Biochemical and Biophysical Research Communications 388 (2009) 35–40

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Functional elucidation of MiR-34 in osteosarcoma cells and primary tumor samples

Chunlei He [a], Jianyi Xiong [b], Xiaoping Xu [b], Wei Lu [b], Lijun Liu [b], Deming Xiao [b], Daping Wang [b,*]

[a] Guangzhou Medical College, Guangzhou 510182, Guangdong Province, China
[b] Department of Traumatic Orthopeadics, Shenzhen Second People's Hospital, Shenzhen 518035, China

ARTICLE INFO

Article history:
Received 16 July 2009
Available online 24 July 2009

Keywords:
MiR-34
p53
Osteosarcoma

ABSTRACT

MiR-34s have been characterized as direct p53 targets, which induce apoptosis, cell cycle arrest, and senescence. MiR-34s were found to associate with tumorigenesis. Thus far, there is no study on the role of MiR-34s in osteosarcoma. In the current study, we intensively investigated the function of MiR-34s in two osteosarcoma cell lines: U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$). We found that MiR-34s affect the expression of its target genes partially in a p53-dependent manner. And p53 also partially contributes to the MiR-34s induced cell cycle arrest and apoptosis. Finally, we examined the expression, genetic and epigenetic alterations of MiR-34 gene in 117 primary osteosarcoma samples. Expression of MiR-34s was decreased in tumor samples, and MiR-34 genes underwent minimal deletions and epigenetic inactivation in osteosarcomas.

Introduction

Micro-RNAs (MiRNAs) are small non-coding RNAs that regulate gene expression mainly through affecting productive translation and mRNA stability [1–3]. Nascent MiRNA transcripts (pri-MiRNAs) are transcribed by RNA polymerases II or III to yield primary transcripts (pri-MiRNAs), which are processed by the nuclear RNase III enzyme Drosha to form hairpin products (pre-MiRNAs). These pre-MiRNAs are transported to the cytoplasm where the RNase III enzyme Dicer cleaves off the double-stranded (ds) portion of the hairpin and generates a short-lived dsRNA of about 20–25 nucleotides in size [1–3]. After maturation, these small RNAs are incorporated into the RNA-induced silencing complex (RISC). The MiRNA complex binds to the partially complementary binding sites located in the 3′ untranslated region (UTR) of target mRNAs by imperfect base pairing that depends mainly on the "seed" sequences comprising bases 2–7 of the mature MiRNA [2,3]. MiRNAs mediate post-transcriptional gene silencing of specific mRNA targets by inhibiting translation or destabilizing target mRNAs [4,5]. However, recent findings indicate that MiRNA-mediated repression can be reversed, prevented or even act as translational activators [6].

MiRNAs play important roles in several cellular processes, such as proliferation, differentiation, apoptosis and development, by simultaneously controlling the expression levels of hundreds of genes [2,7]. Similar to mRNA-encoding genes, several MiRNA-encoding genes have been meanwhile classified as oncogenic or tumor-suppressive genes according to their function in cellular transformation and expression in tumors [8–11]. In human cancer, recent studies have shown that MiRNA expression profiles differ between normal tissues and derived tumors and between tumor types [12,13]. MiRNAs can act as oncogenes or tumor suppressors, exerting a key function in tumorigenesis [10,14]. Furthermore, tumor cells seem to undergo a general loss of MiRNA expression, and forced reduction of global MiRNA expression promotes transformation [15]. Interestingly, MiRNAs cluster within fragile sites and other genomic regions frequently altered in cancers [16,17]. Besides their causal involvement in tumor formation, MiRNAs may be very useful for the classification, diagnosis, prognosis, and therapy of malignancies [8,9,12].

Recently, reports from several laboratories surfaced that the MiR-34 family are direct p53 targets, which induce apoptosis, cell cycle arrest, and senescence [18–24]. In vertebrates, MiR-34 diverged into a family of three homologous MiRNAs: MiR-34a, MiR-34b and MiR-34c. The mature MiR-34a sequence is located within the second exon of its non-coding host gene which contains a predicted p53 binding site [18,19,22,23,25]. Both MiR-34b and MiR-34c are located within a single non-coding precursor (MiR-34b/c), whose transcriptional start site is adjacent to a predicted p53 binding site [18,25]. Ectopic expression of MiR-34a and MiR-34b/c caused a cell cycle arrest in the G1 phase [18,23,25], and inhibited proliferation and colony formation in soft agar [20]. Introduction of MiR-34a and MiR-34b/c into primary

* Corresponding author. Address: Department of Traumatic Orthopeadics, Shenzhen Second People's Hospital, No. 3002, Sungang Road West, Shenzhen 518035, Guangdong Province, China.
E-mail address: daping-wang@hotmail.com (D. Wang).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.07.101

human diploid fibroblasts induced cellular senescence [25]. Interestingly, tumor cells also showed signs of senescence after introduction of ectopic MiR-34a [24]. Furthermore, expression of MiR-34a induced apoptosis [19,22,23,26].

