microRNA-139-5p exerts tumor suppressor function by targeting NOTCH1 in colorectal cancer

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Abstract

Background: miR-139-5p was identified to be significantly down-regulated in colon tumor tissues by miRNA array. We aimed to clarify its biological function, molecular mechanisms and direct target gene in colorectal cancer (CRC).

Methods: The biological function of miR-139-5p was examined by cell growth, cell cycle and apoptosis analysis in vitro and in vivo. miR-139-5p target gene and signaling pathway was identified by luciferase activity assay and western blot.

Results: miR-139-5p was significantly down-regulated in primary tumor tissues (P < 0.0001). Ectopic expression of miR-139-5p in colon cancer cell lines significantly suppressed cell growth as evidenced by cell viability assay (P < 0.001) and colony formation assay (P < 0.01) and in xenograft tumor growth in nude mice (P < 0.01). miR-139-5p induced apoptosis (P < 0.01), concomitantly with up-regulation of key apoptosis genes including cleaved caspase-8, caspase-3, caspase-7 and PARP. miR-139-5p also caused cell cycle arrest in G0/G1 phase (P < 0.01), with upregulation of key G0/G1 phase regulators p21Cip1/Waf1 and p27Kip1. Moreover, miR-139-5p inhibited cellular migration (P < 0.001) and invasiveness (P < 0.001) through the inhibition of matrix metalloproteinases (MMP)7 and MMP9. Oncogene NOTCH1 was revealed to be a putative target of miR-139-5p, which was inversely correlated with miR-139-5p expression (r = −0.3862, P = 0.0002).

Conclusions: miR-139-5p plays a pivotal role in colon cancer through inhibiting cell proliferation, metastasis, and promoting apoptosis and cell cycle arrest by targeting oncogenic NOTCH1.

Keywords: miR-139-5p, Colorectal cancer, NOTCH1, Tumor suppressor

Background

Colorectal cancer (CRC) is the third most common malignancy worldwide and the second leading cause of tumor-related death in Western countries [1]. According to International Agency for Research on Cancer, approximately 1.24 million new cases of CRC were reported worldwide in 2008 [1]. CRC incidence rates are rapidly rising in Asian countries [2], which were formerly considered as low-risk area. So there is a growing need for understanding the molecular pathogenesis of CRC. Over the last decade, microRNAs (miRNAs) have emerged as key players in carcinogenesis. Aberrant expression of miRNAs has been demonstrated to play a critical role in the initiation and progression of several human cancers through post-transcriptional regulation of gene expression [3-5]. It has been shown that miRNAs play an important role in the development of CRC. miRNAs in CRC exhibit oncogenic or tumor suppressive role by directly regulating oncogenes or tumor-suppressor genes [6,7].

Our previous work on miRNA microarray analysis revealed that miR-139-5p was downregulated in colon cancers (0.10 ~ 0.15-fold of the non-tumor miR-139-5p)
However, the functional role and mechanistic action of miR-139-5p in CRC remain largely unclear. In this study, we aimed to determine its biological function, molecular basis and target gene in CRC.

**Results**

**miR-139-5p is frequently downregulated in primary CRC and colon cancer cell lines**

We previously performed miRNA arrays based on five CRC patients and two advanced adenoma patients [8,9]. The miRNA array results showed that miR-139-5p was significantly down-regulated in both CRC and advanced adenoma compared with their adjacent normal tissues. The Sanger database shows that miR-139-5p shares the same precursor with miR-139-3p. However, miR-139-3p was undetectable in CRC and the adjacent non-tumor tissues. Therefore, our study focused on the miR-139-5p. The downregulation of miR-139-5p in CRC was further verified in two independent cohorts of colon cancer patients (cohort 1, n = 45; and cohort 2, n = 50, Additional file 1: Table S1) and 7 colon cancer cell lines. Consistent with the findings from miRNA array, there was a significant decrease in the expression level of miR-139-5p in both cohorts of CRC tumors when compared with their matched non-tumorous tissues ($P < 0.0001$ in both cohorts) (Figure 1A). We had performed qRT-PCR to determine the level of miR-139-5p. Among the paired samples, 89% (40/45) of cohort 1 and 98% (49/50) of cohort 2 CRC samples showed lower miR-139-5p levels than in the adjacent normal tissue. In addition, we examined miR-139-5p expression levels in seven colon cancer cell lines (Caco2, DLD1, HCT116, HT29, LoVo, LS180 and SW620). We observed that miR-139-5p was downregulated in all seven colon cancer cell lines relative to nine normal colon tissues ($P < 0.0001$) (Figure 1B). Moreover, miR-139-5p expressions were restored in 5 CRC cell lines upon administration of DNA methylation inhibitor 5-aza-2'-deoxycytidine (Additional file 2: Figure S1A), which suggests that epigenetic silencing may at least contributed to the down-regulation of miR-139-5p. The correlation of miR-139-5p expression with clinicopathological features of 44 CRC patients was investigated. Based on univariate analysis of this cohort, miR-139-5p was not associated with clinicopathological features including gender, TNM stage, tumor size and recurrence. Kaplan-Meier survival analysis showed that miR-139-5p expression level was not
miR-139-5p suppresses colon cancer cell growth in vitro and in vivo

