MicroRNA 345, a methylation-sensitive microRNA is involved in cell proliferation and invasion in human colorectal cancer

Jie-Ting Tang, Ji-Lin Wang, Wan Du, Jie Hong, Shu-Liang Zhao, Ying-Chao Wang, Hua Xiong, Hui-Min Chen and Jing-Yuan Fang[*]

Digestive Department, Shanghai Institute of Digestive Disease, Shanghai Jiaotong University School of Medicine Renji Hospital, 145 Middle Shandong Road, Shanghai 20001, China

[*]To whom correspondence should be addressed. Tel: +86 21 53882450;
Fax: +86 21 63266027;
Email: jingyuanfang@yahoo.com

Aberrant methylation has been shown to trigger the inactivation of tumor suppressor genes during tumorigenesis. MicroRNAs (miR-NAs) have been found deregulated in human colorectal cancer (CRC), and some of them may function as tumor suppressor genes. Here, we investigated CpG island promoter hypermethylation as a potential mechanism underlying miRNA disruption and identified methylation-sensitive miRNAs that might repress CRC development. We compared differential expression of miRNAs after 5-aza-2′-deoxycitidine (5-aza-dC) treatment using microarrays. DNA methylation status of the candidate miRNA was analyzed. The candidate miRNA was transfected into CRC cells and growth-suppressive mechanisms were explored. Luciferase reporter assay and western blot were used to identify the target genes of the candidate miRNA. The expression of mir-345 was significantly increased after 5-aza-dC treatment. DNA methylation analyses of mir-345 showed high methylation levels in tumor versus normal tissues. Expression of mir-345 was significantly down-regulated in 51.6% of CRC tissues compared with corresponding non-cancerous tissues. Low expression of mir-345 was associated with lymph node metastasis and worse histological type. Increased mir-345 function was sufficient to suppress colon cancer cell proliferation and invasiveness *in vitro*. Furthermore, we identified BCL2-associated athanogene 3 (BAG3), an anti-apoptosis protein, to be a target of mir-345. These results suggested as a methylation-sensitive miRNA in CRC, mir-345 may play an important role of antineoplastic as a growth inhibitor in the development of CRC.

Introduction

Currently, cancer is recognized as both a genetic and an epigenetic disease. Epigenetic changes in tumor cells include alterations in DNA methylation, histone modifications and changes induced by non-coding RNAs (1). MicroRNAs (MiRNAs), which are similar to siRNAs in some aspects, are ~22 nt non-coding RNAs that regulate gene expression in animals, plants and viruses. The mature miRNA strands are incorporated into an RNA-induced silencing complex, guiding it to the messenger RNA (mRNA) targets. miRNAs with perfect or near-perfect complementarity to the 3′-untranslated regions (UTRs) of specific mRNAs induce the degradation of these mRNAs and down-regulate the target genes (2–4). Over 500 miRNA sequences of the human genome have been annotated in the latest version of the miRBase (5). These small RNAs are involved in cell proliferation, cell differentiation, apoptosis and organ development (6–9). The emerging knowledge on the functions and roles of miRNAs has opened a new area in cancer research (10–12). miRNAs have been found deregulated in human colorectal neoplasia

samples, and some of them may function as tumor suppressor genes (13). Despite the growing evidence of their significant involvement in cancer, little is known about the causes of altered miRNA expression.

CpG island hypermethylation is thought to be a mechanism of tumor suppressor gene silencing, and studies were undertaken to identify potential targets of methylation-induced gene silencing by screening for genes whose expression was down-regulated in response to hypermethylation (14). Interestingly, recent evidence has suggested that epigenetic alterations such as DNA methylation could regulate miRNA expression during tumorigenesis (15–20), and ~47% of human miRNA genes are related to CpG islands (21).

Accordingly, we investigated in this study whether the miRNAs expressed in colorectal cancer (CRC) involve CpG island and digged out the novel methylation-sensitive miRNAs that might be potential tumor suppressors in CRC pathogenesis. Different expressions of miRNAs were analyzed via miRNA microarrays after 5-aza-2′-deoxycitidine (5-aza-dC), a DNA methyltransferase inhibitor, treatment in SW1116 and HT29 CRC cell lines. With good reproducibility, this method is easily performed and extensively used to identify epigenetic genes (15). We further focused on mir-345, which is embedded or near a CpG island and highly induced by 5-aza-dC treatment. We found that mir-345 was highly methylated with low expression in CRC tissues compared with non-cancerous tissues. Increased mir-345 function was sufficient to suppress colon cancer cell proliferation and invasiveness *in vitro*. A molecule that regulates apoptosis, BCL2-associated athanogene 3 (BAG3), was identified to be a target of mir-345. Our data indicated that the epigenetic regulation of miRNAs may be regarded as the new anticancer drug targets in the future exploration.

Materials and methods

Cell lines and 5-aza-dC treatment

SW1116 and HT29 (colon adenocarcinoma cell lines) were obtained from Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS) before the start of study. SW1116 cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithersburg, Maryland) supplemented with 10% fetal bovine serum, whereas the HT29 line was cultured in McCOY's 5A medium supplemented with 10% fetal bovine serum, under 5% CO_2 humidified atmosphere at 37°C. Cells were seeded 24 h prior to treatment with 5-aza-dC (2 μM; Sigma–Aldrich, St Louis, Missouri) or isovolumetric dimethyl sulfoxide (DMSO) control for 72 h.

Tissue sample preparation

Thirty-one patients with previously untreated CRC, including 11 women and 20 men, with a median age of 62 years (range 34–85), were selected in a single study center. The protocol was approved by the Ethics Committee of Shanghai Jiao-Tong University School of Medicine Renji Hospital, and the research was carried out according to the provisions of the Helsinki Declaration of 1975. None of the patients received preoperative treatments such as radiotherapy or chemotherapy. The clinical stages of the tumors were defined according to American Joint Committee on Cancer/International Union Against Cancer staging system (AJCC/TNM). Under the supervision of a pathologist, tissue samples from primary tumors, para-cancer tissues (histopathologically dysplasia tissues) and adjacent histopathologically non-tumor mucosal tissues (>5 cm from the border of tumor area) were collected from surgically resected tissues before any treatment was initiated. Immediately after surgical removal, tissue samples were snap-frozen in liquid nitrogen then maintained at –80°C until use. Normal colon tissues and adenoma tissues were obtained during an endoscopic procedure.

RNA and DNA extraction from cells and tissues

Total RNA was extracted using the TRIzol® Reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. The concentration and purity of RNA were determined using the NanoDrop® ND-1000 spectrophotometer. Denaturing agarose gel electrophoresis was also performed to visually assess the RNA, which contains highly abundant 28S and 18S ribosomal RNA bands. For all experiments, genomic DNA was prepared using the DNeasy kit (Qiagen, Dusseldorf, Germany).

miRNA microarray analysis

miRNA microarrays were performed using the miRCURY LNA TM Array micro-RNA Profiling Service (Exiqon, Copenhagen, Denmark). The total RNAs were labeled with Cy3 dye using the miRCURYTM LNA Array labeling kit (catalogue No. 208032; Exiqon) per the manufacturer's instructions. The Cy3-labeled RNA molecules were hybridized to the miRCURYTM LNA Arrays (Exiqon), consisting of control probes, mismatch probes and >2000 capture probes. The sequences of the perfectly matched probes for human miRNAs are registered and annotated in the miRBase release 9.2 at the Wellcome Trust Sanger Institute. GenePix 4000B scanner and GenePix Pro 6.0 software (Axon Instruments, Union city, CA) were used to scan images for the analysis. Each chip was normalized to the U6 signal intensity. The U6 signal intensity with a median value of ~13 000 was used to normalize intensity values and obtain the geometric means for each miRNA on the chip. The arithmetic means and standard errors for the two groups in triplicate were calculated, and the fold changes were measured. Each miRNA signal was transformed to logarithm base 1.5, and a two-sample *t*-test was conducted. miRNAs with a significant value of 0.05 or lower and a fold change value of 1.5 or higher were considered to be differentially expressed. Statistical analyses were performed with MATLAB 7.5 (MathWorks, Natick, MA).