MiR-34 was found to associate with tumorigenesis. For example, loss of MiR-34a expression was observed in neuroblastoma, which may be due to the relatively common deletion of a region on chromosome 1p36, which encompasses MiR-34a [26]. 1p36 deletions frequently encompass very large numbers of genes; however, primary neuroblastomas and cell lines often showed low MiR-34a expression [26]. Artificial elevation of MiR-34a in these cells inhibited proliferation and activated cell death pathways [26]. Furthermore, expression of MiR-34a was low or undetectable in 11 of 15 pancreatic cancer cell lines [19]. The expression level of MiR-34b was decreased by more than 90% in 6 out of 14 non-small-cell lung cancers (NSCLCs) [18]. Minimal deletions containing MiR-34b and MiR-34c have also been found in breast and lung cancer [17]. Epigenetic inactivation of MiR-34b/c was also found to associate with cancer metastasis [27]. A combination of bioinformatic predictions and experimental analysis led to the notion that MiR-34s control broad programs of targets involved in cell cycle control, apoptosis, and DNA repair, among which CDK4/6, Cyclin E2, E2F3/5, cMET, and Bcl-2 were shown as possible candidates [18,24,25].

Thus far, there is no study on the role of MiR-34 in osteosarcoma. In the current study, we intensively investigated the function of MiR-34 in two osteosarcoma cell lines: U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$). We also examined the genetic and epigenetic alterations of MiR-34 gene in primary osteosarcoma samples, providing more evidence on understanding MiR-34 in osteosarcoma.

Materials and methods

Cell culture, reagents and tumor samples. Human osteosarcoma cell lines U2OS and SAOS-2 were cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). Antibodies against CDK6, E2F3, Cyclin E2, Bcl-2 and Actin were from Santa Cruz. MiR-34 mimics, antagonists and control MiRNA mimic were obtained from Dharmacon with the following sequences: hsa-MiR-34a-5′-uggcagugucuuagcugguugu-3′; hsa-MiR-34b-5′-caaucacuaacuccacugccau-3′; hsa-MiR-34c-5′-aggcaguguaguuagcugauugc-3′. Surgically resected osteosarcoma tumor tissues and adjacent normal tissues were collected from 117 primary gastric cancer patients. The study was approved by the ethical committee of the Shenzhen Second People's Hospital. The individuals gave their written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

Quantification of MiR-34s with real-time PCR. Total RNA was isolated using the mirVana MiRNA isolation kit (Applied Biosystems). TaqMan Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis according to the manufacturer's protocol. TaqMan MiRNA assays were used to quantify the level of mature MiRNAs as described previously [28]. TaqMan Micro-RNA assay kits for hsa-MiR-34s (Applied Biosystems) were used for real-time PCR. Human GAPDH served as an internal control for RNA normalization. Random hexamers (Invitrogen) were used for the RT-reaction for GAPDH. Synthetic MiR-34s were used as the standards for copy quantification. The primers for GAPDH were: forward-5′-TCCACC ACTGACACGTTGG-3′, and reverse-5′-AAGGCTGTGGGCAAGGTC-3′. The probe for GAPDH was 5′-6FAM-ACACGGAAGGCCATGCCAG T-BHQ1-3′. Data from three independent experiments were analyzed by student *t*-test and $p < 0.05$ was considered statistically significant.

MiR-34 mimics transfection. Osteosarcoma cells were transfected 24 h after being seeded in 6-well plates. MiRNA mimics (100 pmol) or antagonists in 200 μl of serum-free, antibiotic-free, medium were mixed with 5 μl of Lipofectamine 2000 transfection reagent (Invitrogen) dissolved in 200 μl of the same medium and allowed to stand at room temperature for 20 min. The resulting 400 μl transfection solutions were then added to each well containing 1.6 ml of medium. Six hours later, the cultures were replaced with 2 ml fresh medium supplemented with 10% FBS and antibiotics.