The frequent downregulation of miR-139-5p in both primary CRC tumor tissues and colon cancer cell lines indicates that miR-139-5p might play a role in CRC tumorigenesis. To explore the potential tumor suppressive effect of miR-139-5p, miR-139-5p precursor (pre-miR-139) was transfected into colon cancer cell lines (DLD1, HCT116 and SW1116). We performed real-time PCR to check the expression level of miR-139-5p and miR-139-3p after transfected with pre-miR-139 mimics. We observed an increase of miR-139-5p post-transfection, while miR-139-3p showed no significantly changes (Additional file 2: Figure S1B). Our results demonstrated that pre-miR-139 mimic conferred to up-regulation of miR-139-5p, but not miR-139-3p. Ectopic expression of miR-139-5p in DLD1 and HCT116 cells caused a significant decrease in cell viability ($P < 0.0001$ in both cell lines) (Figure 2A and B). The inhibitory effect of miR-139-5p on cancer cell growth was further confirmed by colony formation assay. The colonies formed in pre-miR-139-transfected cells were significantly less than in control-transfected cells in DLD1 and a borderline statistically significant was observed in HCT116 as compared with control ($P = 0.07$) (Figure 2C). To further confirm the growth inhibitory effect of miR-139-5p on colon cancer cells, a xenograft tumor growth assay was performed. The subcutaneous tumor growth curve of HCT116 stably expressed miR-139-5p or control in vivo was shown in Figure 2D. The tumor volume was
significantly lower in miR-139-5p nude mice as compared to the control mice \((P < 0.01)\). These results provided further evidence that miR-139-5p plays a tumor suppressive role in colon cancer.

**miR-139-5p induces G0/G1 cell cycle arrest in colon cancer cells by induction of p53, p21Cip1/Waf1, and p27Kip1**

We investigated the effect of miR-139-5p on cell cycle distribution. Concomitant with this inhibition of cell proliferation, there was a significant increase in the number of cells accumulating in the G0/G1 phase in DLD1 and HCT116 (Figure 3A). p21Cip1/Waf1 and p27Kip1 are two important cyclin-Cdk inhibitors that negatively regulate G1 phase progression. Therefore, we determined the expression levels of these two cell cycle inhibitors in pre-miR-139-transfected. Ectopic expression of miR-139-5p induced p53 and p27Kip1 protein expression in both DLD1 and HCT116 cells (Figure 3B); however, upregulation of p21Cip1/Waf1 was only observed in HCT116 cells (Figure 3B). Thus, miR-139-5p suppressed cell proliferation by inducing a G0/G1 cell cycle arrest mediated by induction of p21Cip1/Waf1 and p27Kip1.

**miR-139-5p induces cell apoptosis by regulating caspase-dependent apoptosis pathway**

In order to determine whether the decrease in colon cell growth by miR-139-5p was due to an induction of apoptosis, we evaluated the rate of cellular apoptosis using Annexin V-APC and 7-AAD staining by flow cytometry. As shown in Figure 3C, the number of both early and late apoptotic cells at 48–96 h post-transfection of pre-miR-139 in DLD1 and HCT116 cells was substantially increased as compared to control pre-miRNA transfected DLD1 and HCT116 cells. Induction of apoptosis was further confirmed by the expression of apoptosis-related proteins using western blot. As shown in Figure 3D, the protein levels of the active forms of caspase-3, caspase-7, caspase-8 were enhanced. To further confirm their activities in active form, the cleavage of nuclear poly (ADP-ribose) polymerase (PARP) was determined. As expected, the level of cleaved PARP was enhanced in the pre-miR-139-transfected cells compared to the control cells both in DLD1 and in HCT116 (Figure 3D). These results inferred that apoptosis concomitant with cell cycle arrest induced by miR-139-5p