Quantitative real-time reverse transcription–polymerase chain reaction

miRNA levels were determined by quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) performed on complementary DNA (cDNA) generated from 1 μg of total RNA. Expression of the mature miRNA was assayed using stem-loop reverse transcription followed by PCR analysis as described previously (22). PCR reactions were performed in triplicate for each sample. The relative amounts of miRNAs were normalized against U6 snRNA, and the fold change for each miRNA was calculated by the 2-ΔΔCt method (23). The primer sequences for qRT–PCR are listed in supplementary Table 1, available at *Carcinogenesis* Online. Amplification and detection of specific products were performed with the ABI PRISM7900 system (Applied Biosystems, Foster City, CA).

Rapid amplification of 5′ cDNA ends

5′ Rapid amplification of cDNA ends (RACE) system was carried out using 5′-Full RACE (TaKaRa, D315, Tokyo, Japan) TaKaRa kit and according to the manufacturer's instruction. Briefly, 2.4 μg of colon total RNA was reverse transcribed into cDNA using Reverse Transcriptase M-MLV. After that cDNAs were amplified by outer PCR using 5′ RACE outer primer and mir-345 R1 (5′-CAGCAGACCTAGGGTTTGGGT-3′). Then, this was followed by inner PCR amplification using 5′ RACE inner primer and mir-345 R2 (5′-AGGCTGTC-GATATTCAAACCC-3′). Amplified PCR products were subcloned into the PUC19-T vector (Takara, D3219). The isolated clones were sequenced and corresponding genomic sequences were searched for using the BLAST program.

DNA methylation analyses

DNA methylation was determined by PCR analysis of bisulfite-modified genomic DNA. Genomic DNA (1.5 μg) was treated, as described previously, with sodium bisulfite, which induces conversion of unmethylated, but not methylated, cytosine to uracil (24). Then, methylation-specific PCR (MSP) was performed using primers specific for either the methylated or modified unmethylated DNA. The methylation status was also analyzed by bisulfite genomic sequencing of both strands of the corresponding CpG islands, as described previously (25). The primers used and corresponding Tm are described in supplementary Table 2, available at *Carcinogenesis* Online.

Luciferase assay

Luciferase constructs were made by ligating oligonucleotides containing the wild-type (WT) or mutant putative target site of the BAG3 3′-UTR into the multi-cloning site of the pcDNA3-EGFP (TaKaRa). Clones were identified by restriction enzyme digestion (EcoRI and XhoI; TaKaRa) and sequencing (primers used to amplify BAG3 3′-UTR are described in supplementary Table 3, available at *Carcinogenesis* Online). HT29 cells were transfected in 48-well plates with 80 pmol miR-345 Antisense Oligo (miR-345 ASO, 5′-GAGCCCTG-GACTAGGAGTCAGC-3′) and control ASO or 0.8 μg pri-mir-345 and pri-NC (empty vector pcDNA3.1) using 0.8 μl LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instruction. Changing culture medium after 4 h. After 24 h, the cells were transfected with 0.4 μg Luciferase reporter vector using 0.8 μl Lipofectamine™ 2000 (Invitrogen). Changing culture medium after 4 h. Luciferase activity was measured 48 h after the final transfection using a Fluorescence Spectrophotometer F-4500 (HITACHI, Tokyo, Japan).

Proliferation and invasion assays

For these studies, we selected HT29 and SW1116 cell lines for *in vitro* characterization. Number of viable cells was assayed using water-soluble tetrazolium tetrazolium salt (CCK-8, Dojindo) as per the manufacturer's instructions. All the experiments were performed in triplicate. Results were expressed as mean ± SD. Matrigel invasion assay were conducted at 24 h after transfection. Control or pri-mir-345 plasmid-treated HT29 and SW1116 cells were trypsinized and resuspended in fully supplemented medium. Cells were then seeded at 20 000 cells per well for invasion assay into transwell inserts (8 μm pore size; BD Falcon, Corvallis, OR). For invasion assay, the transwell inserts were coated with 40 μl/well of Matrigel (BD Falcon). Complete culture medium was used as chemo-attractant in the lower chamber. The assays were taken down with three ddH2O washes followed by staining with hematoxylin and eosin for 5 min after 48 h (SW1116 cells)/72 h (HT29 cells) for invasion assay. The cells migrated to the basal side of the porous membrane was visualized with a microscope at ×400 magnification. Ten random fields from three replicate wells were counted and the number of cells that had migrated or invaded was presented as number of cells counted per field of the porous membrane.

Western blot and qRT–PCR of BAG3

Protein extracts were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were immunoprobed with antibodies against BAG3 (1:300; ENZO, Alexis-Biomol, Lausen, Switzerland). The relative expression of BAG3 was determined by the density of the specific band in a western blot normalized to the amount of total protein as determined by the density of the glyceraldehyde-3-phosphate dehydrogenase or β-actin band, i.e. if the glyceraldehyde-3-phosphate dehydrogenase control value was 30 000 U (pixels of brightness), then the calculation used to normalize BAG3 to glyceraldehyde-3-phosphate dehydrogenase can be expressed as [30 000/(density of GAPH)] × (density of BAG3). Total RNA was used for qRT–PCR detection of BAG3, which was performed at least three times. The primers used are described in supplementary Table 3, available at *Carcinogenesis* Online.

Immunohistochemistry

BAG3 protein expression was investigated by immunohistochemical staining in a couple of adenomas, three CRC tissues and corresponding normal surrounding tissues. Briefly, the specimens were isolated, rinsed with phosphate-buffered saline, fixed in 4% buffered neutral formalin and embedded in paraffin. Paraffin sections were then deparaffinized, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in phosphate-buffered saline before immunoperoxidase staining. Slides were then incubated overnight at 4°C in a humidified chamber with rabbit polyclonal anti-BAG3 antibody (1:200; Abcam, Cambridge, UK) and subsequently with antirabbit IgG for 20 min. Negative controls were performed by omitting the primary antibody. After incubation in a solution containing 0.06 mM diaminobenzidine and 2 mM hydrogen peroxide in 0.05% phosphate-buffered saline (pH 7.6) for 5 min, slides were washed, dehydrated with alcohol and xylene and mounted with coverslips using a permanent mounting medium.

Databases and GenBank accession numbers

The miRNA sequences were analyzed using miRbase (http://microrna.sanger.-ac.uk/), University of California at Santa Cruz Human Genome Browser (http://genome.cse.ucsc.edu/) and CpG Island Searcher Program (http://cpgi-slands.usc.edu/). Detailed information of base pairing comparisons between mir-345 and its target site in the 3′-UTR of BAG3 mRNA is available at miRBase Targets (http://microrna.sanger.ac.uk/). The GenBank accession number of BAG3 mRNA is NM_004281.

Data analysis

Calculations were carried out using the statistical package for social sciences (SPSS) software. Fisher exact test, chi-square test and non-parametric tests were used, depending on the data set of concern. Wilcoxon test was used for two-related groups. Comparisons between independent groups were made using Mann–Whitney *U*-test between two groups and Kruskall–Wallis test for three or more groups. A *P* value of <0.05 was accepted as statistically significant.