3′UTR luciferase reporter assay. Osteosarcoma cells were transfected in 6-well plates with 2 μg of E2F3 or Bcl-2 3′UTR luciferase reporter plasmid per well, using Lipofectamine 2000 (Invitrogen). Cells in each well were also co-transfected with 100 pmol of each MiR-34 mimics or control mimic as indicated. Luciferase assays were performed 24 h after transfection using Bright-Glo Luciferase Assay System (Promega). Luciferase activity was normalized relative to control.

FACS analysis. For cell cycle analysis by flow cytometry, osteosarcoma cells were transfected with MiR-34 mimics or control mimic in 6-well plates, trypsinized 24 h later, washed with PBS, and fixed in 70% ethanol on ice. After centrifugation, cells were stained with 50 μg/ml propidium iodide and 0.1 μg/ml RNase A, and analyzed by FACS (BD).

Apoptosis analysis. Osteosarcoma cells were transfected with MiR-34 mimics or control mimic. After an additional incubation of 72 h, the cells were harvested, stained with propidium iodide and anti-annexin-V antibody, and visualized by fluorescence microscope.

DNA methylation analysis. Genomic DNA was isolated from the tissues, and was subjected to bisulfite conversion with the EZ DNA methylation kit (Zymo research, USA). The eluted DNA (40 μl volume) was used for the HRM analysis. PCR amplification and HRM were performed on the ABI7500 (Applied Biosystems) as adapted from the published protocol [29]. CpGenome Universal Methylated and unmethylated DNA (Chemicon, Millipore Billerica, MA, USA) were used as 100% and 0% methylated control DNA, respectively. See Supplementary information for detailed methods.

Results and discussion

Expression and induction of MiR-34s in osteosarcoma cells

Since Mir-34s are direct p53 targets, we took advantage of two osteosarcoma cell lines, U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$), to investigate the function of MiR-34s in osteosarcoma cells. We examined the expression of MiR-34s in these cells and found that both cell lines express MiR-34s at similar levels (Fig. 1A), suggesting that basal Mir-34s levels in these cells are p53-independent.

The transcriptional activity of p53 can be readily induced in cells by exposure to genotoxic stress, such as irradiation and the chemotherapeutic agent adriamycin. So, next we treated the cells with adriamycin and measured the expression of MiR-34s by quantitative PCR. There was an obvious increase of the MiR-34 expression in U2OS cells, while this increase was nearly completely abolished in SAOS-2 cells (Fig. 1B). A marked increase in MiR-34a levels was also seen when U2OS cells, but not SAOS-2 cells, were treated with gamma-irradiation (Fig. 1C). This confirms that MiR-34s are the downstream targets of p53, which is consistent with pervious reports [18–24].

Mir-34s affect target genes expression partially in a p53-dependent manner

The well characterized target genes of MiR-34s include CDK4/6, Cyclin E2, E2F3/5, cMET, and Bcl-2 [18,24,25]. First, we evaluated the effect of MiR-34s restoration on the expression of these genes. Basal levels of CDK6, E2F3 and Cyclin E2 were similar in U2OS and