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**Figure 3** miR-139-5p induced colon cancer cell apoptosis and cell cycle arrest at G0/G1 phase. (A) miR-139-5p caused cell cycle arrest at G0/G1 phase in DLD1 and HCT116 cells (left panel). Quantitative analysis of G0/G1 proportion in DLD1 and HCT116. The experiments were repeated three times in triplicate. Data are mean ± SD. (B) G0/G1 phase cell cycle related protein expression including p21Cip1/Waf1, p27Kip1 and p53 were determined by western blot in DLD1 and HCT116 cells (C) miR-139-5p induced apoptosis in DLD1 and HCT116 cells. Cell apoptosis was examined by flow cytometry analysis of Annexin V-APC and 7-AAD double-staining. Data represented the average of the early and late apoptotic cells, respectively. (D) Apoptotic-related protein expression (PARP, caspase-3, 7, 8) were detected by western blot.
was contributing factors leading to the growth inhibition in miR-139-5p-expressing colon cancer cells.

**miR-139-5p suppresses CRC cell migration and invasiveness of colon cancer cell lines**

We next tested whether miR-139-5p could alter migration and invasiveness of colon cancer cells *in vitro*. DLD1 and SW1116 cells were transfected with pre-miR-139 or control induction of miR-139-5p significantly decreased the migrated cells in DLD1 (*P* < 0.001) and in SW1116 (*P* < 0.001) compared to control cells (Figure 4A). Moreover, miR-139-5p markedly slowed cell migration scratchy “wound” at edges of SW1116 and HCT116 cells (Figure 4B and Additional file 5: Figure S3). The reduction in wound closure by miR-139-5p further confirmed its effect in suppressing cell migration.

To study the effect of miR-139-5p on the invasiveness of colon cancer, DLD1 and SW1116 cells were transfected with pre-miR-139 or Control miRNA using a Matrigel model (Figure 4C). *In vitro* invasively growing colon cancer cells were significantly impaired in DLD1 (*P* < 0.01) and in SW1116 (*P* < 0.01) when transfected with pre-miR-139 (Figure 4C).

The matrix metalloproteinases (MMPs) are family members of extracellular proteinases that regulate basic cellular processes including survival, migration and morphogenesis and degradation extracellular matrix during the cancer metastatic process [10,11]. Among the MMPs members, MMP7 and MMP9 cleave the extracellular domain of NOTCH1 and activate NOTCH1 downstream target gene expression [12,13]. Therefore, we examined the expression levels of these two proteinases and found that ectopic expression of miR-139-5p markedly suppressed protein expression of MMP7 and MMP9 (Figure 4D), concomitant with the inhibition of cell migration and invasion.

**miR-139-5p targets NOTCH1 via binding to its 3’UTR**

We searched for the direct target of miR-139-5p based on the following criteria: the target should have oncogenic property and regulate the cell migration and invasion. Among the top 100 targets of miR-139-5p predicted by 2 bioinformatics algorithms (TargetScan and miRanda), NOTCH1 fit our criteria. The 3’ untranslated region (3’UTR) of NOTCH1 contains a conserved binding site for miR-139-5p (Figure 5A). To test the specific regulation through the predicted binding sites, we constructed a reporter vector which consists of the luciferase coding sequence followed by the 3’UTR of NOTCH1 (Luc-NOTCH1-3’UTR) (Figure 5B). Wild type (Luc-NOTCH1-3’UTR) or mutated sequence (Luc-NOTCH1-mut 3’UTR) within the putative binding sites was cloned into the pMIR-REPORT vector (Figure 5A and B). Co-transfection experiments in HCT116 cells showed that miR-139-5p significantly decreased the luciferase activity of Luc-NOTCH1-3’UTR (*P* < 0.05), but this was not observed in Luc-NOTCH1-mut 3’UTR (Figure 5C). Our data thus demonstrated that NOTCH1 was a direct target of miR-139-5p.

