

miR-34 is associated with poor prognosis of patients with gallbladder cancer through regulating telomere length in tumor stem cells

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Abstract *miR-34a* has been identified as a tumor suppressor in several tumors, but its involvement in gallbladder cancer (GBC) has not been reported. In this study, the *miR-34a* level and telomere length were measured in 77 gallbladder adenocarcinomas and 36 peritumoral tissues by real-time PCR. Forced *miR-34a* expression was established by an adenovirus carrying a *miR-34a* expression cassette. The colony-forming ability of isolated CD44⁺CD133⁺ GBC tumor stem-like cells was measured by *matrigel colony assay*. The xenograft tumor models were established by inoculating nude mice with CD44⁺CD133⁺ cells. Results showed that significantly lower *miR-34a* expression and longer telomere length were observed in gallbladder adenocarcinoma tissues, which correlated with poor prognosis of GBC patients. Forced overexpression of *miR-34a* inhibited the colony-forming ability of CD44⁺CD133⁺ GBC tumor stem-like cells in vitro and xenograft tumor growth in vivo. Injection of Ad-miR-34a downregulated PNUMS expression and reduced telomere length in xenograft GBC tumor cells. In conclusion, *miR-34a* is a tumor suppressor in gallbladder cancer. Both low *miR-34a* expression and long telomere length are markers for poor prognosis of patients with gallbladder adenocarcinoma. Our study also suggests that the *miR-34a* gene could be a target for targeting therapy of GBC.

Keywords Gallbladder cancer · miR-34a · Telomere length · Prognosis

Introduction

Gallbladder cancers (GBCs) are highly aggressive cancers with most patients succumbing to the disease within a year of diagnosis [1, 2]. Early diagnosis of GBC is difficult due to a lack of specific signs or symptoms, and a majority of GBC patients are diagnosed at an advanced stage with serious invasion and metastasis to other organs [3]. At present, cholecystectomy is the only effective treatment for about 10 % of patients with early stage GBC [4–6]. Patients with unresectable or metastatic GBC have extremely poor prognosis due to the lack of treatment options. Generally, palliative chemotherapy and radiation therapy offer limited benefits to patients with GBC [2]. Therefore, there is an urgent need to develop other treatment options, such as gene therapy, for patients with advanced GBC. Currently, a clinically available target for the gene therapy of gallbladder cancers has not been identified.

MicroRNAs (miRNA) make up a class of endogenously expressed, small non-coding RNA molecules whose primary function is to down-regulate gene expression by binding specifically to the 3'-untranslated region of mRNAs [7, 8]. It has been widely demonstrated that miRNAs can function as oncogenes or tumor suppressors to take part in multiple oncogenic cellular processes, including cell proliferation, invasion, angiogenesis, and metastasis [9]. Recent studies revealed that elevated expression of *miR-34* family members induces apoptosis and cell-cycle arrest [10–12]. In mammalian cells, the *miR-34* family comprises 3 members: *miR-34a*, *miR-34b*,

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and *miR-34c*. *miR-34a* is encoded by its own transcript, whereas *miR-34b/c* is encoded by one gene. Generally, *miR-34a* is ubiquitously expressed, whereas *miR-34b/c* is mainly expressed in lung tissues [13]. The expression of *miR-34a* has been demonstrated to be low or undetectable in a variety of cancer cells, such as pancreatic cancer cells [14], non-small lung cancer [13], and melanoma [15]. A recent study demonstrated that *miR-34a* suppresses the expression of phosphatase nuclear targeting subunits (PNUTS), which inhibit telomere erosion, DNA damage response, and apoptosis in cardiomyocytes [16]. Telomere shortening is observed to be accompanied by increased p53 activity in resident stem and progenitor cells of the intestines, skin, and bone marrow [17]. *miR-34a* has been revealed to be a direct target of *TP53* gene [10–12]. We therefore hypothesize that *miR-34a* may be involved in tumor growth through regulating telomere erosion in gallbladder cancer cells.

In this study, we investigated *miR-34a* levels and telomere length in 77 gallbladder adenocarcinomas and 36 peritumoral tissues and their correlation with the prognosis of GBC patients. Using adenovirus-carried *miR-34a* expression, we determined the tumor inhibitory effect of *miR-34a* in xenograft GBC tumors.