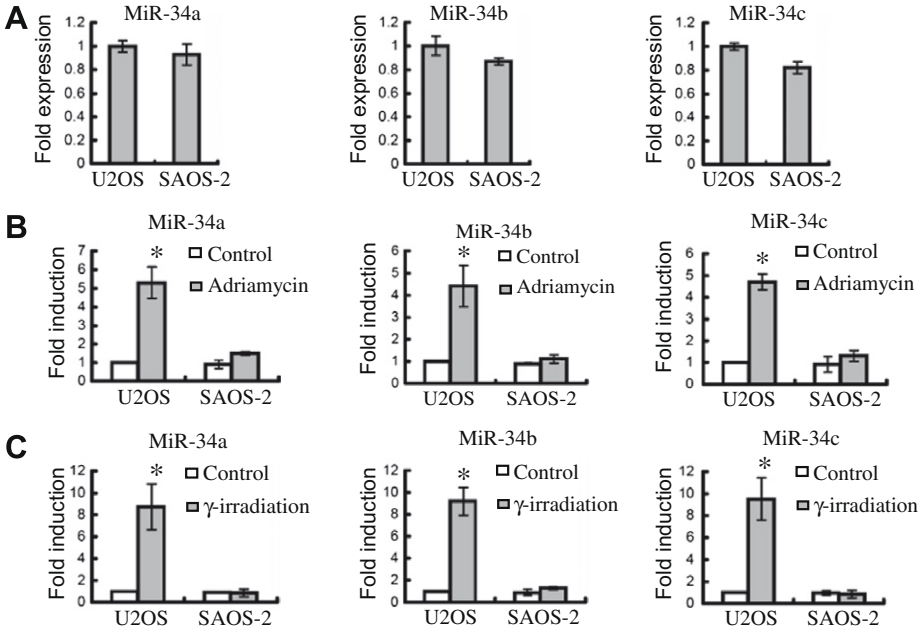


Fig. 1. Expression and induction of MiR-34s in osteosarcoma cells. RNA was extracted from U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$) cells. Expression of MiR-34s was measured by TaqMan based real-time PCR. Relative expression levels of MiR-34s in untreated U2OS cells were set as 1. (A) No treatment. (B) Cells were treated with 200 nM adriamycin for 24 h. (C) Cells were treated with with 8 Gy γ-irradiation and harvested 12 h later.

SAOS-2 cells. In contrast, basal expression of Bcl-2 was lower in the $p53^{-/-}$ cells, SAOS-2 (Fig. 2A and B). As expected, transfection of MiR-34a mimic into U2OS cells inhibited the expression of CDK6, E2F3, Cyclin E2 and Bcl-2, while transfection of MiR-34a antagonist upregulated their protein levels (Fig. 2A and B). As MiR-34s act downstream of p53, effect of MiR-34 on these genes should be independent of p53. To our surprise, MiR-34a mimic inhibited the expression of these genes in the $p53^{-/-}$ cell line, SAOS-2, to a lesser degree compared with that in U2OS, while induction of these genes by MiR-34a antagonist was also alleviated in SAOS-2 cells (Fig. 2A and B).

To further prove that the changes of these genes at protein levels were a direct effect of MiR-34, we fused the 3'UTRs of E2F3 and Cyclin E2, respectively, to a luciferase reporter construct. Consis-

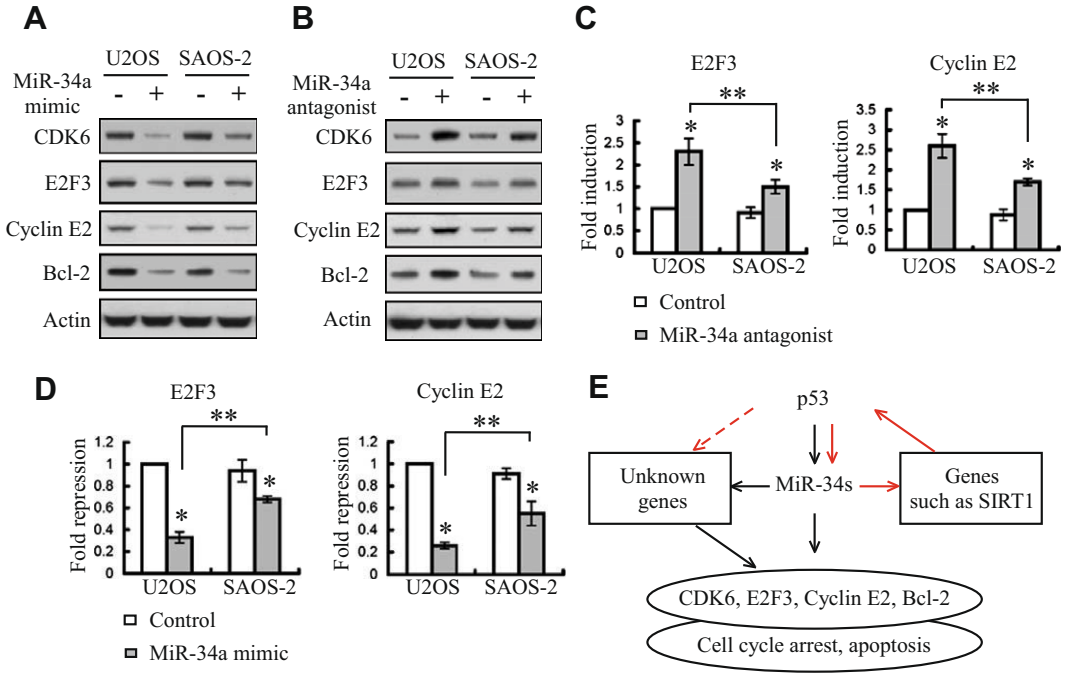


Fig. 2. Mir-34s affect target genes expression partially in a p53-dependent manner. (A,B) U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$) cells were transfected with either MiR-34a mimic or antagonist as indicated and harvested 24 h later. Expression of proteins was detected by Western blot using antibodies as indicated. (C,D) U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$) cells were co-transfected with luciferase reporter constructs together with either MiR-34a mimic or antagonist as indicated and harvested 24 h later. Luciferase assay was performed and relative luciferase activity in control U2OS cells was set as 1. Data were presented as mean + SD from three independent experiments. Asterisk: $p < 0.05$ versus control. Double asterisks: $p < 0.05$ versus the other. (E) A model showing the relationship between p53 and MiR-34s.

