Downregulation of microRNA-124 is an independent prognostic factor in patients with colorectal cancer

Mo-Jin Wang, Yuan Li, Rui Wang, Cun Wang, Yong-Yang Yu, Lie Yang, Yi Zhang, Bin Zhou, Zong-Guang Zhou and Xiao-Feng Sun

Linköping University Post Print

N.B.: When citing this work, cite the original article.

The original publication is available at www.springerlink.com:

Mo-Jin Wang, Yuan Li, Rui Wang, Cun Wang, Yong-Yang Yu, Lie Yang, Yi Zhang, Bin Zhou, Zong-Guang Zhou and Xiao-Feng Sun, Downregulation of microRNA-124 is an independent prognostic factor in patients with colorectal cancer, 2013, International Journal of Colorectal Disease, (28), 2, 183-189.

<http://dx.doi.org/10.1007/s00384-012-1550-3>

Copyright: Springer Verlag (Germany)

<http://www.springerlink.com/?MUD=MP>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-90208>

Downregulation of microRNA-124 is an independent prognostic factor in patients with colorectal cancer

Mo-Jin Wang • Yuan Li • Rui Wang • Cun Wang • Yong-Yang Yu • Lie Yang • Yi Zhang • Bin Zhou •
Zong-Guang Zhou • Xiao-Feng Sun

M.-J. Wang • C. Wang • Y.-Y. Yang • L. Yang • Y. Zhang • B. Zhou • Z.-G. Zhou (✉)

Department of Gastrointestinal Surgery, Institute of Digestive Surgery and National Key Laboratory of
Biotherapy, West China Hospital, Sichuan University, 37 Guo Xue Xiang, Chengdu 610041, China
e-mail: zhou767@163.com

R. Wang

Department of Gastroenterology, West China Hospital, Sichuan University, Chengdu, China

Y. Li

Department of Pediatric Surgery, Institute of Digestive Surgery and State Key Laboratory of Biotherapy,
West China Hospital, Sichuan University, Chengdu, China

X.-F. Sun

Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences,
University of Linköping, Linköping, Sweden

Abstract

Purpose Recently, microRNA-124 (miR-124) has been demonstrated as a potential tumor suppressor in several types of cancers. However, the role of miR-124 in colorectal cancer remains unclear. This study was aimed at investigating the clinicopathological significance of miR-124 expression in colorectal cancer.

Methods Quantitative Real-time PCR was used to analyze miR-124 expression in 96 colorectal cancers and individual-matched normal mucosa samples. The expression of miR-124 was assessed for associations with clinicopathological characteristics using Chi-square test. The survival curves were calculated by the Kaplan–Meier. The influence of each variable on survival was examined by the Cox multivariate regression analysis.

Results The miR-124 expression was significantly downregulated in colorectal cancer compared to normal mucosa ($P=0.001$). In colorectal cancer, miR-124 decreased expression correlated significantly with the grade of differentiation ($P=0.021$). Univariate survival analysis showed that the downregulated miR-124 was significantly correlated with worse prognosis, both in terms of overall survival ($P=0.017$) and disease-free survival (DFS) ($P=0.014$). Further, the downregulated miR-124 was demonstrated a prognostic factor for overall survival [hazard ratio (HR) =4.634, 95% confidence interval (CI) 1.731-12.404, $P=0.002$] and DFS (HR=4.533, 95% CI 1.733-11.856, $P=0.002$), independently of gender, age, location, maximum tumor size, depth of invasion, differentiation and TNM stage.

Conclusions MiR-124 may play a certain role in the development of colorectal cancer. The downregulation expression of miR-124 is an independent prognostic factor in patients with colorectal cancer.