Materials and methods

Case selection

This study was conducted ethically with pre-approval from the Ethics Committee for Human Studies at Xiangya Hospital, Central South University. Written consent was obtained from all patients before using their tissues for this study. A total of 77 gallbladder adenocarcinomas that underwent surgical resection or biopsy and 36 peritumoral tissues from the 77 gallbladder tumors were collected from January 2007 to December 2010. All diagnoses were based on clinical findings, tumor morphological criteria, and immunohistochemical staining. Among the 77 gallbladder cancer patients, 26 patients were female and 51 patients were male with an age variation from 32 to 79 (52.8 ± 9.7) years. Among the 77 adenocarcinoma, 22 were well-differentiated, 23 were moderately differentiated, and 32 were poorly-differentiated. Invasion of gallbladder surrounding tissues and organs was found in 47 patients, while 49 patients had regional lymph node metastasis. Surgery included radical resection for 8 patients, palliative surgery for 19 patients, and no operation for 50 patients with only biopsy. The 2-year survival information of 77 patients was obtained through letters and phone calls. Resected tumors and peritumoral tissues were snap frozen and kept at -80 °C or embedded in paraffin.

Real-time polymerase chain reaction of *miR-34* and PNUTS mRNA expression

Frozen tumor tissues and peritumoral tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was isolated by following the manufacturer's instructions. Reverse transcription was performed using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Science, Waltham, MA, USA). Quantitative PCR reactions were carried out using the SYBR Premix Ex Taq™ kit (TaKaRa, Shiga, Japan). *miR-34a* mRNA was amplified using a forward primer with the DNA sequence of mature miRNA (*hsa-miR-34a*: 5'-TGGCAGTGTCTTAGCTGGTTGT-3') and common reverse primer provided in the kit. Human *PNUTS* gene was amplified using forward primer: 5'-GAAAGATGGTGAGTCGATGC-3' and reverse primer: 5'-GAGATGGTCTACAGTGAGC-3'. *GAPDH* gene was amplified as an internal control by forward primer: 5'-GGTGAAGGTCGGTGTGAACG-3' and reverse primer: 5'-TGGAGGCATGTAGGCCATG-3'. An automated thermocycler (Prism 9700 Sequence Detection System, Applied Biosystems) was used. Relative quantification (RQ) of *miR-34* and *PNUTS* mRNA expression was determined by comparative CT method ($RQ = 2^{-\Delta\Delta CT}$) and normalized to the *GAPDH* mRNA level.

Telomere measurement by real-time PCR

The telomere length was measured from total genomic DNA of tumor tissues and peritumoral tissues using a real-time quantitative PCR method as previously described [18]. The acidic ribosomal phosphoprotein PO (36B4) gene was chosen as a control. The primer sequences used for the telomere measurement were as follows: 5'-CGGTTTGTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and 5'-GGCTTGCCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. Human 36B4 gene was amplified by forward and reverse primers: 5'-CAGCAAGTGGGAAGGTGTAATCC-3' and 5'-CCCACTTATCATCAACGGGTACAA-3'. *GAPDH* was used as a loading control. The reactions for telomere length, 36B4, and *GAPDH* were performed in triplicate. The threshold cycle value (Ct) of telomere and 36B4 was normalized to the Ct of *GAPDH*. The relative T/S ratio (telomere PCRs/ single copy gene of 36B4) was calculated for each sample to reflect the relative length difference of telomeric DNA.

Isolation of CD44⁺CD133⁺ cell subsets from tumor tissues

Telomere shortening is observed to be accompanied by increased p53 activity [17], while *miR-34a* is a direct target of *TP53* gene [10–12]. Therefore, 6 GBC tumor tissue specimens with p53 positive expression were dissected using scissors and scalpels, mixed with collagenase IV (Invitrogen) in

DMEM (collagenase 200 U/ml, Invitrogen, 11150059), and incubated at 37 °C for 3 h. Cells were then filtered through a 40- μ m nylon mesh and washed twice with phosphate-buffered saline (PBS)/10 % fetal bovine serum (FBS) (Gibco-BRL). Cells were then resuspended in DMEM with 2 % FBS at concentration of $10^6/100 \mu\text{l}$ and incubated for 30 min at room temperature with 100-fold dilutions of the following antibodies: anti-CD24-fluorescein isothiocyanate (BD Biosciences), anti-CD133/-phycoerythrin (eBioscience), and anti-CD44-allophycocyanin (BD Biosciences). After incubation, the samples were washed twice with PBS/2 % FBS and resuspended in PBS/2 % FBS. Flow cytometry analysis was performed using FACSaria (BD Immunocytometry Systems, Franklin Lakes, NJ). Cells were routinely sorted twice, and the cells were reanalyzed for purity, which was typically >95 %.