To further confirm that miR-139-5p targets NOTCH1, pre-miR-139 or control was transfected into DLD1 and HCT116 cells. Transfection of pre-miR-139 resulted in

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**Figure 4** miR-139-5p inhibited colon cancer cell migration and invasion. (A) Re-expression of miR-139-5p in DLD1 and SW1116 cells significantly inhibited cell migration ability as determined by cell migration assay (left panel) Quantitative analysis of migrated cell in DLD1 and SW1116. The data are means ± SD. (B) The inhibition on cell migration by miR-139-5p was further confirmed by wound-healing assay. (C) Re-expression of miR-139-5p in DLD1 and SW1116 cells significantly suppressed cell invasive ability as determined by cell invasion assay. (D) miR-139-5p significantly downregulated MMP7 and MMP9 protein levels in both colon cancer cell lines.
significant reduction of NOTCH1 mRNA and protein expression by qRT-PCR (Figure 5D) and by western blot (Figure 5E), respectively. Consistent with this finding, ectopic expression of miR-139-5p significantly suppressed NOTCH1 downstream effectors, including hairy and enhancer of split-1 (HES1) \((P < 0.001)\), Cyclin D1 \((P < 0.001)\) and FADD-like apoptosis regulator (CFLAR) \((P < 0.05)\) transcription (Figure 5F). Collectively, these results showed that mir-139-5p could regulate NOTCH1 and its mediated signal transduction.

**NOTCH1 is upregulated in primary CRC tumors and inverse correlated with miR-139-5p expression**

To assess the importance of NOTCH1 in primary CRC, we compared the level of NOTCH1 expression in 45 paired tumor and adjacent normal tissues. The expression of the NOTCH1 mRNA was significantly increased in CRC tumors compared to the adjacent normal tissues \((P < 0.0001; Figure 5G)\). NOTCH1 was overexpressed in 91% \((41/45)\) of tumors compared with their normal counterparts.

We evaluated the correlation between NOTCH1 mRNA and miR-139-5p expression in 45 pairs of primary CRC. Expression of NOTCH1 mRNA and miR-139-5p exhibited a significant inverse correlation as calculated by Pearson correlation \((r = -0.3862, P = 0.0002)\) (Figure 5H). This result further supported that miR-139-5p targets to NOTCH1.

We investigated the effect of NOTCH1 on cell growth by NOTCH1 knockdown experiment. Knockdown of NOTCH1 was evidenced by markedly decrease of NOTCH1 protein expression (Additional file 6: Figure S4A). Knockdown of NOTCH1 caused a significant reduction in cell viability and an increased G0/G1 phase in HCT116 \((P < 0.0001, Additional file 6: Figure S4B and S4C)\), indicating the potential oncogenic function of NOTCH1 in colon cancer.

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**Figure 5** NOTCH1 is a direct target of miR-139-5p in colon cancer. (A) Human NOTCH1 3’UTR binding site for miR-139-5p. (B) The miR-139-5p wild type binding sequence or its mutated form was inserted into C-terminal of the luciferase gene to generate pMIR-NOTCH1-3’UTR or pMIR-NOTCH1-mut-3’UTR, respectively. (C) miR-139-5p targeted the wild-type but not the mutant 3’UTR of NOTCH1. The data are means ± SD. (D) Ectopic expression of miR-139-5p downregulated NOTCH1 mRNA expression in DLD1 and HCT116 cells as determined by quantitative RT-PCR. (E) miR-139-5p decreased NOTCH1 protein level in DLD1 and HCT116 by western blot. (F) miR-139-5p significantly suppressed NOTCH1 downstream effectors including hairy and enhancer of split-1 (HES1), cyclin D1 and FADD-like apoptosis regulator (CFLAR) transcription. (G) Expression of NOTCH1 is significantly upregulated in primary colorectal tumors compared with their adjacent normal tissues \((n = 45)\). (H) Scatter plots showing the inverse association between miR-139-5p level and NOTCH1 mRNA expression.
Discussion

miR-139-5p is down-regulated in inflammatory bowel disease-associated neoplastic transformation, primary CRC and the metastasis site [14-18]. Our data also indicated that miR-139-5p showed a reduced expression in advanced adenoma, suggesting the dysregulation of miR-139-5p is an early event of colorectal tumorigenesis. Further work on two cohorts of CRC patients evaluated by qRT-PCR verified that miR-139-5p was indeed dramatically downregulated in tumor samples compared to the adjacent normal tissues (P < 0.0001 in both cohorts) (Figure 1). In addition to CRC, down-regulation of miR-139-5p has been reported by miRNA profile studies on gastric cancer [19], endometrial serous adenocarcinoma [20] and HCC [21]. Epigenetic silencing of miRNAs with tumor suppressor features is a common hallmark of human tumors. Human miR-139-5p is embedded in the intronic region of PDE2A gene on chromosome 11q13.4. Most recently it was documented that miR-139 was silenced with its host gene PDE2A through histone methylation in lung cancer [22], EZH2 was also reported to silence miR-139-5p through H3K27 methylation in human hepatocellular carcinoma [23]. In gastric cancer, HER2 cooperates with CD44 and down-regulates miR-139 via histone H3K9 deacetylation in the miR-139 promoter region [24]. In colorectal cancer, we have found that miR-139-5p expression is restored in CRC cell lines upon administration of DNA methylation inhibitor 5-aza-2’-deoxycytidine (Additional file 2: Figure S1A). This suggests that epigenetics regulation could contribute to the down-regulation of miR-139-5p. Except for the epigenetics regulation, Schepeler and colleagues reported that WNT pathway suppressed the expression of miR-139-5p in CRC. Abrogation of the WNT pathway induced the expression of miR-139-5p but not miR-139-3p in DLD1 cell [18]. Collectively, silencing or downregulation of miR-139-5p can be mediated by different mechanisms in human cancer.