Keywords MicroRNA-124 • Colorectal cancer • Quantitative Real-time PCR • Survival

Introduction

MicroRNAs (miRNAs) are endogenous non-coding, single-stranded RNAs of ~22 nucleotides which are involved in the regulation of human gene expression. Through basing pair with the 3'- untranslated region (3'-UTR) of target messenger RNA, miRNAs prevent gene expression by inhibiting protein translation. Several miRNAs have been reported to participate in diverse essential biological processes including: cell proliferation, apoptosis, development and differentiation [1]. Since the first study of miRNAs involvement in chronic lymphocytic leukemia in 2002 [2], there is growing evidence that miRNAs implicated in human carcinogenesis by functioning as either oncogenes or tumor suppressors through their interactions with target genes critical to development and progression of various cancers, including colorectal cancer [3, 4].

Colorectal cancer is a major cause of cancer mortality worldwide [5]. It is the third most commonly diagnosed cancer and the third leading cause of cancer death in the United States [6]. In China, its incidence has increased in the past few years. In 2003, the miR-143 and miR-145 were ascertained as first two novel dysregulated miRNAs in colon cancer [7]. From then on, subsequent authors identified additional miRNAs by various screening tools, and found several dysregulated miRNAs implicated in colorectal cancer. A multitude of experimental studies have described the ability of miRNAs in the detection, treatment and prognosis of colorectal cancer [8, 9].

Recently, as one of the most abundantly expressed miRNAs in the central nervous system, miR-124 has been shown to play a significant role in the pathogenesis of various cancers [10-15]. Lower levels of miR-124 expression were frequently detected in hepatocellular cancer and related to tumor metastasis [15]. The down-regulation of miR-124 has also been observed in gastric, cervical and breast cancer, which may be caused by DNA methylation-based silencing [11, 16, 17]. Additionally, miR-124 has been involved in invasion and metastasis process of hepatocellular cancer, by directly targeting the rho-kinase2 (ROCK2) and an enhancer of the zeste homologue 2 (EZH2) genes [15]. Over-expression of miR-124 attenuated integrin beta-1(ITGB1) expression and suppressed the adherence and motility of oral squamous cancer cells [14]. To date, several lines of evidence have shown that miR-124 may be a novel prognostic factor and therapeutic target for some types of cancers [12, 13, 15]. These data demonstrated the potential tumor suppressor role of miR-124, however, the relationship between expression of miR-124 and colorectal cancer remains unclear.

-

In the present study, we used Real-time PCR to quantify the expression of miR-124 in 96 colorectal cancers and individual-matched normal mucosa samples. Furthermore, we investigated its relationship with clinicopathological features and survival in patients with colorectal cancer.

Materials and methods

Patients and tissues

All patients included in the study underwent surgical resection procedures at the Gastrointestinal Surgery Department, Affiliated West China Hospital of Sichuan University, Chengdu City, China, from July 2006 to December 2007. The eligible cases were primary colorectal cancer without preoperative radiotherapy or chemotherapy. The patients with hereditary syndroms (e.g. FAP, HNPCC) or inflammatory bowel syndroms had been excluded. The patients consisted of 54 men and 42 women with a median age of 55 years (range 24-81 years). Once the surgical specimens were obtained, individual-matched normal mucosa adjacent to the proximal excision margin was harvested. All specimens were snap-frozen immediately in liquid nitrogen and stored at -80°C freezer until further analysis. The specimens were examined for the presence of tumor cells, which usually were at least around 80%, at the Department of Pathology in the West China Hospital. The growth pattern was based on the patterns of growth and invasiveness. Differentiation was graded as well, moderate or poor differentiated. The study was approved by the SiChuan University Ethics Committee and written informed consent was obtained from all the participants. As a regular follow-up program for patients with colorectal cancer, a combination of physical examination (including digital examination), blood chemistry test (including measurement of carcinoembryonic antigen levels), chest X-ray, surveillance colonoscopy and abdominal CT was performed. At the end of 2011, the mean follow-up period of was 4.33 years.