Preparation of adenovirus to express miR-34a

The *miR-34a* expression cassette was constructed by following a protocol published by Hu et al. [19]. Briefly, a minigene which can transcribe a 22 bp *miR-34a* was constructed using pSilencer-2.0 vector (Ambion Inc.). The control plasmid is a plasmid carrying a nontargeted sequence (5'-AATTCTCCGAACGTGTACCGT-3') [19]. The *miR-34a* expression cassette, including the U6 gene promoter, *miR-34a* minigene sequence, and the polythymidine stop sequence, was subcloned into the pAdTrack vector. The adenovirus that carries the *miR-34a* gene was produced as previously described [20]. The resultant adenoviral vector was named Ad-miR-34a. A control adenovirus called Ad-NT was produced with the nontargeted sequence as described above.

Matrigel colony assay

About 2.5×10^6 isolated CD44⁺CD133⁺ GBC tumor cells were seeded in 10-cm plates and cultured in DMEM (Dulbecco's Modified Eagle Medium) with 2 % fetal bovine serum (FBS) at 37 °C and 5 % CO₂. Two days later, cells were infected with 100 MOI (multiple of infection) of Ad-miR-34a or Ad-NT control viruses for 12 h. After replacing the medium with fresh DMEM containing 2 % FBS, cells were irradiated at 4 Gy or sham irradiated using a ⁶⁰Co irradiator at a dose rate of 0.9 Gy/min. Two hours later, single cell suspensions were resuspended in a 1:1 mixture of Matrigel (BD Sciences) to medium and plated on 6-cm dishes in a limiting dilution. After 2 weeks of incubation, cells were incubated with 1 mg/ml of iodinitrotetrazolium chloride solution (Sigma-Aldrich), and colonies were counted. Five replicate dishes were plated for each dilution.

Tumor growth study

Single CD44⁺CD133⁺ GBC tumor cells were resuspended in a 1:1 mixture of Matrigel and serum free medium. A 100- μ l

suspension containing 1×10^5 cells was injected s.c. into the right hind limbs of Balb/C nude mice (BALB/c, *nu/nu*) weighing 20–22 g (Shanghai Biological Science Institution, Shanghai). After the tumors grew to about 8 mm in diameter, mice were randomly divided into 4 groups: Ad-NT virus injection, Ad-miR-34 injection alone, radiation alone, and radiation plus Ad-miR-34a group. Each group contained ten animals. In the radiation and radiation plus Ad-miR-34a injection groups, 7 Gy of radiation was given to the local tumor mass at day 1, 3, 5 using 320 kV X-rays (Therapax 320, Pantak Inc., East Haven, CT). Mice in Ad-NT group received sham-irradiation and intratumoral injection of Ad-NT once a week for 4 weeks. In the Ad-miR-34 injection and radiation plus Ad-miR-34a injection groups, Ad-miR-34a viruses (1×10^8 pfu in 50 μ l saline) were intratumorally injected once a week for 4 weeks. Tumors were measured in two dimensions every 5 days, and tumor volume (*V*) was calculated using the following formula: $V = (1/2) S^2 \times L$ (*S*, the shortest dimension; *L*, the longest dimension) [20]. Animals were euthanized when the subcutaneous tumor reached a size that required euthanasia (45 days after first injection). Tumors were excised and weighted after euthanasia. All animal experiments were conducted under an approved protocol from Central South University and performed in accordance with the animal care guidelines of the Chinese Council.

Western blot

The tumor tissues were homogenized and Western blot was performed as previously described [20]. Briefly, 10 μ g of homogenate was applied to 10 % SDS-PAGE gel and transferred to PVDF membranes (BioRad, Hercules, CA). After blocking, the membranes were incubated with anti-PNUTS monoclonal antibody (1:100, Santa Cruz Bio. Inc., USA) overnight at 4 °C, followed by incubation with HRP-conjugated anti-mouse secondary antibody (1:1000, Santa Cruz Bio. Inc., USA) for 2 h. The immunoreaction was visualized using an enhanced chemiluminescence kit (Millipore, USA). For loading control, the membranes were stripped and incubated with α -tubulin antibody. The densitometric analysis of the PNUTS and α -tubulin bands was performed using Adobe Photoshop software.

