supplementary Table S2). This observation was confirmed by investigating the integrin and laminin expression profile in HCT-116 cells stably transfected with IC53 or control vector by immunocytochemistry or Western blot (Figure 5 and Supplementary Figure S3).



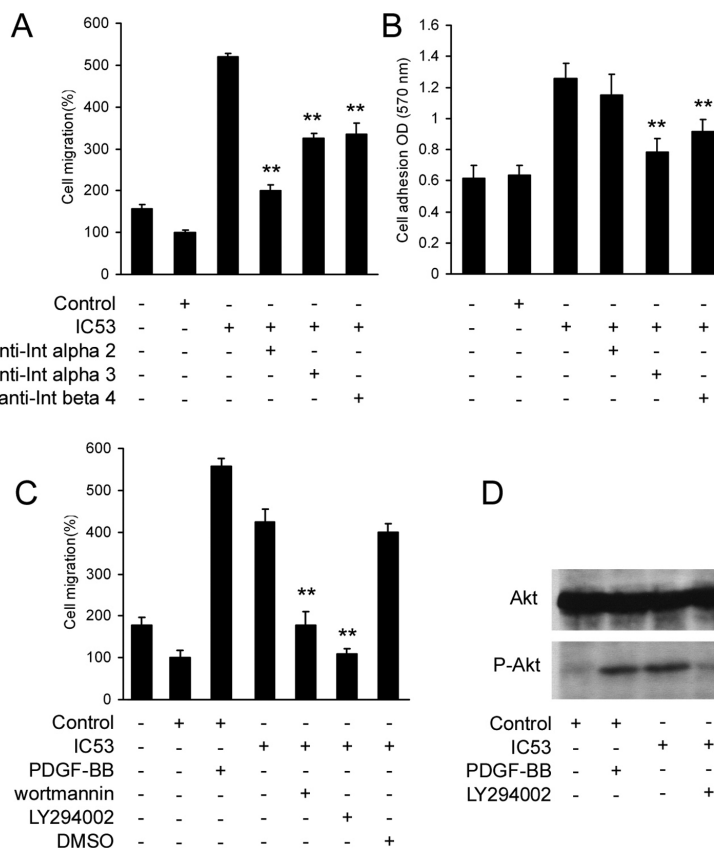
**Figure 5.** IC53 modulated expression of integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$ . IC53 modulated the expression of integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$ . Total protein was isolated from serum-starved HCT-116 cells treated with IC53 plasmids (IC53) or stealth siRNA against IC53 (siRNA) for 48 h, and the expression of integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$  was analyzed by Western blotting. As shown in the lowest panel, an anti-GAPDH antibody was included in the analysis as a loading control.

**Integrin-Mediated IC53-Induced Cell Invasive Growth**

To confirm whether upregulation of integrins mediates IC53-induced tumor cell invasive growth, we treated the cells stably expressing IC53 with antibodies against integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$ , which are the most upregulated integrins (3.3- to 5.5-fold) in HCT-116 cells. As shown in Figure 6A, antibodies against integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$  partially but significantly blocked IC53-mediated HCT-116 cell migration by 60%, 37% and 36% ( $P = 7 \times 10^{-7}$ ), respectively, compared with the controls (not treated with specific antibodies). Similar results were observed in the cell adhesion assay, except that treatment with the antibody against integrin  $\alpha_2$  did not result in a significant blockade of cell adhesion (Figure 6B,  $P = 0.23$ ).