We therefore characterized the putative tumor suppressive function of miR-139-5p in human colon cancer cell lines. We found that the restoration of miR-139-5p in the colon cancer cell lines DLD1 and HCT116 significantly inhibited cell proliferation as evidenced by cell viability and colony formation assays (Figure 2). In nude mice, colon cancer cells over-expressing miR-139-5p displayed a significantly lower growth rate than the control cancer cells (Figure 2D). Furthermore, we performed FACS to evaluate the effects of miR-139-5p on the cell cycle in colon cancer cells. We revealed a concomitant increase of cells in G0/G1, which lead to inhibition of cell proliferation (Figure 3A). Previous study suggested that miR-139-5p did not affect the proliferation phenotype and DNA profile of breast cancer cell MDA-MB-231 based on Doxycycline-induced plasmid expression [25]. However, miR-139-5p shows an anti-proliferative effect in colorectal cancer. This discrepancy may be attributed to a tissue specific function of miR-139-5p signaling in CRC compared with other solid tumors. On the basis of the immunoblot analysis of negative cell-cycle regulators, G0/G1 arrest by miR-139-5p was most likely associated with the induction of p53, p21$^{Cip1/Waf1}$ and p27$^{Kip1}$ (Figure 3B). It is well known that p53 acts as a putative mediator to induce cell cycle arrest and to allow either DNA repair or apoptosis through transcriptional regulation of the cyclin dependent kinase (CDK) inhibitor p21$^{Cip1/Waf1}$, an active inhibitor of CDKs [26]. p21$^{Cip1/Waf1}$ is a direct regulator of the cell cycle, inducing growth arrest in G1-phase of the cell cycle by binding to and thus inhibiting the activity of cyclinD-CDK2/4 complexes [27]. Increased protein expression of p21$^{Cip1/Waf1}$ prevents cyclinD-CDK2/4 mediated phosphorylation of retinoblastoma protein (pRb), thus, inhibiting E2F transcriptional activity and cell cycle progression to S-phase [28]. p27$^{Kip1}$ is a potent inhibitor of cyclin D/CDK4 and cyclin E/CDK2 activities. The role of p27$^{Kip1}$ as a major player in G1 arrest has been well accepted [29]. In this regard, miR-139-5p mediated G1 cell cycle arrest in colon cancer may be partly contributed by a mechanism involving up-regulation of protein expression of p21$^{Cip1/Waf1}$ and p27$^{Kip1}$.

In the present work, we showed that, in addition to inhibition of cell proliferation, the growth inhibitory effect of miR-139-5p was also related to induction of apoptosis (Figure 3C). We observed that induction of miR-139-5p mediated apoptosis occurs by the modulation of extrinsic apoptosis pathway. Apoptosis induced by extrinsic pathways has been considered to be an important antitumor mechanism [30-32]. After transfection with miR-139-5p, the protein expression of the downstream active apoptosis executors caspase-8, caspase-7, caspase-3 was upregulated and enhanced level of cleaved PARP indicated caspase 3 was functionally active (Figure 3D). The extrinsic apoptosis pathway is initiated by the binding of extracellular death ligands such as TNFα to transmembrane death receptors. Engagement of death receptors with their cognate ligands provokes the recruitment of adaptor proteins such as Fas-associated death domain protein, which in turn recruits and aggregates several molecules of caspase-8, thereby promoting its auto-processing and activation [33]. Activation of caspase-8 processes other effector caspase members, including caspase-3 and caspase-7 to initiate a caspase cascade. These effectors further initiate the proteolytic cleavage of the nuclear enzyme PARP, causing loss of DNA repair, cellular disassembly and finally apoptosis.