RNA extraction and cDNA synthesis

For RNA extraction, frozen tissues were cut into 5-mm cubic blocks and pulverised in a mortar and pestle. RNA was isolated using TRIzol reagent (Molecular Research Center, Inc., OH, USA) according to the manufacturer's protocol. RNA was quantified at 460 nm using the spectrophotometer (Beckman Coulter, Fullerton, CA, USA), its integrity was checked by agarose gel electrophoresis. For specifically quantization mature miR-124 expression level, RNA was reverse transcribed to cDNA of miR-124 using the stem-loop RT primers (Invitrogen, Carlsbad, CA, USA, Table 1) which were designed according to early description [18, 19]. Total RNA (2.5 μl) was first denatured at 70°C with 50nM

-

stem-loop RT primer for 5 min, and then a 10 µl reaction mix was assembled using 1 mM final of each of the four deoxynucleotide triphosphates (dNTPs), 1 U/µl RNase inhibitor, 10 U/µl M-MLV reverse transcriptase and 1×M-MLV RT buffer (TaKaRa Biotechnology Co., Ltd, Dalian China). The reaction proceeded for 30 min at 16°C, 30 min at 42°C followed by 5 min incubation at 85°C. To synthesize cDNA of endogenous control 5S rRNA, 5 µg of RNA was first denatured at 70°C with 2.5 mM random hexamers (TaKaRa) for 5 min before quenching on ice, and then 1 mM final of each of the four dNTPs, 1 U/µl RNase inhibitor, 10 U/µl M-MLV reverse transcriptase and 1×M-MLV RT buffer were added together to make up a final volume of 20 µl reaction mix. The reaction mix was incubated using MJ Research PTC-100TM Programmable Thermal Controller (MJ Research, MA, USA) for 10 min at 20°C and 1 h at 42°C. The reverse transcriptase was inactivated at 70°C for 10 min and then held at 4°C.

Quantitative Real-time PCR

The gene-specific primers and probes for quantitative Real-Time PCR (Table1) were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA) based on the sequences of mature miRNA downloaded from the miRNA Registry database (<http://miRNA.sanger.ac.uk>). All quantitative Real-Time PCR reactions were performed in a total volume of 30 µl. The PCR-mix consisted of 1×PCR buffer (Mg^{2+} free), 2.5 mM $MgCl_2$, 0.3 mM dNTP, 1.5 U of rTaq DNA polymerase (TaKaRa), 0.33 µM Taqman probes, 0.33 µM each primer (Invitrogen), 17.1 µl ddH₂O and 1 µl of cDNA. The iCycler iQ System (Bio-Rad, Hercules, CA) was used as Real-Time PCR platform and the PCR conditions for miRNAs and 5S rRNA were as follows: a single predenaturation step of 3 min at 94 °C followed by 40 cycles of 20s at 94 °C and 40 s at 60 °C. All reactions, including no-target control consisted of PCR master mix alone, were run in triplicate. To determine PCR efficiency, five-fold dilutions of colorectal cancer cell cDNA were diluted for four times (1, 1:5, 1:25, 1:125 and 1:625). Both miRNAs and 5S rRNA gene in the diluted cDNA were amplified by real-time PCR using the identical conditions established for the gene expression analysis. Plots were made of the log of the template concentration vs the Ct, and the PCR efficiency was calculated by the iCycler iQ fluorescence PCR machine automatically from the slope of the line using Equation 2 [21]: $E = 10^{[-1/slope]}$. The amplification curve and standard curve also were given at the same time. The $2^{-\Delta\Delta CT}$ method of relative quantification was used for comparing the expression of miR-124 in tumour samples to matched normal mucosa samples [20]. First, the ΔC_T between the CT values for target and reference genes for

-

each tumour (t) and normal (n) sample were calculated: $\Delta C_T(t) = C_T(t \text{ target}) - C_T(t \text{ reference})$; $\Delta C_T(n) = C_T(n \text{ target}) - C_T(n \text{ reference})$. The $\Delta\Delta C_T$ for the tumour (t) and normal (n) were then calculated: $\Delta\Delta C_T(t) = \Delta C_T(t) - \Delta C_T(n)$. Therefore, $2^{-\Delta\Delta C_T(t)}$ is the fold change of the miR-124 expression in the tumour sample relative to the normal sample, when normalized to the reference gene.