Statistical analysis

Data was analyzed using SPSS 13.0 (statistical package for the Social Sciences Version 13.0). Gene expression and telomere length were analyzed using Student's *t* test. Kaplan–Meier analysis and the log rank test were performed to identify survival differences in gallbladder cancer patients. A $p < 0.05$ was considered statistically significant.

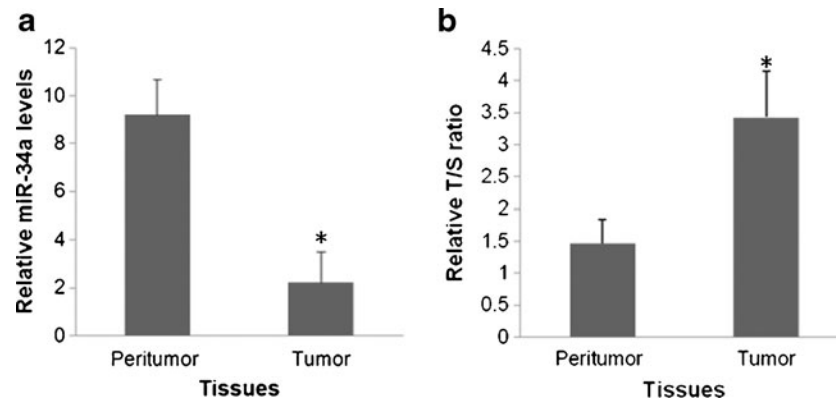


Fig. 1 *miR-34a* expression and telomere length in gallbladder adenocarcinoma and peritumoral tissues. **a** Real-time PCR of *miR-34a* expression. *miR-34a* expression is significantly lower in tumor tissues than in peritumoral tissues. **b** Relative *T/S* ratio examined by real-time PCR. The relative *T/S* ratio reflects relative length difference in telomeric DNA.

Results

miR-34a levels and telomere lengths in GBC and peritumoral tissues

Real-time PCR revealed that *miR-34a* expression was significantly lower in adenocarcinoma tissues ($RQ=2.22\pm 0.14$) than in peritumoral tissues ($RQ=9.21\pm 0.24$) (Fig. 1a, $p < 0.001$). The telomere length was measured by real-time PCR and the relative *T/S* ratio was calculated for each tumor tissue and peritumoral tissue. Results demonstrated that the relative *T/S* ratio was significantly smaller in peritumoral tissues than in tumor tissues (Fig. 1b, $p < 0.001$).

Significant shorter telomere length was observed in tumor tissues compared to peritumoral tissues. $*p < 0.001$ vs. peritumor (Peritumoral tissue). $N=77$ for gallbladder adenocarcinomas and $n=36$ for peritumoral tissues

miR-34a level and telomere length correlated with survival in patients with GBC

Survival information of 77 patients with gallbladder adenocarcinomas was obtained through letters and phone calls over a period of 2 years. Among the 77 GBC patients, 55 patients survived < 1 year and 22 patients survived ≥ 1 year (8 cases survived > 2 years) with an average survival time of 10.3 ± 0.77 months. The Kaplan–Meier survival analysis of GBC patients revealed that patients with lower *miR-34a* expression survived significantly shorter than patients having higher *miR-34a* expression ($p < 0.001$) (Fig. 2a). Cox multivariate analysis of cancer patients revealed that *miR-34a* expression positively correlated with