Results

5-Aza-dC treatment up-regulates mir-345 transcripts and decreases promoter methylation of mir-345 in HT29 cell

Expression profiling of human miRNAs in SW1116 and HT29 CRC cells was performed using miRNA microarrays. SW1116 cells treated with 5-aza-dC (Group A2) showed >2-fold induction of four miRNAs compared with SW1116 cells treated with isovolumetric DMSO (Group A1) (see supplementary Table 4 is available at *Carcinogenesis* Online). HT29 cells treated with 5-aza-dC (Group B2) showed >2-fold induction of six miRNAs compared with HT29 cells treated with isovolumetric DMSO (Group B1) (see supplementary Table 5 is available at *Carcinogenesis* Online). The up-regulated folds of miRNAs were different between different CRC cells. For instance, hsa-mir-129-5p was up-regulated >2-fold

in SW1116 cells but only 1.68-fold in HT29 cells. Among these miR-NAs, hsa-mir-345 was most highly up-regulated after the treatment in HT29. To confirm the results of the miRNA microarray analyses, we analyzed the expressing level of hsa-mir-345 and hsa-mir-129-5p by qRT–PCR in SW1116 and HT29 cells before and after 5-aza-dC treatment. As shown in supplementary Table 6, available at *Carcinogenesis* Online, the real-time PCR results were consistent with the microarray data, which demonstrated the reliability of microarray. Programs at http://genome.ucsc.edu/ and http://www.ncbi.nlm.nih.gov/ were used to analyze gene structures of the 10 miRNAs up-regulated >2-fold and to identify the transcriptional start sites. We input the DNA sequence of the miRNA genes (including upstream 1000 bp, 5′-UTR, CDS, 3′-UTR) into the CpG Island Searcher program (http://www.cpgislands.com/) and found that hsa-mir-345 (near), hsa-mir-129-2 (stem-loop sequence of hsa-mir-129-5p) (embedded), hsa-mir-320a (embedded) and hsa-mir-200c (near) were embedded in or near a canonical CpG island. Among these miRNAs, hsa-mir-200c was previously reported to have no methylation changes in CRC cells (19). No related information was available about the other three miRNAs. To further investigate CpG island promoter methylation alterations as a potential mechanism underlying miR-NAs deregulation in CRC cells, MSP analysis and Bisulfite genome sequencing were performed. MSP analysis showed that only the mir-129-2 methylated PCR product was detected both in 5-aza-dC-treated cells and control cells. The promoter region of the mir-129-2 gene was 94.95% methylated in DMSO-treated control cells and stays the same after 5-aza-dC treatment, which was consistent with the result of MSP (supplementary Figure 1A and D is available at *Carcinogenesis* Online).

mir-320a unmethylated PCR product was detected both in 5-aza-dC-treated cells and control cells. Bisulfite genome sequencing showed that the methylation level of the promoter region of the mir-320a gene was 0.37 and 0.53% in control cells and treated cells, respectively (supplementary Figure 1A and E is available at *Carcinogenesis* Online). Since mir-345 gene was not embedded but near a CpG island, we mapped by 5′ RACE the transcriptional start site of mir-345 and it was found indeed in the analyzed CpG island (Figure 1A). MSP analysis showed that only the mir-345 methylated PCR product was present in control cells, whereas an unmethylated PCR product was detected in 5-aza-dC-treated cells (Figure 1B). Bisulfite genome sequencing showed that the promoter region of the mir-345 gene was heavily methylated in the untreated cells (64.73%), while the methylation level decreased to 35.78% after 5-aza-dC treatment (Figure 1D and E). Therefore, we selected hsa-mir-345 as our candidate miRNA, which expression was significantly increased after 5-aza-dC treatment (Figure 1C) and the induction of mir-345 was accompanied by a prominent decrease in DNA methylation of the mir-345 gene promoter region.

The promoter of mir-345 is hypermethylated and the expression of mir-345 is down-regulated in CRC tissues

In order to further detect the promoter methylation level of mir-345 in the human CRC, we analyzed DNA methylation levels in the promoter region of the mir-345 gene in several normal colon tissues (Group NC), adenoma (Group A) and CRC [cancerous samples (Group Ca) and the corresponding non-cancerous samples (Group N)] by MSP and bisulfite genomic sequencing. MSP testing results
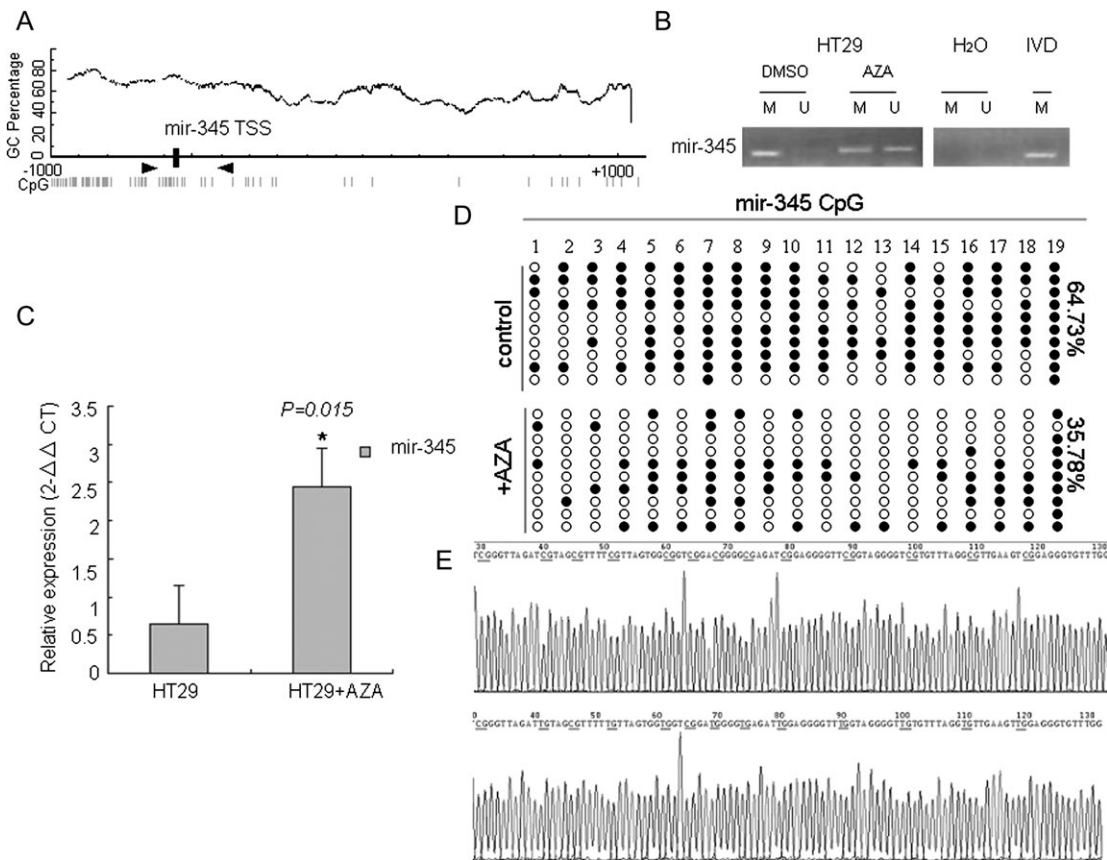

Fig. 1. Alterations in DNA methylation around the promoter region of mir-345 induced by 5-aza-dC in HT29 cell. (**A**) mir-345 is near in a CpG island. The black vertical line indicates the transcription start sites of primary transcripts of mir-345 determined by 5′ Race. The percentage of G + C over a 100-bp window. Vertical red lines, position of individual CpG dinucleotides; arrowheads, bisulfite PCR primers. (**B**) MSP analyses for mir-345 methylation in HT29 cells treated with 5-aza-dC (AZA) and DMSO as control. Unmethylated (U) or methylated (M) sequences. *In vitro* methylated DNA (IVD) and H_2O are shown as controls. (**C**) Analysis of mir-345 expression levels between HT29/HT29+AZA by qRT–PCR. *P = 0.015. (**D**) DNA methylation status of HT29 cells untreated or treated with 5-aza-dC was determined by bisulfite genomic sequencing. Open circle, unmethylated CpG; filled circle, methylated CpG; AZA, 2 μM 5-aza-dC. Percentage of methylated CpG sites is shown for each analysis. (**E**) Bisulfite genomic analyses of mir-345 CpG island methylation status. After treatment, most of 'CG' are converted to 'TG', highlighted by '—' in red.