Quantitation of PCR products

The PCR products were subjected to electrophoresis on a 2% agarose gel, to which ethidium bromide (10 mg/ml) had been added. Electrophoresis was carried out in Tris-Borate-EDTA (TBE) buffer at 120 V for 30 min. The bands were visualised under UV illumination and a densitometer (Bio-Rad) was used to scan the intensity of the bands.

Statistical analysis

The relative expression analysis of miR-124 was performed by a randomization test using the Relative Expression Software Tool (REST) 2009 (<http://gene-quantification.com/rest-2009.html>). As for the $2^{-\Delta\Delta C_T}$ method, the fold change less than one in expression was defined as decreased expression. The expression of miR-124 was assessed for associations with clinicopathological characteristics using Chi-square test. The survival curves were calculated by the Kaplan–Meier method and the difference by the log rank test. The influence of each variable on survival was examined by the Cox multivariate regression analysis. All the analyses were performed with SPSS 18.0 software (Chicago, IL, USA). All p-values cited were two-sided and p-values < 0.05 were judged as statistically significant.

Results

Expression of miR-124 in colorectal cancer and individual-matched normal mucosa

The expression of miR-124 was examined in 96 colorectal cancers and individual-matched normal mucosa samples. The amplification efficiency was 1.93 for 5S RNA and 1.89 for miR-124. No primer-dimer was generated during the 40 real-time PCR amplification cycles. Among 96 paired samples, 71 (73.9%) cases showed the relative expression ratio (R) <1 which means miR-124 downexpression in those cancers. Fifty-six (58.3%) cancers showed strong down-regulated expression of miR-124 (>2-fold), of which 29 (30.2%) cancers had >10 times lower expression and 8 (8.3%) cancers had >100 times lower expression, relative to the normal mucosa. To further compare the overall level of miR-124 expression in colorectal cancer relative to normal mucosa, we put the Ct values of all the samples into the REST 2009 software, and chose 3000 as the randomization number. The results of the randomization test showed that the miR-124 was significantly downregulated in colorectal cancer compared to normal mucosa (R=0.390, $P=0.001$, Table 2).

Relationship between expression of miR-124 and clinicopathological characteristics

The relationship between miR-124 expression and clinicopathological characteristics is shown in Table 3. The expression of miR-124 differed significantly according to the grade of differentiation ($P=0.021$). There were 20/26 patients with down-regulated level in poor differentiated cancer samples, 39/47 in moderate and 12/23 in well differentiated cancer samples. No significant relationship was observed between the miR-124 expression and gender ($P=0.618$), age ($P=0.977$), location ($P=0.690$), maximum tumor size ($P=0.992$), depth of invasion ($P=0.692$), venous invasion ($P=0.166$), lymph node metastasis ($P=0.618$), distant metastasis ($P=0.589$) or TNM stage ($P=0.236$).

Relationship between expression of miR-124 and prognosis of colorectal cancer

During follow-up, 43 patients died of colorectal cancer. Univariate survival analysis showed that the group with downregulated miR-124 was significantly correlated with worse prognosis, both in terms of overall survival ($P=0.017$, Fig.1) and disease-free survival (DFS) ($P=0.014$, Fig.2), than the

-

upregulated group. Three-year overall survival and DFS were 88.0% and 76.0% in the miR-124 upregulated group, but only 62.0% and 47.9% in the downregulated group. In the further Cox multivariate regression analysis, the prognostic significance still remained after adjusting for patients' gender, age, location, maximum tumor size, depth of invasion, differentiation and TNM stage. Besides TNM stage, miR-124 was demonstrated an independent prognostic factor for overall survival [hazard ratio (HR) =4.634, 95% confidence interval (CI) 1.731-12.404, $P=0.002$, Table 4] and DFS (HR=4.533, 95% CI 1.733-11.856, $P=0.002$, Table 5).