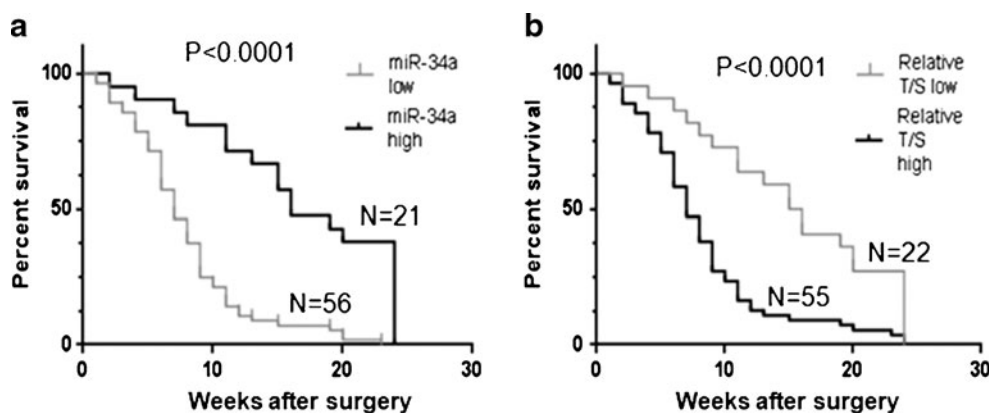


Fig. 2 *miR-34a* expression and relative *T/S* ratio and survival in patients with gallbladder adenocarcinoma. **a** Kaplan–Meier plots of overall survival in patients with gallbladder adenocarcinoma and with low and high level of *miR-34a*. The low and high *miR-34a* level was determined by comparing with the mean level. Cases with a *miR-34a* level higher than

the mean *miR-34a* of all patients was designed as high *miR-34a* level. **b** Kaplan–Meier plots of overall survival in patients with gallbladder adenocarcinoma and with high and low relative *T/S* ratio. Low and high *T/S* ratio was determined by comparing with the mean ratio

overall survival in GBC patients. The role of telomere length in the survival of patients with gallbladder adenocarcinoma was also investigated. The Kaplan–Meier survival analysis revealed that patients with lower relative *T/S* ratio survived significantly longer than patients having higher relative *T/S* ratio ($p < 0.001$) (Fig. 2b). Cox multivariate analysis revealed that telomere length negatively correlated with overall survival in GBC patients.

miR-34a expression significantly decreased Matrigel colony formation and sensitized the role of radiation

Matrigel colony assay (Fig. 3) showed that Ad-miR-34a infection significantly decreased the number of colonies formed

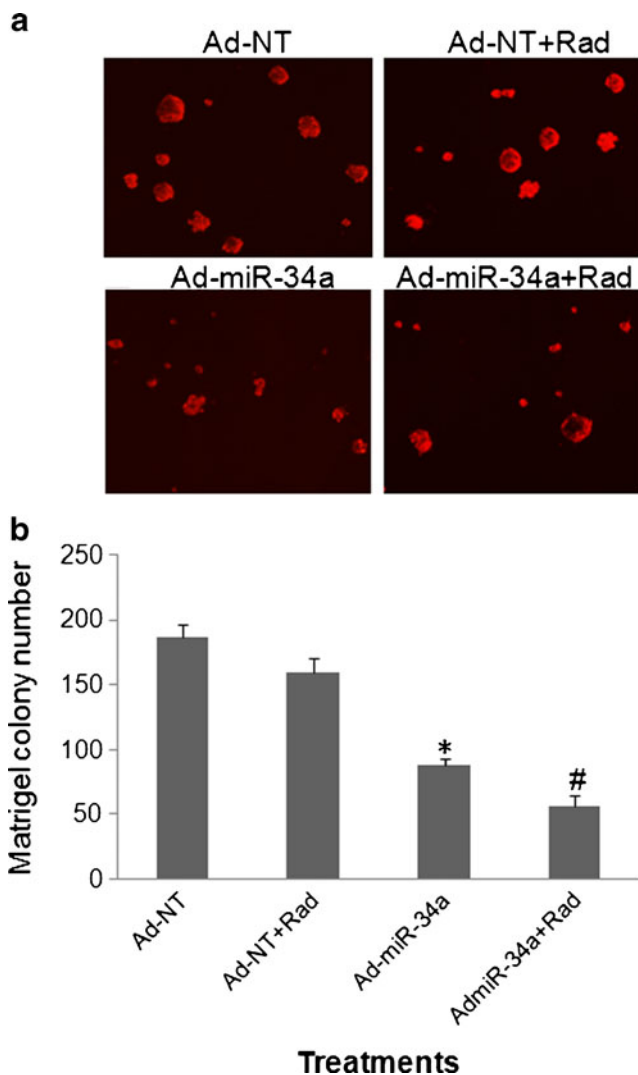


Fig. 3 Matrigel colony formation assay. CD44⁺CD133⁺ cells were isolated from p53 positive GBC tumors and treated with Ad-NT infection, radiation, Ad-miR-34a infection, and combined radiation and Ad-miR-34a infection. The treated cells were then incubated with Matrigel for 2 weeks. **a** Representative colony formation. **b** Colony counts. * $p < 0.01$, # $p < 0.001$ vs. Ad-NT group. $N = 5$