of mir-345 showed 13 cases with methylated PCR products (M) only, 4 cases with unmethylated PCR products (U) only and 14 cases with both methylated (M) and unmethylated PCR products (U) in the 31 samples of CRC. Electrophoretogram of some representative cases was showed in Figure 2A. Thus, mir-345 hypermethylation was observed in 87.1% (27 of 31) of CRC patients. Bisulfite genomic sequencing was performed in 10 normal colon tissues, 10 adenoma and 10 pairs of CRC (including cancerous samples and the corresponding non-cancerous samples), partial sequencing maps of some representative cases were shown in supplementary Figure 2, available at *Carcinogenesis* Online. We analyzed the methylation status of 10 colons of each sample and found that the relative promoter methylation level of mir-345 was 0.8 and 2.0% in normal colon tissues and adenoma, respectively, whereas it was 8.7% in the corresponding non-cancerous samples and 16% in the CRC samples (Figure 2B). The DNA methylation level of mir-345 was significantly higher in CRC samples than in the corresponding non-cancerous samples, adenoma and normal colon tissues ($P = 0.041$; <0.001; <0.001, respectively). Meanwhile, no significant differences of DNA methylation level were found between adenomas and normal colon tissues ($P = 0.739$). The expression levels of mir-345 in CRC samples were examined by qRT–PCR. As expected, compared with adjacent non-tumor mucosal tissues, expression level of mir-345 was significantly down-regulated in 51.6% (16/31, $P < 0.001$), up-regulated in 25.8% (8/31, $P = 0.012$) and unchanged in 22.6% (7/31, $P = 0.075$) of CRC tissues (Figure 4A). The relationship between the expression levels and methylation status of mir-345 in CRC samples were studied using Mann–Whitney U-test. As Figure 3B shows the expression

of mir-345 was significantly decreased in the CRC samples ($n = 13$) with higher promoter methylation level which showed only methylated sequences compared with other CRC samples ($n = 18$) in the 31 patients CRC samples. Thus, the expression of mir-345 was negatively correlated with methylation status.

The expression of mir-345 may be associated with several clinicopathologic features of CRC

Furthermore, we studied the relationship between the expression and tumor size, age, sex, clinical stage, lymph node positivity, localization and serum levels of carcinoembryonic antigen (Mann–Whitney U-test between two groups and Kruskall–Wallis test for three or more groups). Clinical characteristics of CRC samples were showed in supplementary Table 7, available at *Carcinogenesis* Online. Forty-three point eight percent (7/16) of the patients had $>50\%$ lymph node metastasis in the CRC group with significantly down-regulated mir-345 levels, whereas only 6.7% (1/15) of the patients had $>50\%$ lymph node metastasis in other CRC tissues (Figure 3C). The histological type of 62.5% of the CRC tissues with significantly down-regulated mir-345 level was moderately differentiated adenocarcinoma and 37.5% showed worse histological type such as poorly differentiated adenocarcinoma, adenocarcinoma mucocellulae and signet ring cell cancer. Meanwhile, 93.3% of the CRC tissues without down-regulated mir-345 levels were moderately differentiated adenocarcinomas (Figure 3D). Thus, CRC tissues with significantly down-regulated mir-345 levels seemed to have higher rate of lymph node metastasis ($P = 0.037$) and worse histological type ($P = 0.040$). On the other hand, we found no significant correlation between the analyzed miRNAs
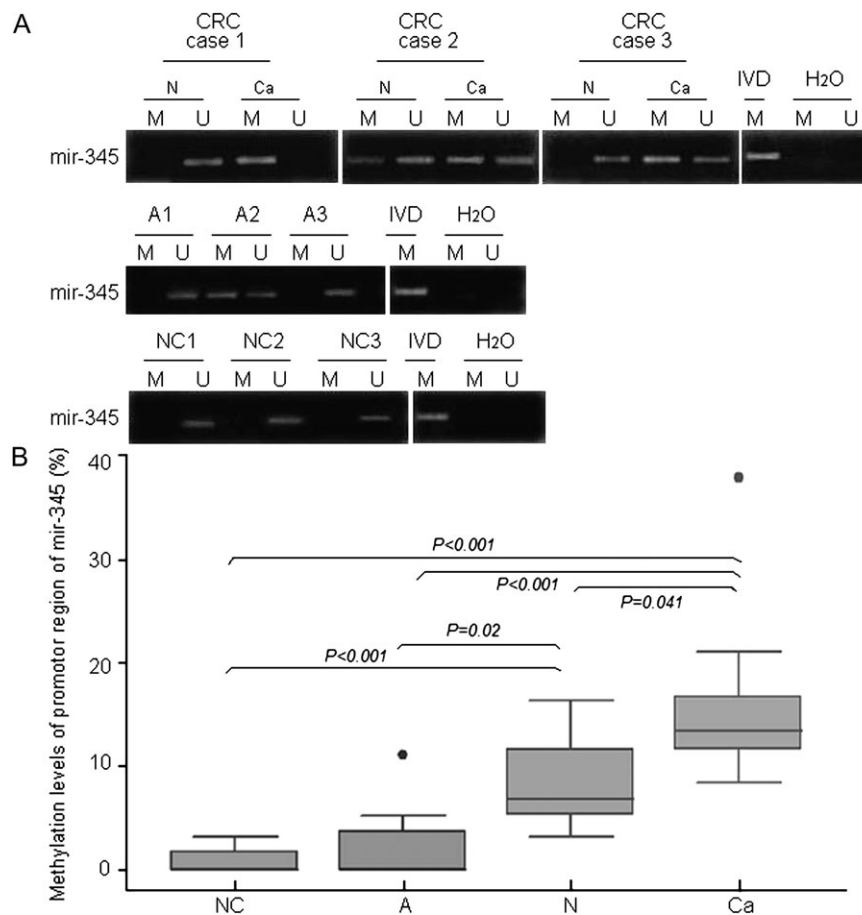
Fig. 2. DNA methylation analyses of mir-345 in CRC, adenoma and normal colon tissues. (**A**) Electrophoretogram of MSP analyses for mir-345 methylation in CRC (case 1–3), adenoma (A1–A3) and normal colon tissues (NC1–NC3). M, methylated sequences; U, unmethylated sequences. *In vitro* methylated DNA (IVD) is shown as a positive control for methylated sequence. (**B**) The methylation levels of the promoter region of mir-345 in CRC (10 pairs), adenoma ($n = 10$) and normal colon tissues ($n = 10$) by bisulfite genomic sequencing. NC, normal colon tissue; A, adenoma; N, the corresponding non-cancerous samples; Ca, the colorectal tumor samples. The filled circles, outliers.
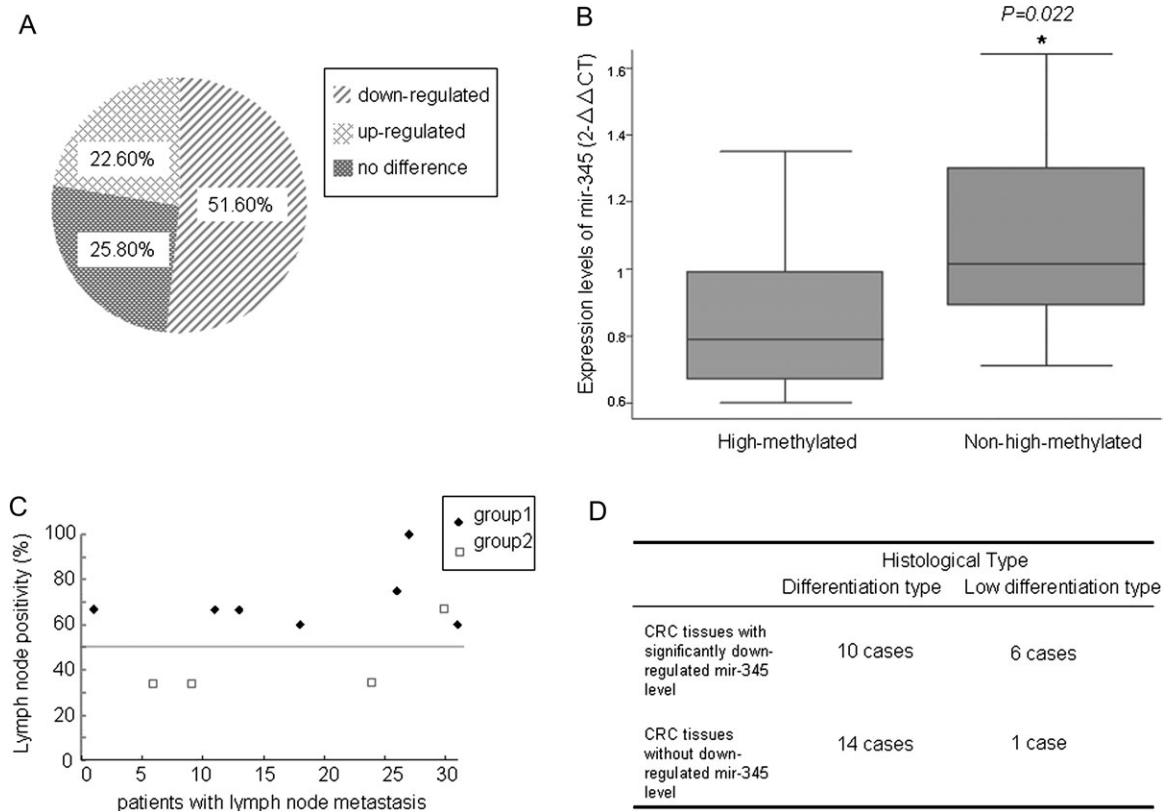
Fig. 3. mir-345 expression level is down-regulated in CRC and correlated with methylation status and lymph node metastasis. (**A**) The rate of change of mir-345 expression level in CRC tissues compared with corresponding non-cancerous tissues. (**B**) 'High-methylated' group, the 13 CRC cases which showed only methylated sequences; 'non-high-methylated' group, the other 18 CRC cases include the cases showing both methylated and unmethylated sequences and the cases showing only unmethylated sequences. *$P = 0.022$. (**C**) mir-345 expression level may be associated with lymph node metastasis in CRC. There were seven patients with >50% lymph node metastasis in the CRC group 1 with significantly down-regulated mir-345 level; in other CRC patients (group 2), there were four patients which had lymph node metastasis, but only one patient had >50% lymph node metastasis among them. '—' is a baseline representing lymph node metastasis 50%. (**D**) Histological type of CRC samples.