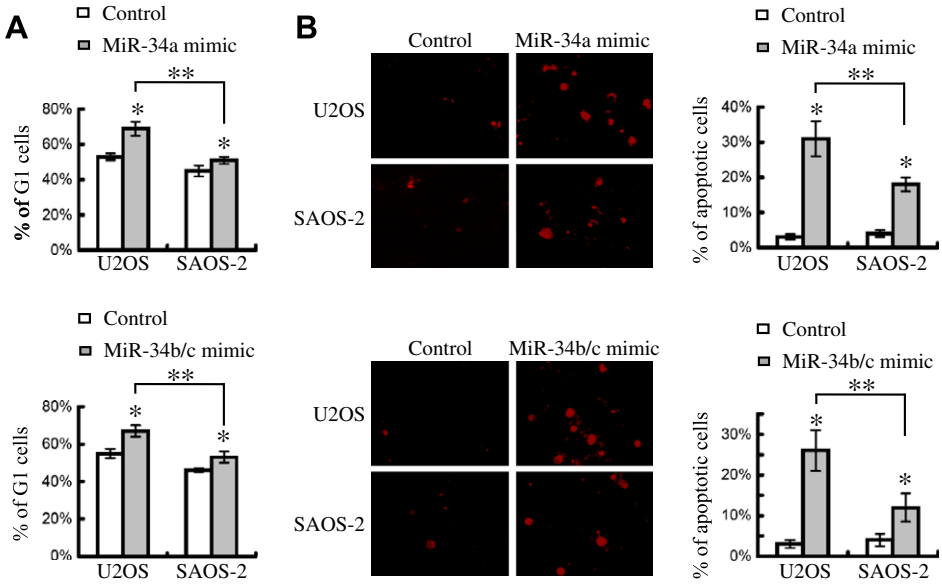


Fig. 3. Mir-34s induce cell cycle arrest and apoptosis in osteosarcoma cells. U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$) cells were transfected with either control MiRNA or MiR-34s mimics as indicated and incubated for 72 h. (A) Cells were trypsinized, washed with PBS, fixed, stained with PI and subjected to FACS analysis. The percentage of G1 cells was presented as mean ± SD from three independent experiments. (B) Cells were stained with Annexin-V antibody and fluorescence-conjugated secondary antibody. The percentage of apoptotic cells was presented as mean ± SD from three independent experiments. Double asterisks: $p < 0.05$ versus the other. Asterisk: $p < 0.05$ versus control.

tent with previous results, transfection of MiR-34a mimic or antagonist effectively modulated the luciferase reporter gene expression (Fig. 2C and D). And the effect of MiR-34a in SAOS-2 cells was not as potent as that in U2OS cells. Similar results were obtained when MiR-34b/c mimics or antagonists were applied to these cells (data not shown).

This suggests that some p53-dependent factors may participate in the MiR-34s mediated gene modulation. One possibility is the existence of a positive feedback loop in which p53 serves as a key component (Fig. 2E, red lines). This has been proved by Yamakuchi et al. who showed that Silent Information Regulator 1 (SIRT1), a target of MiR-34a, increases p53 activity [30,31]. Another possibility is that genes such as E2F3 and Cyclin E2 may be indirectly regulated by some unknown MiR-34s target genes, which are p53-dependent (Fig. 2E, red dash line).[1] Decreased levels of these genes in $p53^{-/-}$ cells may attenuate the effect of MiR-34s. Further evidence is needed to prove this hypothesis, and this also provides a direction for our future study.

Mir-34s induce cell cycle arrest and apoptosis in osteosarcoma cells

To study the effect of MiR-34s on cell cycle in osteosarcoma cells, we transfected MiR-34 mimics to U2OS and SAOS-2 cells and collected the cells after 24 h for FACS analysis. MiR-34s induced G1 arrest in both cell lines, although the effect of MiR-34s in SAOS-2 cells was not as obvious as that in U2OS cells (Fig. 3A). Next, by annexin-V labeling assay, we found that MiR-34s induced apoptosis in both U2OS and, with a lesser extent, SAOS-2 cells (Fig. 3B). This again suggests that a complex connection between p53 and MiR-34s may exist, as proposed in our model (Fig. 2E).