in CD44⁺CD133⁺ GBC tumor cells compared to the cells infected with Ad-NT control virus ($p < 0.001$). In contrast, radiation only showed a tendency to reduce the colony-forming ability. However, the colony-forming ability was decreased more significantly in cells treated with Ad-miR-34a injection ($p < 0.001$) and radiation compared to cells infected with Ad-NT control virus alone ($p < 0.001$) or cells infected with Ad-miR-34a alone ($p < 0.05$).

Expression of miR-34a inhibits xenograft GBC tumor growth

We further investigated whether the expression of *miR-34a* could inhibit tumor growth in vivo. Balb/C nude mice were inoculated with tumor cells. When the tumors grew to 7–8 mm in diameter, Ad-miR-34a viruses were injected intratumorally and additional injections were given once a week. Ad-miR-34a injection

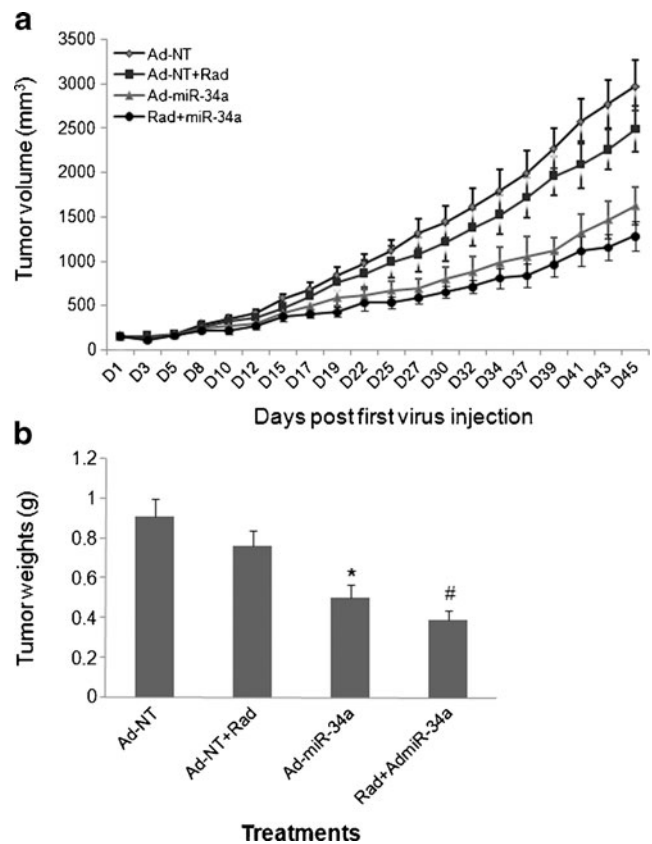


Fig. 4 The role of miR-34a overexpression on tumor growth. The measurement of tumor sizes was conducted every 2 days for 45 days post first irradiation. Bars, \pm SD at each data point. Mice were randomly divided into four groups: Ad-NT virus injection+sham-radiation (*Ad-NT*), Ad-miR-34 injection alone (*Ad-miR-34a*), Ad-NT virus injection+radiation (*Ad-NT+Rad*), and radiation plus Ad-miR-34a group (*Rad+Ad-miR-34a*). **a** Tumor growth curve presented by tumor volume. **b** Tumor weights at sacrifice. * $p < 0.05$, # $p < 0.01$ vs. Ad-NT group. $N = 10$

significantly inhibited tumor growth starting from the 19th day post first irradiation compared to tumors injected with Ad-NT control virus. Ad-miR-34a injection significantly sensitized the inhibitory effect of radiation on tumor growth from the 17th day post first irradiation (Fig. 4a). After euthanasia, tumors were excised and weighted. Radiation and radiation plus Ad-miR-34a injection significantly reduced tumor weights (Fig. 4b, $p < 0.05$ and $p < 0.01$, respectively). Ad-miR-34a plus radiation was more effective in decreasing tumor weight.