Discussion

Currently, a closer link between miRNAs function and colorectal cancer pathogenesis is supported by a number of molecular studies examining the expression of miRNAs in clinical samples. With the investigation of these miRNAs, it would be conducive to expand our view to better understand colorectal carcinogenesis by analyzing mRNA targets associated and miRNA-mediated pathways. As a potential tumor suppressor gene, miR-124 has been shown to regulate growth, proliferation, apoptosis, migration and invasion of tumor cell in certain cancers [11, 14, 17 21, 22]. The tumor-specific hypermethylation-mediated silencing of miR-124 was a relatively frequent molecular event in gastric, cervical and breast cancer [11, 16, 17]. Moreover, some studies suggested that miR-124 have prognostic and therapeutic potential in hepatocellular cancer and non-small cell lung cancer [12, 13, 15]. In the research combining miRNA expression profiles with chromatin signatures, methylation of miR-124 was identified in colorectal cancer cell lines [23]. Although deregulation of miR-124 has been observed in various types of human cancers, for our limited knowledge, the role of miR-124 in colorectal cancer is still unclear.

In this study, we observed that downregulation of miR-124 is a frequent event in colorectal cancer compared to normal mucosa, which is in line with the previous study included 12 pairs of colorectal cancers and normal mucosa samples [24]. Lujambio et al [25] showed methylation-mediated silencing of miR-124 and concomitant reduced miR-124 expression in different human cancer types, reaching the highest frequency in colorectal cancer (75%). In addition, this epigenetic dysregulation of miR-124 was proved to mediate cyclin D kinase 6 (CDK6) activation and retinoblastoma phosphorylation in HCT-116 colon cancer cells, and these processes can be restored by erasing DNA methylation. Based on the data derived from quantitative mass spectrometry [26], the tumor suppressor gene IGFBP7 was identified as another target of miR-124 in colorectal cancer [27, 28].

In line with previous research [29], we indicated that level of miR-124 expression was associated with the grade of differentiation. Lower expression of miR-124 was detected in moderate-poor differentiated colorectal cancer. It has been shown that miR-124 play an important role during neuronal differentiation and neural development [30]. By mediating inhibition of CDK6, which can regulate both cell cycle progression and differentiation [31], the up-regulation of miR-124 induced neuronal differentiation of brain tumor stem cells and inhibited proliferation of glioblastoma multiforme cell

lines [29]. On the other hand, a recent research that knockdown of miR-124 promotes neuroblastoma cell differentiation, cell cycle arrest and apoptosis, indicated that miR-124 might exert diverse functions in differentiation of different cancers [32]. Thus, in agreement with these studies, our results may suggest that miR-124 play a role in the differentiation of colorectal cancer. Further mechanism of the differentiation regulation for miR-124 in colorectal cancer should be explored in the future.

To the best of our knowledge, this is the first study to reveal the relationship between miR-124 and the prognosis of colorectal cancer. We showed that miR-124 was a significant predictor of overall survival and DFS and an independent prognostic factor for colorectal cancer. The similar conclusion was reported by Zheng F et al [15] — that low-level expression of miR-124 was an independent prognostic factor for poor outcome in hepatocellular cancer patients. The miR-124 modulated hepatocellular carcinoma cell aggressiveness by repressing the expression of ROCK2 and EZH2. The administration of miR-124 has been revealed to prevent and suppress hepatocellular carcinogenesis by inducing tumor-specific apoptosis without toxic side effects. The miR-124 may be a clinically viable anticancer therapeutic target for cancer [13]. Taking these results together, miR-124 potentially plays a tumor suppressive function in the development and progression of colorectal cancer through negatively regulating its gene targets. The limitation of our study is that we have not analyzed the relationship between the expression of miR-124 and adjuvant therapy (chemoradiation or chemotherapy) for diverse regimens. As an important prognostic factor for colorectal cancer, adjuvant therapy could not be neglected. The prognostic significance of miR-124 in colorectal cancer patients receiving adjuvant therapy requires a further prospective controlled study.