**IC53-Induced Cell Invasive Growth via the Phosphatidylinositol 3-Kinase (PI3-K)-Akt Pathway**

Next, we investigated which signaling pathway is involved in mediation of the process. It is well established that



**Figure 6.** IC53 promoted colon cancer cell invasive growth via the upregulation of integrins and activation of the PI3-K-Akt signaling pathway. Effects of anti-integrin (anti-Int) antibodies on IC53-induced migration and adhesion in HCT-116 cells. HCT-116 cells were stably transfected with IC53 plasmid or empty vectors. The effects of blocking integrin on cell migration and adhesion were tested in the presence or absence of antibodies against integrin  $\alpha_2$  (10  $\mu\text{g/mL}$ ),  $\alpha_3$  (10  $\mu\text{g/mL}$ ) or  $\beta_4$  (20  $\mu\text{g/mL}$ ). (A) Antibodies against integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$  partially, but significantly, inhibited IC53-mediated HCT-116 cell migration by 60%, 37% and 36%, respectively, compared with the controls (not treated with these specific antibodies) (\*\* $P < 0.01$ ). (B) Similar results were observed in the cell adhesion assay, except that treatment with the antibody against integrin  $\alpha_2$  did not result in significant inhibition of cell adhesion ( $P = 0.23$ ). Three independent experiments were carried out and each sample was tested in triplicate; similar results were obtained in all cases. This graph shows representative results, expressed as the mean  $\pm$  SD (\*\* $P < 0.01$ ). (C) PI3-K inhibitors almost completely blocked IC53-induced HCT-116 cell migration. The migration assay was performed by using serum-starved HCT-116 cell transfectants and control cells. These cells were treated with 30 ng/mL PDGF-BB, the PI3-K inhibitor wortmannin (100 nmol/L), LY294002 (25  $\mu\text{mol/L}$ ) or its vehicle ( $\text{Me}_2\text{SO}$ ). The number of migrated cells was counted in the transwell chambers. The values were presented as the mean  $\pm$  SD ( $n = 6$ ). (\*\* $P < 0.01$ ). (D) Western blotting analysis of Akt phosphorylation. The serum-starved HCT-116 cells stably transfected with plasmid alone or plasmid carrying human IC53 were incubated with PDGF-BB (30 ng/mL) or LY294002 (10  $\mu\text{mol/L}$ ) for 2 h. Akt phosphorylation and Akt expression were analyzed by Western blotting with an Akt-phosphorylated antibody (1:1,000) and an Akt antibody (1:1,000), respectively. OD, optical density.

integrin-mediated activation of the phosphatidylinositol 3-kinase (PI3-K) pathway plays an important role in colon

cancer invasive growth (29). To determine whether the effect of IC53 on cell invasive growth is also mediated by this