The partial abolishment of MiR-34a-induced apoptosis was supported by a previous study in which WT and $p53^{-/-}$ human colon cancer (HCT116) cell lines were used as model [19], further addressing the possibility of both p53-dependent and p53-independent mechanisms of MiR-34a induced cell death.

[1] For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

Expression, genetic and epigenetic alterations of Mir-34 genes in primary osteosarcoma samples

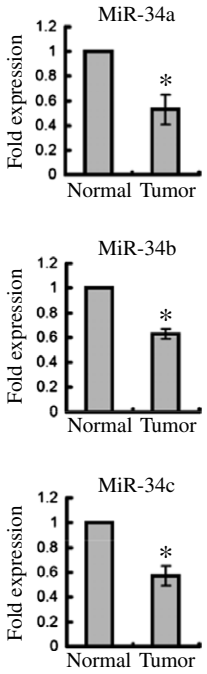
It is known that MiR-34s were correlated with tumor, and decrease of the MiR-34s expression was observed in multiple cancer types [18,19,26]. In order to further explore the function of MiR-34s in tumorigenesis, we collected 117 pairs of primary osteosarcoma tumor samples and adjacent normal tissues. Expression of MiR-34s in these samples was measured by quantitative TaqMan assay. Logarithmic transformation ($\text{Log}_2[1 + X]$) was used to transform primary data to normal distribution before statistical analysis. We found that expression of MiR-34s was lower in tumor samples than that in normal tissues (Fig. 4A).

Since MiR-34 genes are located in the fragile site (FRA) [17], next we want to find out whether minimal deletions happen in these tumor samples. For MiR-34a, loss of heterozygosity (LOH) was found in 7/117 tumor sampes, while no homologous deletion (HD) was observed (Fig. 4B). Interestingly, minimal deletions happen at a much higher frequency in MiR-34b/c gene, and LOH and HD were 21/117 and 1/117, respectively (Fig. 4B). In both cases, no LOH or HD was detected in normal tissues.

Epigenetic inactivation of MiRs in cancers has been reported by many groups. And promoter methylation of MiR-34b/c was also found to associate with cancer metastasis [27]. Thus, we used a recently developed method, high-resolution melting analysis (HRM), to semi-quantitatively detect the methylation levels MiR-34b/c in osteosarcoma samples. We used different combinations of methylated and unmethylated DNA as templates to generate standard curves (Fig. 4C). Methylation levels between 1% and 10% were observed in 47/117 tumors, while 17/117 tumors have high methylation levels (>10%) (Fig. 4D).

Many groups have reported the association of MiR-34s with tumorigenesis. Decreased expression of MiR-34s was found in neuroblastoma, non-small-cell lung cancer (NSCLC) and pancreatic cancer [18,19,26]. Minimal deletions and epigenetic inactivations of MiR-34s were found in breast, colon and lung cancers as well as melanoma [17,27]. Our study showed that expression of MiR-34s was decreased in osteosarcoma samples. And both genetic and epigenetic modulations may contribute the altered expression

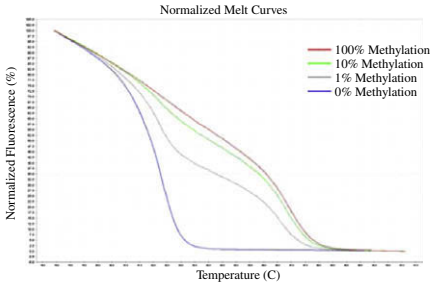
A



B

	Total No.	No deletion	LOH	HD	*P*
MiR-34a					
Tumor	117	110	7	0	0.007
Normal	117	117	0	0	
MiR-34b/c					
Tumor	117	95	21	1	5.3E-06
Normal	117	117	0	0	

C



D

	Total No.	Methylation levels			*P*
		< 1%	1-10%	10-100%	
Tumor	117	53	47	17	4.3E-19
Normal	117	116	1	0	