In conclusion, our results showed that miR-124 expression was significantly downregulated in colorectal cancer compared to normal mucosa. The expression of miR-124 differed significantly according to the grade of differentiation. Lower expression of miR-124 was detected in moderate-poor differentiated colorectal cancer. Further, the downregulation expression of miR-124 is an independent prognostic factor in colorectal cancer patients. We believed that it is just the beginning of a long and still unexplored avenue of highly promising investigation of miR-124. More deeply and larger scale study remains to identify the role of miR-124 in colorectal cancer.

-

Acknowledgments This study was supported by National Natural Science Foundation of China (No. 30830100) and Ph.D. Programs Foundation of Ministry of Education of China (No. 200806100058). We thank Chen Keling and Lv Zhaoying for their technical assistance.

References

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116:281-297
2. Calin GA, Dumitru CD, Shimizu M et al (2002) Frequent deletions and down-regulation of microRNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 99: 15524-15529
3. Lu J, Getz G, Miska EA et al (2005) MicroRNA expression profiles classify human cancers. *Nature* 435:834-838
4. Schetter AJ, Leung SY, Sohn JJ et al (2008) MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 299: 425-436
5. Aaltonen LA et al (2000) World Health Organization Classification of Tumors. Pathology and Genetics of Tumours of the Digestive System. Lyon, France, International Agency for Research on Cancer Press
6. American Cancer Society (2008) Colorectal Cancer Facts & Figures 2008-2010. Atlanta, American Cancer Society
7. Michael MZ, O' Connor SM, van Holst Pellekaan NG, Young GP, James RJ (2003) Reduced accumulation of specific microRNAs in colorectal neoplasia. *Mol Cancer Res* 1:882-891
8. Nugent M, Miller N, Kerin MJ (2011) MicroRNAs in colorectal cancer: function, dysregulation and potential as novel biomarkers. *Eur J Surg Oncol* 37: 649-654
9. Wu WK, Law PT, Lee CW, Cho CH, Fan D, Wu K, Yu J, Sung JJ (2011) MicroRNA in colorectal cancer: from benchtop to bedside. *Carcinogenesis* 32:247-253
10. Pierson J, Hostager B, Fan R, Vibhakar R (2008) Regulation of cyclin dependent kinase 6 by microRNA 124 in medulloblastoma. *J Neurooncol* 90:1-7
11. Wilting SM, van Boerdonk RA, Henken FE, Meijer CJ, Diosdado B, Meijer GA, le Sage C, Agami R, Snijders PJ, Steenbergen RD. (2010) Methylation-mediated silencing and tumour suppressive function of hsa-miR-124 in cervical cancer. *Mol Cancer* 9: 167
12. Patnaik SK, Kannisto E, Knudsen S, Yendamuri S (2010) Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. *Cancer Res* 70: 36-45
13. Hatziaepostolou M, Polytarchou C, Aggelidou E (2011) An HNF4alpha-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. *Cell* 147: 1233-1247
14. Hunt S, Jones AV, Hinsley EE, Whawell SA, Lambert DW (2011) MicroRNA-124 suppresses oral squamous cell carcinoma motility by targeting ITGB1. *FEBS Lett* 585: 187-192
15. Zheng F, Liao YJ, Cai MY et al (2012) The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2. *Cell* 151:278-289
16. Ando T, Yoshida T, Enomoto S, Asada K, Tatematsu M, Ichinose M, Sugiyama T, Ushijima T (2009) DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. *Int J Cancer* 124:2367-2374.
17. Lv XB, Jiao Y, Qing Y, Hu H, Cui X, Lin T, Song E, Yu F (2011) miR-124 suppresses multiple steps of breast cancer metastasis by targeting a cohort of pro-metastatic genes in vitro. *Chin J Cancer* 30: 821-830.
18. Chen C, Ridzon DA, Broomer AJ (2005) Real-time quantification of microRNAs by stem-loop