and tumor size, age, sex, clinical stage, localization and serum levels of carcinoembryonic antigen ($P = 0.377, 0.860, 0.792, 0.584, 0.172, 0.835$, respectively).

Up-regulation of mir-345 decreases CRC cell growth and invasion
To further determine the role of mir-345 in CRC, expression of mir-345 was up-regulated in HT29 and SW1116 cells by transfected with pri-mir-345 plasmid or down-regulated by mir-345 ASO. Cell growth and invasion was evaluated comparing with cells transfecting with pri-NC or negative control RNA. Over-expression of mir-345 significantly repressed the proliferation of CRC cells. Conversely, mir-345 ASO significantly enhanced the proliferation of CRC cells (Figure 4A and C), the data suggest that mir-345 may regulate cell proliferation in CRC HT29 and SW1116 cells. Then, an *in vitro* cell invasion assay was performed. The number of HT29 cells transfected with pri-mir-345 plasmid that invaded the polycarbonate membrane of transwell chamber was significantly less than control group [(16.00 ± 2.83)/HPF versus (63.00 ± 5.22)/HPF, $P = 0.01$]. The number of SW1116 cells transfected with pri-mir-345 plasmid that invaded the polycarbonate membrane of transwell chamber was significantly less than control group [(17.33 ± 8.71)/HPF versus (59.07 ± 2.08)/HPF, $P = 0.036$]. The data suggest that mir-345 may inhibit cell invasion. However, there was no significant difference between mir-345 ASO group and negative control group (Figure 4B and D).

BAG3 is identified to be a target of mir-345
The database of computationally predicted human miRNA targets, miRBase Targets (http://microrna.sanger.ac.uk/), indicated that BAG3 is a potential target of mir-345. To determine whether mir-345 can

regulate the expression of BAG3, we performed a luciferase reporter assay with a vector containing the WT or mutant (MUT) putative BAG3 3′-UTR target site downstream of the luciferase reporter gene. Then, we co-transfected these vectors into HT29 cells with pri-mir-345 or mir-345 ASO, respectively. The luciferase activity of HT29 cells co-transfected with BAG3-WT and pri-mir-345 was significantly lower than that of HT29 cells co-transfected with BAG3-WT and mir-345 ASO ($P = 0.006$). In contrast, the luciferase activity of HT29 cells co-transfected with BAG3-MUT and pri-mir-345 showed no significant difference from the HT29 cells co-transfected with BAG3-MUT and mir-345 ASO ($P = 0.111$) (Figure 5A and B). The protein expression level of BAG3 was down-regulated in HT29 cells after pri-mir-345 and pri-NC control transfection, indicating that mir-345 may regulate the expression of BAG3 in HT29 cells (Figure 5C). On the other hand, there was no statistically significant difference of the BAG3 mRNA expression levels after pri-mir-345 and pri-NC control transfection in HT29 cells, suggesting that the expression of BAG3 is inhibited by mir-345 via blocking translation rather than mRNA degradation.