Fig. 4. Expression, genetic and epigenetic alterations of Mir-34 genes in primary osteosarcoma samples. (A) RNA was exacted from primary osteosarcoma samples and adjacent normal tissues. Expression of MiR-34s was measured by TaqMan based real-time PCR. Relative expression levels of MiR-34s in normal tissues were set as 1. Data were presented as mean ± SD. Asterisk: $p < 0.05$ versus normal (B) DNA was exacted from tumor samples and normal tissues. Copy number of DNA was measured by TaqMan assay. *P* value was obtained from Chi-square test. LOH, loss of heterozygosity; HD, homozygous deletion. (C) Different combinations of methylated and unmethylated DNA as indicated were used as templates to generate standard curves for high-resolution melting (HRM) analysis. See Supplementary information for a high-resolution picture. (D) DNA was exacted from tumor samples and normal tissues. Methylation levels of MiR-34b/c were determined by HRM assay. *P* value was obtained from Chi-square test.

of MiR-34s in osteosarcoma. We noticed that the frequencies of LOH and HD for MiR-34a were lower than that for MiR-34b/c (Fig. 4B). This may be due to the different location of MiR-34a (1p36) and MiR-34b/c (11q23). On the other hand, the frequency of minimal deletions for both MiR-34a and MiR-34b/c was not high, suggesting that epigenetic alterations may play an important role in modulating MiR-34s expression. Furthermore, many other transcriptional factors besides p53 may also contribute to the expression of MiR-34s, adding more complexity to the network. In the future, it would be interesting to explore such factors.

Conclusion

Our study showed that: (a) both U2OS ($p53^{+/+}$) and SAOS-2 ($p53^{-/-}$) cells express MiR-34s at similar levels; (b) irradiation and adriamycin induce MiR-34s expression in U2OS cells but not in SAOS-2 cells; (c) MiR-34s affect the expression of CDK6, E2F3, Cyclin E2 and Bcl-2 partially in a p53-dependent manner; (d) MiR-34s induce G1 arrest and apoptosis partially in a p53-dependent manner; (e) expression of MiR-34s in osteosarcoma samples was decreased; and (f) MiR-34s undergo genetic and epigenetic alterations in osteosarcoma samples.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.101.

References

[1] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.
[2] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[3] P.D. Zamore, B. Haley, Ribo-gnome: the big world of small RNAs, Science 309 (2005) 1519–1524.
[4] G. Meister, T. Tuschl, Mechanisms of gene silencing by double-stranded RNA, Nature 431 (2004) 343–349.
[5] R.S. Pillai, S.N. Bhattacharyya, W. Filipowicz, Repression of protein synthesis by MiRNAs: how many mechanisms?, Trends Cell Biol 17 (2007) 118–126.
[6] W. Filipowicz, S.N. Bhattacharyya, N. Sonenberg, Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight?, Nat Rev. Genet. 9 (2008) 102–114.
[7] L. He, G.J. Hannon, MicroRNAs: small RNAs with a big role in gene regulation, Nat. Rev. Genet. 5 (2004) 522–531.
[8] G.A. Calin, C.M. Croce, MicroRNA signatures in human cancers, Nat. Rev. Cancer 6 (2006) 857–866.
[9] J.M. Cummins, V.E. Velculescu, Implications of micro-RNA profiling for cancer diagnosis, Oncogene 25 (2006) 6220–6227.
[10] A. Esquela-Kerscher, F.J. Slack, Oncomirs – microRNAs with a role in cancer, Nat. Rev. Cancer 6 (2006) 259–269.
[11] R. Garzon, M. Fabbri, A. Cimmino, G.A. Calin, C.M. Croce, MicroRNA expression and function in cancer, Trends Mol. Med. 12 (2006) 580–587.
[12] J. Lu, G. Getz, E.A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B.L. Ebert, R.H. Mak, A.A. Ferrando, J.R. Downing, T. Jacks, H.R. Horvitz, T.R. Golub, MicroRNA expression profiles classify human cancers, Nature 435 (2005) 834–838.
[13] S. Volinia, G.A. Calin, C.G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R.L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C.C. Harris, C.M. Croce, A microRNA expression signature of human solid tumors defines cancer gene targets, Proc. Natl. Acad. Sci. USA 103 (2006) 2257–2261.
[14] S.M. Hammond, MicroRNAs as tumor suppressors, Nat. Genet. 39 (2007) 582–583.
[15] M.S. Kumar, J. Lu, K.L. Mercer, T.R. Golub, T. Jacks, Impaired microRNA processing enhances cellular transformation and tumorigenesis, Nat. Genet. 39 (2007) 673–677.
[16] G.A. Calin, C.G. Liu, M. Ferracin, T. Hyslop, R. Spizzo, C. Sevignani, M. Fabbri, A. Cimmino, E.J. Lee, S.E. Wojcik, M. Shimizu, E. Tili, S. Rossi, C. Taccioli, F. Pichiorri, X. Liu, S. Zupo, V. Herlea, L. Gramantieri, G. Lanza, H. Alder, L. Rassenti, S. Volinia, T.D. Schmittgen, T.J. Kipps, M. Negrini, C.M. Croce, Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas, Cancer Cell 12 (2007) 215–229.
[17] G.A. Calin, C. Sevignani, C.D. Dumitru, T. Hyslop, E. Noch, S. Yendamuri, M. Shimizu, S. Rattan, F. Bullrich, M. Negrini, C.M. Croce, Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers, Proc. Natl. Acad. Sci. USA 101 (2004) 2999–3004.
[18] G.T. Bommer, I. Gerin, Y. Feng, A.J. Kaczorowski, R. Kuick, R.E. Love, Y. Zhai, T.J. Giordano, Z.S. Qin, B.B. Moore, O.A. MacDougald, K.R. Cho, E.R. Fearon,