- RT-PCR. *Nucleic Acids Res* 33: e179.
19. Wang CJ, Zhou ZG, Wang L, Yang L, Zhou B, Gu J, Chen HY, Sun XF (2009) Clinicopathological significance of microRNA-31, -143 and -145 expression in colorectal cancer. *Dis Markers* 26: 27-34
 20. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Method* 25: 402-408
 21. Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J (2010) miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. *Carcinogenesis* 31:766-776.
 22. Ponomarev ED, Veremeyko T, Barteneva NS (2011) Visualization and quantitation of the expression of microRNAs and their target genes in neuroblastoma single cells using imaging cytometry." *BMC Res Notes* 4: 517
 23. Suzuki H, Takatsuka S, Akashi H, Yamamoto E, Nojima M, Maruyama R, Kai M, Yamano HO, Sasaki Y, Tokino T, Shinomura Y, Imai K, Toyota M (2011) Genome-wide profiling of chromatin signatures reveals epigenetic regulation of MicroRNA genes in colorectal cancer. *Cancer Res* 71:5646-5658
 24. Bandrés E, Cubedo E, Agirre X, Malumbres R, Zárate R, Ramirez N, Abajo A, Navarro A, Moreno I, Monzó M, García-Foncillas J (2006) Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Mol Cancer* 5: 29
 25. Lujambio A, Ropero S, Ballestar E et al (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Res* 67: 1424-1429
 26. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP (2008) The impact of microRNAs on protein output. *Nature* 455:64-71
 27. Suzuki H, Igarashi S, Nojima M et al (2010) IGFBP7 is a p53 Responsive Gene Specifically Silenced in Colorectal Cancer with CpG Island Methylator Phenotype. *Carcinogenesis* 31:342-349
 28. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR (2008) Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell* 132:363-374.
 29. Silber J, Lim DA, Petritsch C et al (2008) miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells. *BMC Med* 6:14
 30. Makeyev EV, Zhang J, Carrasco MA, Maniatis T (2007) The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol Cell* 27:435-448
 31. Grossel MJ, Hinds PW (2006) From cell cycle to differentiation: an expanding role for cdk6. *Cell Cycle* 5:266-270
 32. Huang TC, Chang HY, Chen CY, Wu PY, Lee H, Liao YF, Hsu WM, Huang HC, Juan HF (2011) Silencing of miR-124 induces neuroblastoma SK-N-SH cell differentiation, cell cycle arrest and apoptosis through promoting AHR. *FEBS Lett* 585: 3582-3586

Fig. 1 The expression of miR-124 in relation to overall survival in the patients with colorectal cancer

Fig. 2 The expression of miR-124 in relation to disease-free survival in the patients with colorectal cancer

Table 1 Stem-loop RT primers for miR-124, primers and probes for miR-124 and 5S rRNA

	Sequence
miR-124	
Stem-loop RT primers	5'-GTCGTATCCAGTGTGGGTCCGAGTGATTTCGCACTGGATACGACGGCATTTC-3'
Forward primer	5'-GCTTTCTAAGGCACGCGGT-3'
Reverse primer	5'-CAGTGCTGGGTCCGAGTGA-3'
Probe	5'-FAM-TCGTATCCAGTGCGAATGACTC-TAMRA-3'
5S rRNA	
Forward primer	5'-TACGGCCATACCACCCTGA-3'
Reverse primer	5'-GGCGGTCTCCCATCAA-3'
Probe	5'-FAM-CCCGACCCTGCTTAGCTTCCGA-TAMRA-3'

-

Table 2 Output of randomization test by REST 2009 software in pair-matched samples of primary colorectal cancer and adjacent normal mucosa

	5S RNA	miR-124
PCR Efficiency	1.930	1.890
Control Means	17.004	22.233
Sample Means	16.537	23.229
Expression Ratio	1.000	0.390
<i>P</i> value		0.001

-

Table 3 Relationship between expression of miR-124 and clinicopathological characteristics in patients with colorectal cancer