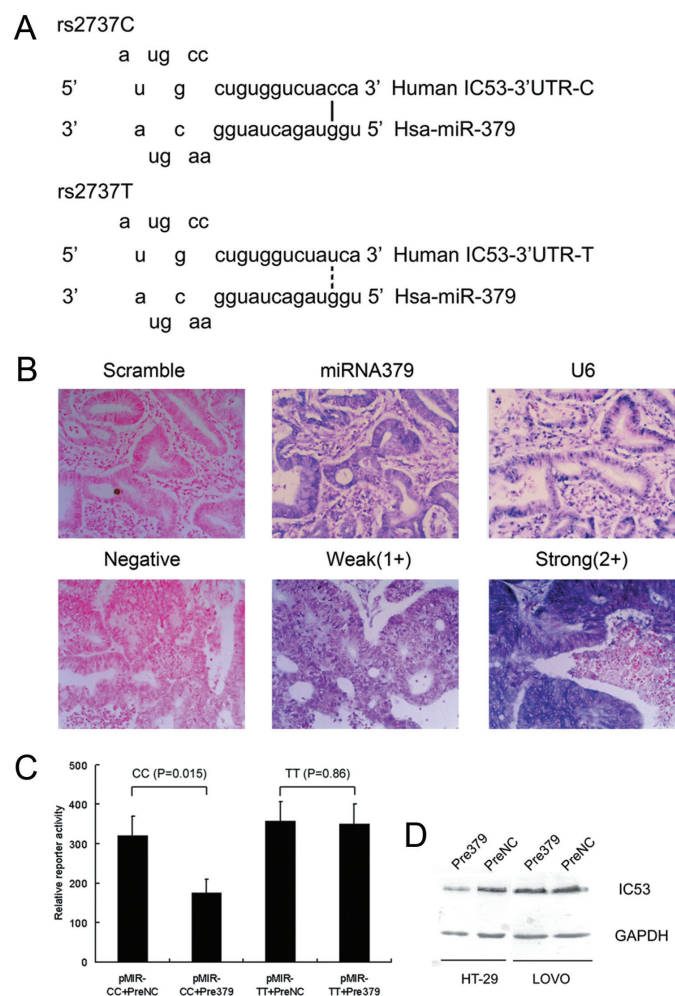


pathway, we examined cell invasive growth after blocking the PI3-K pathway in HCT-116 cells stably transfected with IC53. The two PI3-K-specific inhibitors, wortmannin and LY294002, used to block activation of the PI3-K pathway, almost completely abolished IC53-induced HCT-116 cell migration (Figure 6C,  $P = 2 \times 10^{-7}$ ), indicating the involvement of the PI3-K pathway. Because Akt is an important downstream effector of the PI3-K pathway, we next investigated Akt expression and phosphorylation. As indicated in Figure 6D, Western blot results showed that transfection of IC53 did not alter the expression of Akt protein in HCT-116 cells, but dramatically increased the phosphorylation of Akt.

Immunoblot analysis with antibodies against serine-473 of Akt showed that the level of Akt phosphorylation was comparable in the HCT-116 cells overexpressing the IC53 gene to that in the cells treated with PDGF-BB, a known activator of Akt. To confirm whether Akt phosphorylation induced by IC53 is indeed due to PI3-K activity, we used the PI3-K inhibitor LY294002 to treat HCT-116 cells overexpressing IC53 and found that Akt phosphorylation could be blocked by LY294002 at a concentration of 25  $\mu\text{mol/L}$  (see Figure 6D, *last lane*). This finding supported the possibility that IC53 upregulates the expression of integrins, which activate PI3-K, and PI3-K then enhances the phosphorylation of Akt, a cell growth signal.

### rs2737 C/C Genotype Was Associated with the Late Onset of CRC

Taken together, our results indicate that the IC53 gene is a new mediator for colon cancer progression. Using a bioinformatics approach, we found that rs2737 (a T-C substitution) in the IC53 gene created a potential miR-379 target site (Figure 7A), leading us to hypothesize that rs2737 could modify the colon cancer incidence as well as the timing of colon cancer onset. To test our hypothesis, we first examined miR-379 expression in colon cancer tissue. The expression of miR-379 was confirmed by *in situ*



**Figure 7.** Variant rs2737 created a potential miR-379 target site in the IC53 gene and affected IC53 translation. (A) The IC53 rs2737 T/C variant occurred in the miR-379-binding site. Variant rs2737 is a T to C change (mRNA sequence shown as reference) located in the predicted binding site for miR-379 at the 3'UTR of the IC53 gene. At rs2737, allele C base-paired with G in the Watson-Crick mode (shown with a solid line), whereas allele T wobble base-paired with G (shown with a dashed line). (B) *In situ* hybridization analysis of miR-379 expression in the colon cancer samples. The expression of miR-379 was detected by *in situ* hybridization in the tissue microarray of clinical colon cancer samples by using a monoclonal antibody, and representative images are shown in the upper panel. The intensity of miR-379 staining was scored as negative (0), weak (1+) or moderate (2+) (200 $\times$  magnification), and representative images from each of these scores are shown in the lower panel. (C) Testing the interaction between miR-379 and IC53 by using a reporter assay. The results of luciferase assays showing the allele-specific targeting of miR-379 to the IC53 gene in HCT-116 cells. The luciferase plasmid, pMIR-TT or pMIR-CC, was cotransfected with the negative control miRNA (PreNC) or miR-379 (Pre379). At least six replicate assays were performed for each transfection. For each sample, luciferase activity was normalized by Renilla activity. The  $P$  values for the difference in luciferase activity of the two plasmids were as follows. For pMIR-CC transfection: PreNC versus Pre379,  $P = 0.015$ ; for pMIR-TT transfection: PreNC versus Pre379,  $P = 0.86$ . (D) Immunoblot analysis of the translation levels of IC53 in different genotypic colon cancer cell lines. MiR-379 (20 pmol) significantly suppressed IC53 protein levels in HT-29 cells harboring the heterozygote allele C at rs2737, but did not change the IC53 protein levels in LOVO cells harboring the homozygote allele T at rs2737. The blot was reprobed with GAPDH antibody showing the same level of loading.