BAG3 is highly expressed in CRC tissues
We used qRT–PCR and western blot analysis to detect the expression level of BAG3 in CRC tissues, para-cancer tissues and adjacent non-tumor mucosal tissues. The mRNA and protein expression level of BAG3 showed significantly higher level in CRC tissues, compared with para-cancer tissues and adjacent non-cancerous mucosal tissues ($P < 0.001$) (Figure 6A and B). Then, we selected a couple of adenomas, three carcinomas and normal surrounding tissues to verify that anti-BAG3 antibody stained CRC cells in immunohistochemistry (Figure 6C). The data showed the dramatical brown staining of BAG3
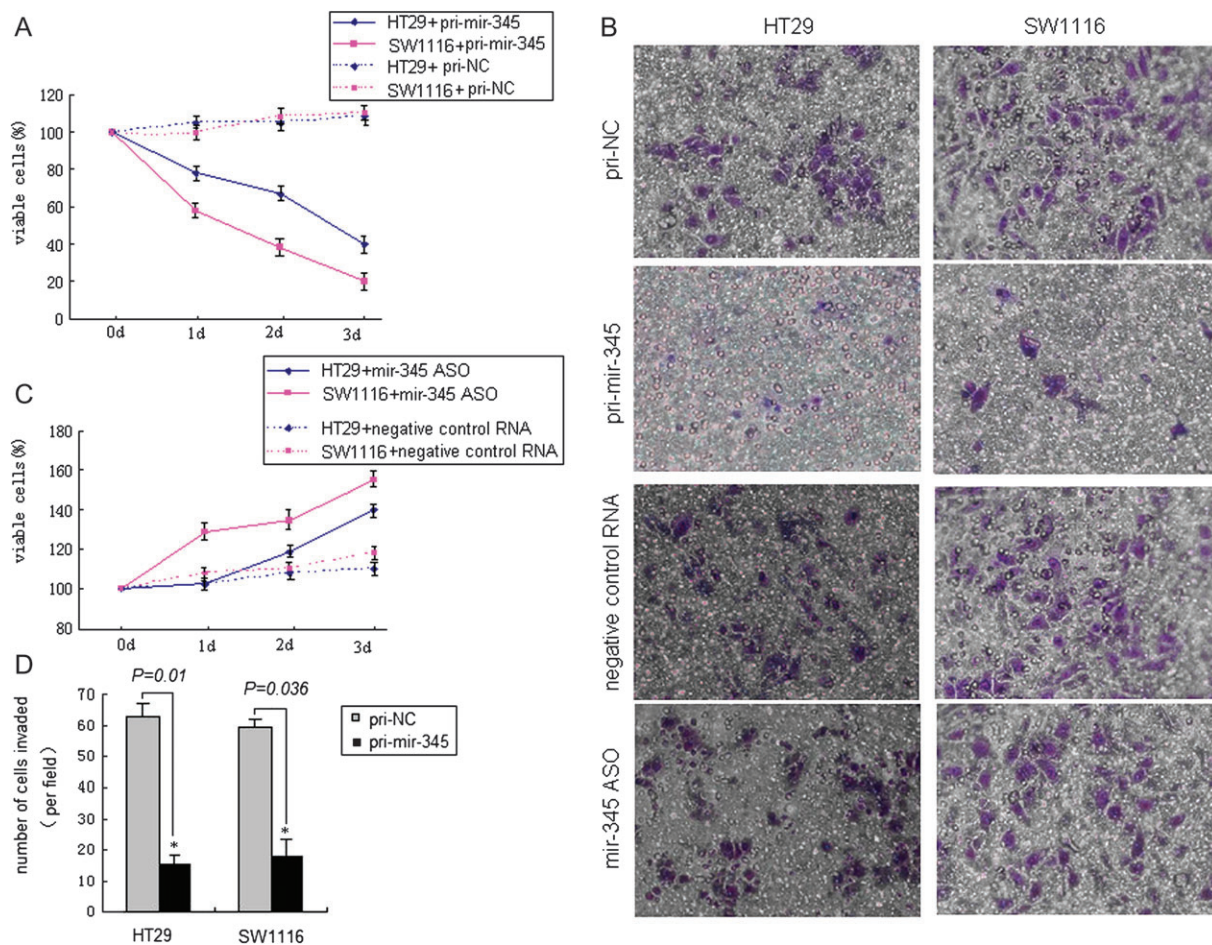
Fig. 4. Up-expression of mir-345 decreases CRC cells proliferation and the invasion potential of CRC cells (**A**) Alteration of viable cells after the transfection of pri-mir-345 plasmid. (**B**) HT29 and SW1116 cells that invaded the polycarbonate membrane of transwell chamber (gentian violet staining, magnification ×400). (**C**) Alteration of viable cells after the transfection of mir-345 ASO. (**D**) The number of HT29 and SW1116 cells that invaded the polycarbonate membrane of transwell chamber. *P =0.01 and 0.036, respectively.

protein in tumor tissue (Ca) but not in its normal surrounding tissue (N). Adenomas were weak positive for BAG3 expression.

Discussion

Due to the high incidence of CRC, the pathogenesis, prevention and treatment of CRC have received considerable attention. miRNAs may function as tumor suppressors or proto-oncogenes during tumorigenesis and may contribute to the diagnosis and prognosis of CRC (16,26–34). These evidences indicate that miRNA could be a good candidate as biomarkers for CRC diagnosis and prognosis. Meanwhile, miRNA-based cancer therapies could be new strategies for CRC prevention and treatment. However, little is known to date about the causes of disturbed miRNA expression. Interestingly, recent studies have suggested that other epigenetic alterations such as DNA methylation and histone modifications could regulate miRNA expression during tumorigenesis (17–19,35). However, it should be noted that not all miRNAs are regulated by epigenomics (36). Since the regulation of miRNA expression by DNA methylation is complex, further studies on the methylation patterns of miRNA in CRC are warranted. Here, we showed that microarray analysis combined with 5-aza-dc treatment can be used to screen methylation-sensitive miRNAs. As shown in supplementary Tables 4 and 5, available at *Carcinogenesis* Online, the microarray analysis indicated that a small part of the miRNAs were up-regulated by 5-aza-dC treatment in CRC cells. The up-regulated miRNAs were quite different between SW1116 and HT29 cells. We think it is mainly due to the different drug sensitivity between SW1116 and HT29 cells. In spite of the same concentration and administration

time, different cell types may achieve different biological drug sensitivity. Besides, the DNA methylation status of miRNA genes may be different between different CRC cells.

The DNA demethylating agent 5-aza-dC is a similar molecule to cytidine. Covalently binding to DNA methyltransferase, 5-aza-dC decreases methylation levels, thus regulating genetic expression. 5-aza-dC can induce the demethylation of several tumor-associated genes promoters, and changed their expression levels in CRC cell lines including SW1116 or HT29 (37,38). DNA methylation analyses showed that the promoter region of the mir-345 gene was heavily methylated in HT29 cells (64.73%), and the methylation level was decreased to 35.78% after 5-aza-dC treatment (Figure 2D), which is consistent with the up-regulation of mir-345 expression after the treatment (Figure 2C). In spite of the up-regulation of mir-129-2 and mir-320a after treatment, the methylation status of these two miRNAs stays the same. We presume that the up-regulated miRNAs found by microarray combined with 5-zaz-dC treatment were probably modulated by methylation directly or indirectly. mir-129-2 and mir-320a might be involved in other signal pathway which participate in methylation modification instead of being induced directly by methylation. The complex network between them still need further study. Furthermore, mir-345 was methylated significantly higher in CRC tissues than adenoma and normal colon tissues. These results indicate that the hypermethylation of mir-345 was a CRC-related DNA methylation event. 5-aza-dC was effective in reducing the methylation level in the promoter region of mir-345, especially in association with its activation. Therefore, mir-345 could be a methylation-sensitive miRNA in CRC.

Accumulating studies have determined that the transcriptional silencing of tumor suppressor genes by CpG island hypermethylation
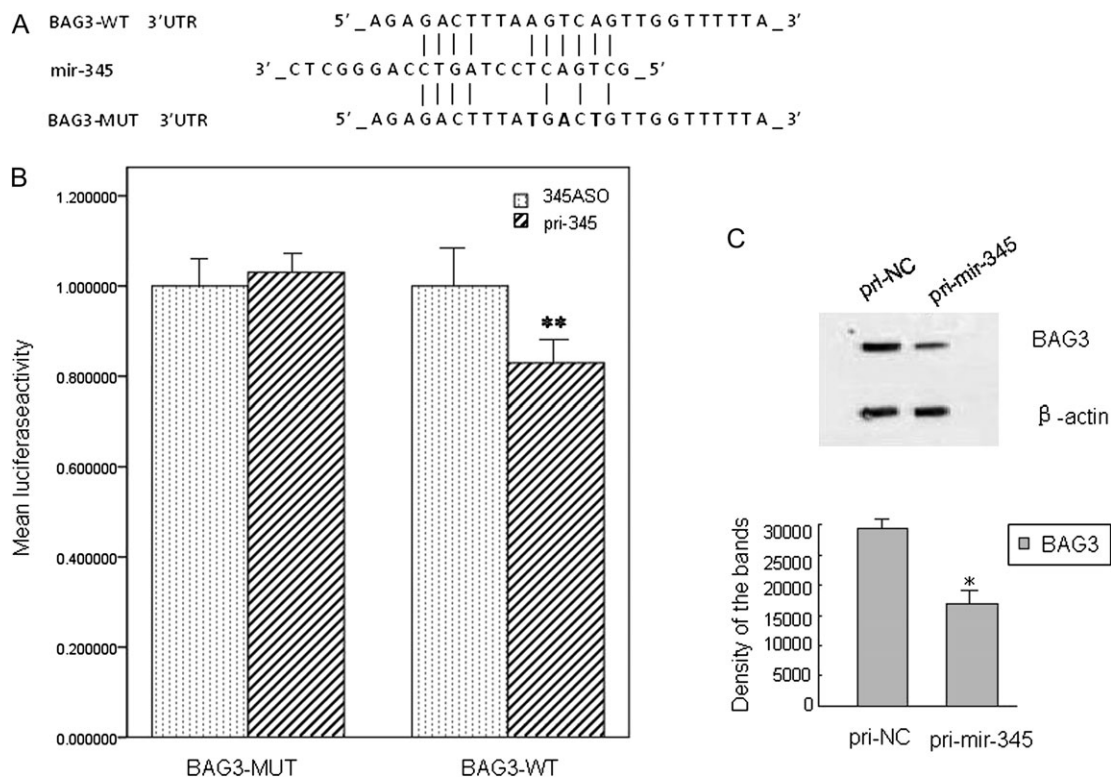
Fig. 5. BAG3 was identified to be a target of mir-345. (**A**) Base pairing comparison between mature mir-345 and the WT or mutant (MUT) putative target site in the 3′-UTR of BAG3 mRNA. The mutant sequence is identical to the WT construct except for mutations highlighted in bold disrupting base pairing at the 5′ end of mir-345. (**B**) Dual luciferase assay of HT29 cells co-transfected with vector containing the WT or mutant (MUT) putative BAG3 3′-UTR target site and pri-mir-345 or mir-345 ASO. The luciferase values of pri-mir-345 transfections were normalized to the average values for the corresponding mir-345 ASO transfections. $^{**}P < 0.01$. (**C**) Western blots of BAG3 in HT29 cells transfected with pri-mir-345. β-actin was used as a loading control. The density of the western blot bands was normalized to the amount of BAG3 protein as determined by the density of the band obtained by western blot for β-actin. $^{*}P < 0.05$.