In vitro assays showed that re-expression of miR-139-5p inhibited the cell migration and invasive capabilities
miR-139-5p expression in CRC patients \( (r = 0.3862, P = 0.0001) \) (Additional file 6: Figure S4B). In agreement with our results, it has been shown that suppression of NOTCH1 inhibits tumor growth in human HCC [44] and in osteosarcoma [45] by regulating cell proliferation and cycle. However, in the Kaplan-Meier survival analysis, there is no association between miR-139-5p or NOTCH1 mRNA and patient disease-free survival and overall survival in colorectal patients of our cohort (Additional file 4: Figure S2 and Additional file 8: Table S3).

Recent studies have reported that miR-139-5p was a potential prognostic marker for renal cell carcinoma and endometrial serous adenocarcinoma [46,20]. The clinical value of miR-139-5p in CRC is still controversial. It was reported that higher level of miR-139-5p was associated with more aggressive disease of colorectal cancer [16], whereas a recent study suggested that decreased miR-139-5p was associated with CRC disease progression and metastasis based on a cohort of 34 CRC patients [17]. In our study, miR-139-5p was not found to be associated with clinicopathological features including survival of CRC patients. The clinical value of miR-139-5p needs future investigations on larger cohorts of CRC samples.

**Conclusions**

In conclusion, we have identified that miR-139-5p was frequently under-expressed in CRC. miR-139-5p inhibits cell growth both \textit{in vitro} and \textit{in vivo}. The mechanisms by which miR-139-5p acts as a tumor suppressor in CRC involve inhibition of cell proliferation and metastasis, and induction of G0/G1 arrest and apoptosis through repression of oncogenic NOTCH1.

**Methods**

**Tumor cell lines**

Eight colon cancer cell lines (Caco2, DLD1, HCT116, HT29, LoVo, LS180, SW620 and SW1116) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in DMEM (Gibco BRL, Rockville, MD) and McCoy's 5A modified (Invitrogen; Life Technologies, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT) and incubated in 5% CO$_2$ at 37°C.

**Primary tumor and adjacent non-tumorous tissue samples**

Two cohorts of totally 95 histologically confirmed sporadic CRC patients diagnosed in the Prince of Wales Hospital (between November 1999, and January 2011) were included in this study (Additional file 1: Table S1). In addition, nine biopsies of normal colon mucosa from healthy controls during colonoscopy were obtained in...
the Prince of Wales Hospital. All of the patients were given written informed consent according to the Helsinki declaration and the study protocol was approved by the Clinical Research Ethics Committee of the Chinese University of Hong Kong.

Real-time quantitative PCR for miRNA expression analyses
Total RNA was extracted from cells pellets and tissue samples using miRNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative reverse transcription (qRT)-PCR of miR-139-5p was performed using the TaqMan miRNA reverse transcription kit (Applied Biosystems; Life Technologies) and the TaqMan human miRNA assay kit (assay ID: miR-139-5p: 002289, and RNU6B: 001093) [8]. The comparative Ct method was used to calculate the relative abundance of miRNA compared with RNU6B expression (Fold difference relative to RNU6B).

miRNA transfection and RNA interference
Human miR-139-5p precursor (pre-miR-139, MC11749) and the miRNA Mimic Negative Control (control) were purchased from Ambion (Ambion Life Technologies, Austin, TX) and then transiently. Cell was seeded in 24-well plate at 60% confluence and transfected with 15 pmole miRNA per well using Lipofectamine 2000 (Invitrogen). siRNAs against human NOTCH1 (Santa Cruz Biotechnology, Santa Cruz, CA) were delivered into cell using Lipofectamine 2000. Cells transfected with miRNA or siRNA were harvested 12 h to 48 h post-transfection.

Cell viability assay
After 24 h of transfection of miRNA/siRNA, the cells were digested and re-seeded in 96-well plates (1.5 × 10^3 per well) for cell viability assay using MTS (Promega, Madison, WI). 20 μL of reaction solution was added to cultured cells in 100 μL culture medium and incubated at 37°C for 1.5 h. The optical density was measured at a wavelength of 492 nm. The cell viability assay was carried out in four wells for three independent experiments.