**Table 2.** Expression pattern of miR-379 was negatively correlated with that of IC53.

	miR-379 levels	IC53 levels						
		+	2+	3+	<i>P</i>	+	2+ and 3+	<i>P</i>
Total (n = 152)	+	52	51	19	0.029	52	70	0.018
	2+	20	7	3		20	10	
TC + CC (n = 105)	+	26	41	15	0.009	26	56	0.003
	2+	15	6	2		15	8	
TT (n = 47)	+	23	10	4	0.73	23	14	0.66
	2+	7	2	1		7	3	

hybridization in 84% (152/182) of independent clinical colon cancer samples in the tissue microarray described above (Figure 7B). The expression pattern of miR-379 was negatively correlated with that of IC53 (correlation coefficient  $-0.192$ , odds ratio  $0.37$ , 95% confidence interval  $0.15-0.86$ ,  $P = 0.018$ ; Table 2), and the correlation was stronger in the subjects possessing allele C of rs2737, which created a potential target site of miR-379 (correlation coefficient  $-0.284$ , odds ratio  $0.25$ , 95% confidence interval  $0.09-0.66$ ,  $P = 0.003$ ; see Table 2), whereas the correlation was absent in the subjects possessing the TT genotype (correlation coefficient  $-0.067$ , odds ratio  $0.70$ , 95% confidence interval  $0.16-3.12$ ,  $P = 0.66$ ; see Table 2). Next, we tested the interaction between IC53 transcripts and miR-379 directly. We cloned a 75-bp fragment, which contained the predicted binding site of miR-379 around rs2737 into the 3'UTR of the luciferase reporter vector pMIR-REPORT to generate the wild-type (pMIR-TT) or mutant (pMIR-CC) rs2737 constructs. Using HCT-116 cells cotransfected with pMIR-CC reporter constructs and the pre-miR miRNA precursor of miR-379, we found that the pMIR-CC luminescent signal was about 55% that of the control ( $P = 0.015$ , Figure 7C). There was no significant difference between the signal from the cells cotransfected with the pMIR-TT reporter constructs and those cotransfected with the pre-miR miRNA precursor of miR-379 ( $P = 0.86$ , see Figure 7C). To further explore the effects of miR-379 on the translation of IC53, we used two colon cancer cell lines: LOVO homozygous for the T allele and HT-29 heterozygous for the C allele. Im-

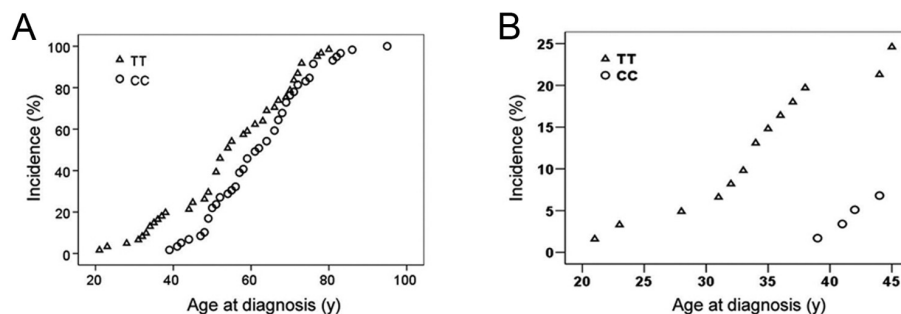
munoblot analysis showed that miR-379 (20 pmol) repressed IC53 translation in HT-29 cells but not in LOVO cells (Figure 7D). This strongly suggested that miR-379 represses IC53 translation in carriers of allele C *in vitro* and *in vivo*.