is a common event (34). Given the hypermethylation and lower expression of mir-345 in CRC cells, we thus focused on the potential role of mir-345 in the pathogenesis of CRC. Although altered expression levels of many miRNAs have been identified in human cancers (39), to our knowledge, very little is reported about mir-345 expression levels and its physiological and pathological roles in CRC tissues. Recently, Chang *et al.* have identified miR-345 as a stable reference miRNA in CRC (40). Our study further analyzed the relationship between the expression levels and methylation status of mir-345 in CRC samples and performed *in vitro* experiments to clarify the potential role of mir-345 in CRC. First, we examined the expression level of mir-345 in clinical samples by qRT–PCR, a quantitative and sensitive assay. As expected, statistically significant differences in the expression of mir-345 was observed in tumors compared with non-tumor adjacent tissues. Furthermore, the 13 CRC cases, which showed methylated PCR products (M), expressed lower levels of mir-345 than the other 18 cases ($P = 0.022$). CRC tissues with significantly down-regulated mir-345 level seemed to have higher rate of lymph node metastasis ($P = 0.037$) and worse histological type ($P = 0.040$). These findings suggested methylation status of mir-345 was associated with its expression in CRC, and it may be involved in pathogenesis of CRC. Since low expression of mir-345 may be associated with lymph node positivity and worse histological type, mir-345 may have a potential role in CRC invasion and progression. However, no correlations between miRNA levels and tumor size, age, sex, clinical stage, localization or serum levels of carcinoembryonic antigen were found. Thus, an increased number of cases may be necessary and further studies should be done to identify the targets of mir-345 and to clarify the potential role of mir-345 in CRC. Then, we selected HT29 and SW1116 cell lines for *in vitro* characterization. *In vitro*, mir-345 over-expression by pri-mir-345 transfection had negative effect on the proliferation of the two cell lines. In contrast,

decreased mir-345 function led to a significant increase of proliferation of the two cell lines. The results of cell invasion test showed that increased mir-345 function led to a significant decrease of the ability of HT29 and SW1116 cells to invade through Matrigel-coated basement membrane. These results demonstrate that over-expression of mir-345 may suppress colon cancer cell invasiveness *in vitro*. However, mir-345 ASO treatment resulted in no significant changes of cell invasiveness. It may be because of the low expression level of mir-345 in colon cancer cells.

As we known, miRNA is a non-coding RNA that has roles in down-regulating its target genes. Thus, it would be highly useful to identify the potential targets of miRNA. Computational predictions for human miR-NA targets show that BAG3 is a potential target of mir-345. We identified this target through a luciferase reporter assay. As a member of the BAG co-chaperone protein family, BAG3 is one of the molecules that regulates apoptosis. Proteins that share the BAG domain are characterized by their interaction with a variety of partners (heat shock proteins, steroid hormone receptors, Raf-1 and others), involved in regulating a number of cellular processes, including proliferation and apoptosis. Studies have assigned an anti-apoptotic role to BAG3 in some neoplastic cell types and identified the protein as a candidate target of therapy (41). BAG3 over-expression has been observed in many human epithelial cancer cell lines, especially adenocarcinomas and may regulate motility and adhesion of epithelial cancer cells. The high levels of BAG3 protein may be relevant to mechanisms of tumor invasion and metastasis. Silencing BAG3 expression with small interfering RNA increased apoptosis (42,43). Our study determined that BAG3 is one of the targets of mir-345, and for the first time, we report on the expression level of BAG3 in human CRC tissues. The high expression of BAG3 in CRC tissues indicated the potential role of BAG3 in CRC. These results suggested that mir-345 might be involved in pathogenesis of CRC through down-regulation of the BAG3 expression. As a methylation-sensitive miRNA
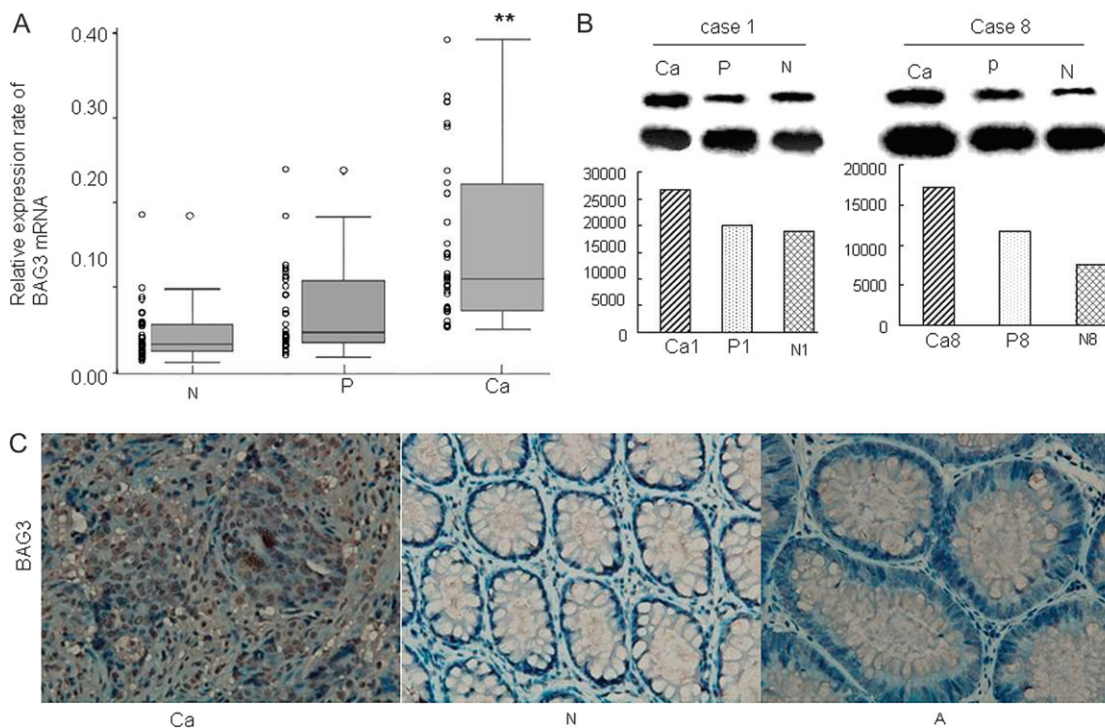
Fig. 6. Western blots and qRT–PCR results of BAG3 in CRC patients. (**A**) qRT–PCR of BAG3 in CRC tissues (Ca), para-cancer tissues (P) and adjacent non-tumor mucosal tissues (N) of 31 patients. **$^{**}P < 0.001$. (**B**) Western blots of BAG3 in CRC cases 1 and 8 with glyceraldehyde-3-phosphate dehydrogenase as a loading control (bottom panel). Western blots of BAG3 in all of the 31 CRC cases were shown in supplementary Figure 3, available at *Carcinogenesis* Online. The density of the western blot bands was normalized to the amount of BAG3 protein as determined by the density of the glyceraldehyde-3-phosphate dehydrogenase band. The three columns in the bar represent the mean density of BAG3 bands in the N, P and Ca groups. The amount of BAG3 protein was significantly higher in the Ca group. (**C**) Immunocytochemistry analysis of BAG3 expression in CRC case (Ca), its normal surrounding tissue (N) and adenoma (A).

in CRC, mir-345 may be a link between chromosome modifying drugs to cancer-related genes.