Characteristics	miR-124			P
	All Cases n=96	Low expression n=71 (%)	High expression n=25 (%)	
Gender				
Male	54	41 (57.7)	13 (52.0)	0.618
Female	42	30 (42.3)	12 (48.0)	
Age (years)				
≤ 55	42	31 (43.7)	11 (44.0)	0.977
>55	54	40 (56.3)	14 (56.0)	
Tumor site				
Colon	39	28 (39.4)	11 (44.0)	0.690
Rectum	57	43 (60.6)	14 (56.0)	
Maximum tumor size (cm)				
≤ 4	50	37 (52.1)	13 (52.0)	0.992
>4	46	34 (47.9)	12 (48.0)	
Depth of invasion				
T ₁	3	2 (2.8)	1 (4.0)	0.692
T ₂	16	10 (14.1)	6 (24.0)	
T ₃	34	26 (36.6)	8 (32.0)	
T ₄	43	33 (46.5)	10 (40.0)	
Venous invasion				
Absent	68	53 (74.6)	15 (60.0)	0.166
Present	28	18 (25.4)	10 (40.0)	
Lymph node metastasis				
Absent	54	41 (57.7)	13 (52.0)	0.618
Present	42	30 (42.3)	12 (48.0)	
Distant metastasis				
Absent	90	66 (93.0)	24 (96.0)	0.589
Present	6	5 (7.0)	1 (4.0)	

-

TNM stage				
I	15	9 (12.7)	6 (24.0)	
II	37	31 (43.7)	6 (24.0)	
III	38	26 (36.6)	12 (48.0)	
IV	6	5 (7.0)	1 (4.0)	0.236
Differentiation				
Well	23	12 (16.9)	11 (44.0)	
Moderate	47	39 (54.9)	8 (32.0)	
Poor	26	20 (28.2)	6 (24.0)	0.021

55 years = median age at operation; 4cm= mean diameter of tumor.

-

Table 4 Cox multivariate regression analysis of miR-124 expression, gender, age, tumor site, maximum tumor size, depth of invasion, grade of differentiation and stage in relation to overall survival in patients with colorectal cancer

Variable	Unfavorable/favorable	β	SE	HR	95% CI	<i>P</i>
MiR-124 expression	(Downregulated/upregulated)	1.533	0.502	4.634	1.731-12.404	0.002
Gender	(Male/ female)	0.602	0.345	1.826	0.928-3.589	0.081
Age	(>55/ ≤55 years)	0.193	0.327	1.213	0.639-2.303	0.556
Tumor site	(Rectal/ colon)	0.041	0.348	1.042	0.527-2.060	0.905
Maximum tumor size	(>4/ ≤4 cm)	0.535	0.333	1.708	0.889-3.282	0.108
Depth of invasion	(T4/ T3/ T2/ T1)	0.448	0.271	1.566	0.920-2.665	0.098
Differentiation	(Poor/ moderate/ well)	0.248	0.263	1.282	0.765-2.147	0.346
Stage	(IV/ III/ II/ I)	1.336	0.342	3.805	1.947-7.434	<0.001

β indicates coefficient; SE, standard error; HR, hazard ratio; CI, confidence interval.

-

Table 5 Cox multivariate regression analysis of miR-124 expression, gender, age, tumor site, maximum tumor size, depth of invasion, grade of differentiation and stage in relation to disease-free survival in patients with colorectal cancer

Variable	Unfavorable/favorable	β	SE	HR	95% CI	<i>P</i>
MiR-124 expression	(Downregulated/upregulated)	1.511	0.491	4.533	1.733-11.856	0.002
Gender	(Male/ female)	0.601	0.357	1.824	0.907-3.670	0.092
Age	(>55/ ≤55 years)	0.184	0.324	1.201	0.637-2.266	0.571
Tumor site	(Rectal/ colon)	0.087	0.345	1.091	0.555-2.144	0.801
Maximum tumor size	(>4/ ≤4 cm)	0.525	0.348	1.691	0.855-3.346	0.131
Depth of invasion	(T4/ T3/ T2/ T1)	0.547	0.266	1.728	1.026-2.908	0.040
Differentiation	(Poor/ moderate/ well)	0.132	0.263	1.141	0.682-1.911	0.615
Stage	(IV/ III/ II/ I)	1.383	0.333	3.988	2.076-7.662	<0.001

β indicates coefficient; SE, standard error; HR, hazard ratio; CI, confidence interval.