To test the hypothesis that IC53-rs2737 can modify colon cancer incidence as well as the timing of cancer onset, we consecutively selected 222 Chinese CRC patients with histologically confirmed colon or rectal adenocarcinoma and 260 age, sex and ethnically matched controls. The results of IC53-rs2737 genotyping are summarized in Supplementary Table S3. No association between IC53-rs2737 and CRC incidence was observed. We next

compared the age distribution at CRC diagnosis for patients who had the homozygous wild-type (T/T) genotype with that of patients harboring the homozygous (C/C) variant genotype at rs2737. Among the 222 patients with CRC, the median age at CRC diagnosis was 55.3 years for patients with the T/T genotype and 63.0 years for patients with the C/C rs2737 genotype (95% confidence interval  $2.6-12.8$  years;  $P = 0.003$  [one-way ANOVA test, two-sided]) (Figure 8A). As seen in Figure 8B, the frequency of the C/C genotype was greatly decreased in those individuals who developed CRC at a young age. Individuals who developed CRC before the age of 45 years showed a homozygous C/C frequency for rs2737 of 10.8%, whereas the homozygous C/C frequency was 26.6% for the whole group ( $P = 0.039$ , see Figure 8B). Our data indicated that rs2737 may correlate with age at CRC onset.

**DISCUSSION**

In this study, we found that the expression level of IC53 correlated with the grade and depth of invasion of adenocar-



**Figure 8.** Variant rs2737 correlated with the onset age of CRC patients. (A) The cumulative number curve of the correlation between the rs2737 allele and the onset age of CRC patients. The incidence of the C homozygous allele of rs2737 (circles) and the wild-type T homozygous allele of rs2737 (triangles) among CRC patients was plotted against the onset age. Among the 222 patients with CRC, the onset median age was 55.3 years among patients with the T/T genotype and 63.0 years among patients with the C/C rs2737 genotype (95% confidence interval  $2.6-12.8$  years;  $P = 0.003$  (one-way ANOVA test, two-sided)). (B) The frequency of the C/C rs2737 allele was low among the population with early-onset CRC. The cumulative number curve of individuals harboring either the homozygous rs2737 allele (CC, circles) or the wild-type homozygous rs2737 allele (TT, triangles) is plotted against the onset age of CRC patients below the age of 45 years (1 SD from the median age at diagnosis). The incidence of the C/C rs2737 allele was significantly decreased among patients below the age of 45 years (10.8%) compared with the whole group (26.6%,  $P = 0.039$ ).

cinoma of the colon, and the involvement of IC53 in regulation of colon cancer cell invasiveness occurred via modulation of the integrin-PI3-K-Akt pathway. Importantly, we found that the C allele of rs2737 creates a miR-379 target site in the *IC53* gene and correlates with the late onset of CRC. These results provide direct evidence for the biological function of IC53 as a positive regulator for tumor invasive growth and a new target for suppressing colon cancer progression.

It is well known that integrins increase tumor cell adhesion and migration and promote invasive growth of cancer cells. We demonstrated *in vitro* that IC53 stimulates colon cancer cell line HCT-116 invasive growth via its effects on integrin production. Blockade of integrins with antibodies against integrin  $\alpha_2$ ,  $\alpha_3$  and  $\beta_4$  suppressed invasive growth. These results suggested that IC53 has the potential to promote the migration of HCT-116 cells and colon cancer cells *in vivo*. The invasive growth effects of IC53 are mediated via integrins. Our results are consistent with several previous studies. For example, in intestinal epithelial cells, the  $\alpha_2\beta_1$  integrins mediate Erk activation, which prevents apoptosis induced by serum deprivation. Integrins can also enhance the survival effect of growth factors by facilitating downstream signaling events, such as the effect of  $\alpha_5\beta_1$  integrins on intestinal epithelial cells (30).