In conclusion, this is the first time to report that mir-345 is a methylation-sensitive miRNA in CRC development. Furthermore, this miRNA may regulate BAG3 expression as a tumor suppressor in human colon carcinogenesis. Further studies in epigenetic regulation of miRNA expression are necessary. An attempt could be made to identify new avenues for anticancer therapy based on the epigenetic regulation of miRNA. With the identification of a greater number of epigenetically silenced tumor suppressor miRNA genes in human cancer, targeting these miRNAs will become a potentially powerful approach to the development of novel epigenetic drugs. We anticipate further research and development in this direction of epigenetic drug development to be explored in the near future.

Supplementary material

Supplementary Tables 1–7 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

References

1. Egger,G. *et al*. (2004) Epigenetics in human disease and prospects for epigenetic therapy. *Nature*, **429**, 457–463.
2. Cho,W.C. (2007) OncomiRs: the discovery and progress of microRNAs in cancers. *Mol. Cancer*, **6**, 60.
3. Zanette,D.L. *et al*. (2007) miRNA expression profiles in chronic lymphocytic and acute lymphocytic leukemia. *Braz. J. Med. Biol. Res.*, **40**, 1435–1440.
4. Lawrie,C.H. (2007) MicroRNAs and haematology: small molecules, big function. *Br. J. Haematol.*, **137**, 503–512.
5. Griffiths-Jones,S. *et al*. (2008) miRBase: tools for microRNA genomics. *Nucleic Acids Res.*, **36**, D154–D158.
6. Lin,S.L. *et al*. (2006) First in vivo evidence of microRNA-induced fragile X mental retardation syndrome. *Mol. Psychiatry*, **11**, 616–617.
7. Van Rooij,E. *et al*. (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc. Natl. Acad. Sci. USA*, **103**, 18255–18260.
8. Calin,G.A. *et al*. (2006) MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res.*, **66**, 7390–7394.
9. Iwai,N. *et al*. (2005) Polymorphisms in human pre-miRNAs. *Biochem. Biophys. Res. Commun.*, **331**, 1439–1444.
10. Blenkiron,C. *et al*. (2007) miRNAs in cancer: approaches, aetiology, diagnostics and therapy. *Hum. Mol. Genet.*, **16**, R106–R113.
11. Cho,W.C. (2010) MicroRNAs: potential biomarkers for cancer diagnosis, prognosis and targets for therapy. *Int. J. Biochem. Cell Biol.*, **42**, 1273–1281.
12. Cho,W.C. (2010) MicroRNAs in cancer—from research to therapy. *Biochim. Biophys. Acta*, **1805**, 209–217.
13. Michael,M.Z. *et al*. (2003) Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol. Cancer Res.*, **1**, 882–891.
14. Conway,K.E. *et al*. (2000) TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. *Cancer Res.*, **60**, 6236–6242.
15. Mori,Y. *et al*. (2006) A genome-wide search identifies epigenetic silencing of somatostatin, tachykimin-1, and 5 other genes in colon cancer. *Gastroenterology*, **131**, 797–808.

16. Díaz,R. *et al.* (2008) Deregulated expression of miR-106a predicts survival in human colon cancer patients. *Genes Chromosomes Cancer*, **47**, 794–802.

17. Han,L. *et al.* (2007) DNA methylation regulates microRNA expression. *Cancer Biol. Ther.*, **6**, 1284–1288.

18. Grady,W.M. *et al.* (2008) Epigenetic silencing of the intronic microRNA hsamiR-342 and its host gene EVL in colorectal cancer. *Oncogene*, **27**, 3880–3888.

19. Lujambio,A. *et al.* (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res.*, **67**, 1424–1429.

20. Kunej,T. *et al.* (2011) Epigenetic regulation of microRNAs in cancer: an integrated review of literature. *Mutat. Res* doi:10.1016/j.mrfmmm.2011.03.008.

21. Weber,B. *et al.* (2007) Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle*, **6**, 1001–1005.

22. Chen,C. *et al.* (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res.*, **33**, e179.

23. Livak,K.J. *et al.* (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T))method. *Methods*, **25**, 402–408.

24. Frommer,M. *et al.* (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA*, **89**, 1827–1831.

25. Daskalakis,M. *et al.* (2002) Demethylation of a hypermethylated p15/INK4B gene in patients with myelodysplastic syndrome by 5-Aza-2′-deoxycytidine (decitabine) treatment. *Blood*, **100**, 2957–2964.

26. Schetter,A.J. *et al.* (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocar cinoma. *JAMA*, **299**, 425–436.

27. Slaby,O. *et al.* (2007) Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology*, **72**, 397–402.

28. Asangani,I.A. *et al.* (2008) MicroRNA-21(miR-21) posttranscriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene*, **27**, 2128–2136.

29. Lanza,G. *et al.* (2007) mRNA/microRNA gene expression profile in microsatellite unstable colorectal cancer. *Mol. Cancer*, **6**, 54.

30. Akao,Y. *et al.* (2006) MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. *Oncol. Rep.*, **16**, 845–850.

31. Bandrés,E. *et al.* (2006) Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and nontumoral tissues. *Mol. Cancer*, **19**, 29.

32. Akao,Y. *et al.* (2006) let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol. Pharm. Bull.*, **29**, 903–906.

33. Lu,J. *et al.* (2005) MicroRNA expression profiles classify human cancers. *Nature*, **435**, 834–838.

34. Xi,Y. *et al.* (2006) Prognostic values of microRNAs in colorectal cancer. *Biomark Insights*, **2**, 113–121.

35. Toyota,M. *et al.* (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. *Cancer Res.*, **68**, 4123–4132.

36. Akao,Y. *et al.* (2007) Downregulation of microRNAs-143 and -145 in B-cell malignancies. *Cancer Sci.*, **98**, 1914–1920.

37. Fang,J.Y. *et al.* (2004) Epigenetic modification regulates both expression of tumor-associated genes and cell cycle progressing in human colon cancer cell lines: Colo-320 and SW1116. *Cell Res.*, **14**, 217–226.

38. Lu,X. *et al.* (2008) Differential regulation of CDX1 and CDX2 gene expression by deficiency in methyl group donors. *Biochimie*, **90**, 697–704.

39. Wu,W. *et al.* (2007) MicroRNA and cancer: current status and prospective. *Int. J. Cancer*, **120**, 953–960.

40. Chang,K.H. *et al.* (2010) MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. *BMC Cancer*, **10**, 173.

41. Rosati,A. *et al.* (2007) Apoptosis inhibition in cancer cells: a novel molecular pathway that involves BAG3 protein. *Int. J. Biochem. Cell Biol.*, **39**, 1337–1342.

42. Iwasaki,M. *et al.* (2007) BAG3 regulates motility and adhesion of epithelial cancer cells. *Cancer Res.*, **67**, 10252–10259.

43. Jacobs,A.T. *et al.* (2009) HSF1-mediated BAG3 expression attenuates apoptosis in 4-hydroxynonenal-treated colon cancer cells via stabilization of anti-apoptotic Bcl-2 proteins. *J. Biol. Chem.*, **284**, 9176–9183.