Cell cycle analysis and apoptosis assay
Cells used for cell cycle analysis were plated 24 h prior to transfection with miRNAs in 24-well plate using Lipofectamine 2000 per manufacturer's instructions. Cells were trypsinized and then collected by centrifugation at 2,000 rpm for 6 min. Cell pellets were rinsed once with phosphate-buffered saline (PBS) and fixed in 70% (v/v) ice-cold ethanol for at least 16 h at −20°C. Cells were resuspended and stained with 50 μg/mL propidium iodide (BD Biosciences, Erembodegem, Belgium) containing RNase A for 30 min at 37°C in the dark. Apoptosis was determined by dual staining with Annexin V-Allophycocyanin (APC) and 7-Aminoactinomycin D (7-AAD) (BD Biosciences, Erembodegem, Belgium). Briefly, cells were collected 48 h after transfection with pre-miR-139 or control, and stained with Annexin V-APC and 7-AAD according to the manufacturer’s instruction. The combination of Annexin V-APC and 7-AAD staining distinguished early apoptotic cells (Annexin V−, 7-AAD+) and late apoptotic cells (Annexin V+, 7-AAD−). The cells were sorted by FACS-Calibur System (BD Biosciences) and cell-cycle profiles and apoptosis were analyzed by ModFit 3.0 software (BD Biosciences). The assay was carried out in triplicate for three times.

Cell migration assays and wound healing assay
To measure cell migration, 8-mm pore-size culture inserts (Transwell; Falcon, BD Biosciences) were placed into the wells of 24-well culture plates, separating the upper and the lower chambers. In the lower chamber, 600 μL DMEM containing 10% FBS was added. Then 1 × 10^5/well cells were added to the upper chamber. After 24 h incubation, cells that had migrated through the transwell membrane were stained with crystal violet, and counted (four high power fields, ×100 magnification). In addition, wound healing assay was also performed for analysis of cell migration in vitro. Briefly, cells transfected with either miR-139 mimics or and negative control miRNA were cultured in six-well plates (5 × 10^5 cells per well). When the cells grew up to 90% confluence, a single wound was made in the center of cell monolayer using a P-200 pipette tip. The wound closure areas were visualized under phase-contrast microscope with a magnification × 100, and the migrated cells were counted. The experiment was performed in triplicate wells for three times.

Cell invasion assay
Cell invasion was performed by Matrigel invasion assay (BD Biosciences). SW1116 and DLD1 cells transfected with pre-miR-139 or control for 48 h were harvested, suspended (1 × 10^5/well) in 500 μL serum-free medium and then loaded onto the upper compartment of chamber. The lower chamber contains 600 μL DMEM media and 10% FBS. After 24 h incubation, cells that had
invaded through the Matrigel membrane were stained with crystal violet, and counted (five high power fields, \( \times 100 \) magnification). Three independent experiments were conducted.

**Vector construction and dual-luciferase reporter assay**

The potential miR-139-5p binding sites were predicted by TargetScan (www.targetscan.org) and miRanda (www.microRNA.org). Sequence of 42 bp segment with the wild-type or mutant seed region of NOTCH1 was synthesized and cloned into pMIR-REPORT luciferase vector (Applied Biosystems; Life Technologies). The mutant NOTCH1 3’UTR sequence was prepared by deleting 10 nucleotides in the seed region. The synthesized oligos were shown as follows: Wild type of NOTCH1: 5’-CTA GTG ACT TTA AAA GTG ATC TAC ATG AGG AAC TGT AGA TGA TGT GAG CT-3’; 5’-CAC ATC ATC AGT GCC TCG TCA TGA TCA CTT TTA AAG TCA-3’; Mutant type of NOTCH1: 5’- CTA GTG ACT TTA AAA GTG ATC TAC ATG AGT GAT GTG AGC T-3’; 5’-CAC ATC ATC ACT CAT GTA GAT CAC TTT TAA AAG TCA-3’.

Cells (1 × 10^5/well) transiently transfected with pre-

\[
\text{miR-139} \\
\text{miR-139}^{-}\text{Ctrl}
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were harvested 48 hours post-transfection and luciferase (195 ng/well) and pRL-TK vector (5 ng/well) were seeded in 24-well plates. pMIR-REPORT vector miR-139 or control (at 30 nM final concentration) was then transfected into cells using Lipofectamine 2000 and stable miR-139-5p expression plasmid pCMV-139-5p and control pCMV-Ctrl plasmid was delivered into cells using Lipofectamine 2000 and stable cell lines were created from puromycin-resistant colonies. HCT116 cells (2 × 10^6) stably expressing pCMV-139-5p or HCT116 control cells (pCMV-Ctrl) were injected subcutaneously into the left flank of the female BALB/c nude mice (4 weeks old), respectively (n = 4 mice per group). Tumour size was measured every 3 day using caliper, and the tumor volume \((V)\) was calculated as \((l \times w \times h)/2\), with \(l\) indicating length and \(w\) indicating width. Mice were killed by cervical dislocation in day 28, and the tumors were excised and snap-frozen for protein extraction. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