The PI3-K signaling pathway has been shown to play a pivotal role in intracellular signal transduction pathways involved in cell growth, cellular transformation and tumorigenesis. Analysis of colon adenocarcinoma cell lines indicates that the PI3-K signaling pathway is up-regulated in colon cancers (31,32), along with the phosphorylation of Akt. Inhibition of the PI3-K pathway with wortmannin resulted in a suppression of the anchorage-independent growth of colon cells in a soft agar assay (33). Integrins have been linked to PI3-K/Akt signaling in promoting tumor cell invasiveness. In the presence of growth factors, integrins

can prevent apoptosis of fibroblasts by focal adhesion kinase (FAK) and mediate activation of PI3-K and Akt (34). Phosphorylation of Akt has been shown to effect Wnt (Wnt-1, Wnt-3a) signaling, a pathway central to the initiation of colorectal carcinogenesis. Akt activation leads to inhibition of the proapoptotic glycogen synthase kinase 3B, with a resultant increase in the level of the anti-apoptotic  $\beta$ -catenin protein (35), and acts synergistically with the Ras and Raf cascades, which are critical in colorectal carcinogenesis (36,37).

We found that IC53 upregulates integrins, stimulates PI3-K activation and increases Akt phosphorylation. Antibodies against integrins could abolish IC53-induced PI3-K activation, and PI3-K-specific inhibitors (wortmannin and LY294002) could block Akt phosphorylation, suggesting that integrins induce PI3-K activation and PI3-K mediates IC53-induced Akt phosphorylation. In our studies, wortmannin and LY294002, two inhibitors of PI3-K, could also inhibit the migration of HCT-116 cells, as they did on Akt phosphorylation, indicating that IC53-induced HCT-116 cell migration *in vitro* occurs via activation of the integrin-PI3-K-Akt pathway.

Our results are consistent with the data reported by Stav *et al.* (23), which showed that IC53 is overexpressed in tumor tissues of lung adenocarcinoma. In contrast, IC53 was reported as a tumor suppressor in Hela, H1299, HT1080 and U2OS cell lines (24–26); however, all the cell lines are not originated from colon cancer; these data indicate that IC53 probably has diverse effects in different cancers. We speculated that IC53, as a cytoplasmic protein (data not shown), may act as a regulator in the signal pathway and demonstrate diverse effects by binding to a different partner. IC53 was first identified as protein binding to p35 (38), the regulatory protein of cdk5, overexpression of the cdk5/p35 complex reversed the inhibitory effects of ciglitazone and promoted cell growth in the colon cancer cell line HT29 (39). Thus, IC53 may promote colon cancer progres-

sion through regulating activity or expression of cdk5.

It is well known that modifier factors, including both environmental and genetic components, influence clinical phenotypes (8). Several reports have shown that genetic polymorphisms may constitute genetic modifiers of age at diagnosis of CRC (12,14,15). In this study, we found that rs2737 in the *IC53* gene, which was a positive mediator for colon cancer progression, created a miR-379 target site, and the rs2737 C/C genotype was associated with late onset of CRC. Furthermore, the frequency of the C/C genotype was much lower in patients with an age of <45 years at diagnosis than in the whole group, indicating that the C allele of rs2737 may be protective versus CRC.

The function of miR-379 remains to be fully elucidated. However, our results showed that the expression pattern of miR-379 was negatively correlated with that of IC53 in colon cancer tissues, and miR-379 inhibited IC53 translation. We also found that the rs2737 C/C genotype created an miR-379 target and resulted in late onset of CRC. To the best of our knowledge, this is the first report to show that miR-379 functions as a novel mediator for CRC progression.

In summary, our results indicate that IC53 is a positive regulator for CRC progression via the upregulation of integrin expression, activation of PI3-K and increase in Akt phosphorylation. Importantly, we demonstrated that the C allele of rs2737 creates a miR-379 target site in the *IC53* gene and correlates with the late onset of CRC. The findings from this study may significantly contribute toward the development of improved preventative or therapeutic strategies for CRC. Future prospective studies could extend these findings by using a greater sample size.

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**DISCLOSURE**

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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