P53-mediated activation of MiRNA34 candidate tumor-suppressor genes, Curr. Biol. 17 (2007) 1298–1307.
[19] T.C. Chang, E.A. Wentzel, O.A. Kent, K. Ramachandran, M. Mullendore, K.H. Lee, G. Feldmann, M. Yamakuchi, M. Ferlito, C.J. Lowenstein, D.E. Arking, M.A. Beer, A. Maitra, J.T. Mendell, Transactivation of MiR-34a by p53 broadly influences gene expression and promotes apoptosis, Mol. Cell 26 (2007) 745–752.
[20] D.C. Corney, A. Flesken-Nikitin, A.K. Godwin, W. Wang, A.Y. Nikitin, MicroRNA-34b and MicroRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth, Cancer Res. 67 (2007) 8433–8438.
[21] L. He, X. He, S.W. Lowe, G.J. Hannon, MicroRNAs join the p53 network – another piece in the tumour-suppression puzzle, Nat. Rev. Cancer 7 (2007) 819–822.
[22] N. Raver-Shapira, E. Marciano, E. Meiri, Y. Spector, N. Rosenfeld, N. Moskovits, Z. Bentwich, M. Oren, Transcriptional activation of MiR-34a contributes to p53-mediated apoptosis, Mol. Cell 26 (2007) 731–743.
[23] V. Tarasov, P. Jung, B. Verdoodt, D. Lodygin, A. Epanchintsev, A. Menssen, G. Meister, H. Hermeking, Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: MiR-34a is a p53 target that induces apoptosis and G1-arrest, Cell Cycle 6 (2007) 1586–1593.
[24] H. Tazawa, N. Tsuchiya, M. Izumiya, H. Nakagama, Tumor-suppressive MiR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells, Proc. Natl. Acad. Sci. USA 104 (2007) 15472–15477.
[25] L. He, X. He, L.P. Lim, E. de Stanchina, Z. Xuan, Y. Liang, W. Xue, L. Zender, J. Magnus, D. Ridzon, A.L. Jackson, P.S. Linsley, C. Chen, S.W. Lowe, M.A. Cleary, G.J. Hannon, A microRNA component of the p53 tumour suppressor network, Nature 447 (2007) 1130–1134.
[26] C. Welch, Y. Chen, R.L. Stallings, MicroRNA-34a functions as a potential tumor suppressor by inducing apoptosis in neuroblastoma cells, Oncogene 26 (2007) 5017–5022.
[27] A. Lujambio, G.A. Calin, A. Villanueva, S. Ropero, M. Sanchez-Cespedes, D. Blanco, L.M. Montuenga, S. Rossi, M.S. Nicoloso, W.J. Faller, W.M. Gallagher, S.A. Eccles, C.M. Croce, M. Esteller, A microRNA DNA methylation signature for human cancer metastasis, Proc. Natl. Acad. Sci. USA 105 (2008) 13556–13561.
[28] C.K. Raymond, B.S. Roberts, P. Garrett-Engele, L.P. Lim, J.M. Johnson, Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs, RNA 11 (2005) 1737–1744.
[29] T.K. Wojdacz, A. Dobrovic, L.L. Hansen, Methylation-sensitive high-resolution melting, Nat. Protoc. 3 (2008) 1903–1908.
[30] M. Yamakuchi, M. Ferlito, C.J. Lowenstein, MiR-34a repression of SIRT1 regulates apoptosis, Proc. Natl. Acad. Sci. USA 105 (2008) 13421–13426.
[31] M. Yamakuchi, C.J. Lowenstein, MiR-34, SIRT1 and p53: the feedback loop, Cell Cycle 8 (2009) 712–715.