**Statistical analysis**

Statistical analysis was carried out using SPSS 16.0 for Windows. All measurements or variables were shown as mean ± SD. Results of miR-139-5p level between paired samples determined by the Wilcoxon matched pairs test. Results of colony formation assays, flow-cytometry analyses, cell growth, migration and invasion assays were analyzed by student T test. Repeated Measures ANOVA was used to compare the tumor growth rate between two groups in the in vivo assay. Based on the expression levels in tumor, the miR-139-5p expression in CRCs was categorized as high (miR-139-5p level in tumor > median) and low (miR-139-5p level in tumor < median). The Pearson’s chi-squared test was used to analyse the association of miR-139-5p expression and clinicopathological parameters. Kaplan-Meier tests were used for survival analysis. Overall survival times were calculated from the date of curative surgery to death or last follow-up of patients. A \( P < 0.05 \) was taken as statistical significance.

**Additional files**

- **Additional file 1:** Table S1. Clinicopathologic Characteristics of 95 patients with colorectal cancer included in the study.
- **Additional file 2:** Figure S1. (A) The expression level of miR-139-5p was restored in CRC cell lines upon administration of DNA methylation inhibitor S-aza-2-deoxycytidine. 5Aza: 5-aza-2-deoxycytidine. (B) Overexpression of Pre-miR-139 increased the miR-139-5p level in HCT116, DLD1 and SW1116.
- **Additional file 3:** Table S2. Correlation between miR-139-5p expression and clinicopathologic parameters in 44 CRC patients (cohort 1).
- **Additional file 4:** Figure S2. Kaplan-Meier survival curves for patients with colorectal cancer in Cohort 1, stratified according to miR-139-5p (A and B) or NOTCH1 (C and D) expression levels. The follow-up data for one patient is not available, n = 44.
- **Additional file 5:** Figure S3. Overexpression of miR-139-5p in HCT116 significantly suppressed cell migration ability as determined by wound-healing.
- **Additional file 6:** Figure S4. Role of NOTCH1 in colon cancer. (A) Knock-down of NOTCH1 was evidenced by markedly decrease of NOTCH1 protein expression in HCT116 cells by si-NOTCH1. (B) NOTCH1 knock-down significantly suppressed cell viability in HCT116 cells. (C) si-NOTCH1 caused cell cycle arrest at G0/G1 phase in HCT116. Quantitative analysis of DNA profile in HCT116 (right panel). The experiments were repeated three times in triplicate. Data are mean ± SD.
Additional file 7: Figure S5. miR-139-5p showed no significant effect on JUN in HCT116. (A) Human JUN 3’UTR binding site for miR-139-5p. (B) The miR-139-5p wild-type binding sequence or its mutated form was inserted into C-terminal of the luciferase gene to generate pmirJUN-3’UTR or pmirJUN-mut-3’UTR, respectively. (C) miR-139-5p showed no significant effect on the wild-type or the mutant 3’UTR of JUN. The data are means ± SD.

Additional file 8: Table S3. Correlation between NOTCH1 mRNA expression and clinicopathological parameters in 44 CRC patients (cohort 1).

Abbreviations
3’ UTR: 3’ untranslated region; 7-AAD: 7-aminoactinomycin D; APC: Annexin V-Allophycocyanin; ATCC: American type culture collection; CDK: Cyclin-dependent kinase; CFLAR: FADD-like apoptosis regulator; CRC: Colorectal cancer; FBS: Fetal bovine serum; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; HES1: Hairy and enhancer of split-1; IQR: Interquartile range; mRNA: Messenger RNA; miRNA: microRNA; MMP: Matrix metalloproteinases; PARP: Poly ADP-ribose polymerase; PCR: Polymerase chain reaction; qRT-PCR: Quantitative real-time polymerase chain reaction; RNU6B: U6 small nuclear 2.

Competing interests
The authors declared that they have no competing interests.

Authors’ contributions
LZ, YD, NZ, HT, KW designed and performed the experiments, analyzed data and wrote the manuscript; CWW, SSMN, SZ, FKLC and JJYS provided technical support; ZZ provided manpower and material support. JY supervised the project, analyzed data and revised the manuscript. All authors read and approved the final manuscript.

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