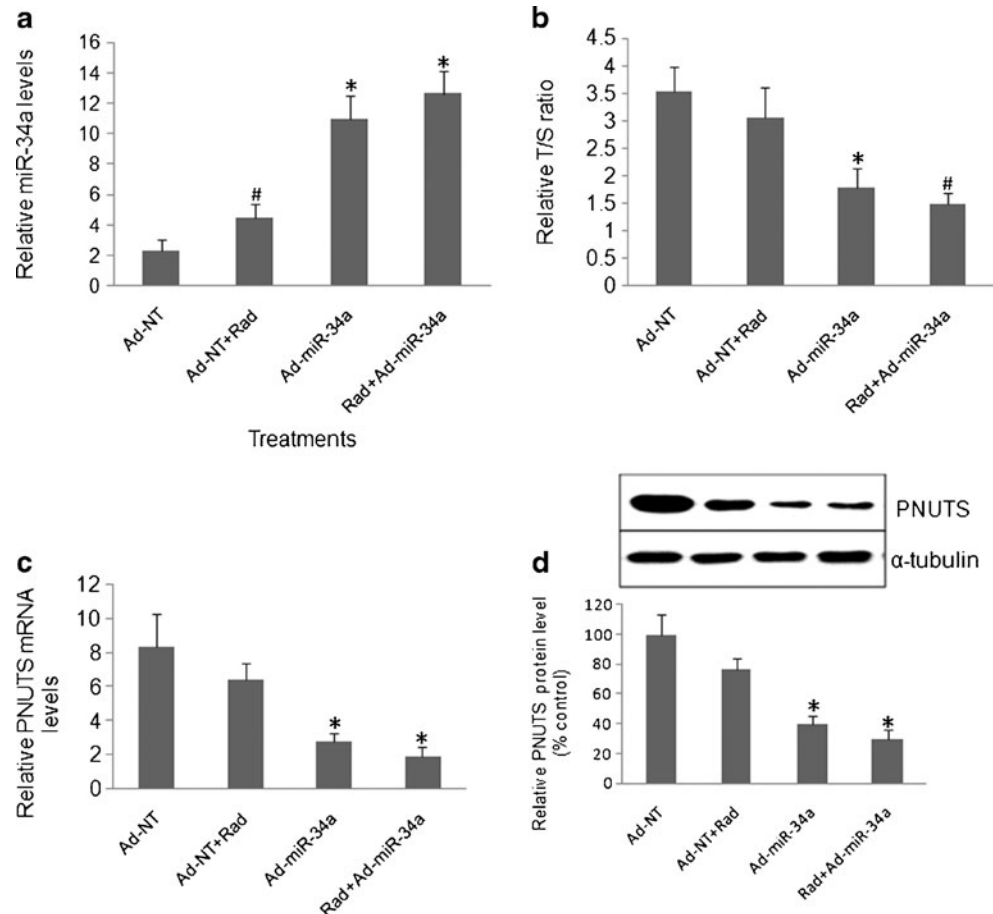
miR-34a inhibited xenograft GBC tumor growth through regulating telomere erosion

Real-time PCR demonstrated that Ad-miR-34a injection significantly increased *miR-34a* level (Fig. 5a, $p < 0.001$) and decreased relative T/S ratio (Fig. 5b, $p < 0.001$) in xenograft tumors compared to Ad-NT control virus-injected tumors. Injection of Ad-miR-34a virus significantly decreased *PNUTS* mRNA (Fig. 5c) and protein expression (Fig. 5d).

Discussion

miR-34a expression has been recently found to be very low or undetectable in several human cancers and to be associated with the progression and poor prognosis of these tumors [21, 22]. In cancer cells, *miR-34a* functions as an inhibitory gene and regulates the expression of a large number of proteins, even after their transcripts have already been synthesized [23]. Recent studies demonstrated that *miR-34a* regulates telomere length [16]. However, the expression pattern of *miR-34a*, its role on telomere length, and its involvement in the survival of gallbladder cancer patients has not been reported. In this study, *miR-34a* expression was found to be absent or reduced in gallbladder adenocarcinoma tissues, which is associated with poor prognosis. Moreover, the telomere length was longer in gallbladder tumor tissues than in peritumoral tissues which correlated with poor prognosis in patients. Forced overexpression of *miR-34a* reduced the colony-forming ability of tumor stem cells and inhibited tumor growth in a xenograft animal model inoculated with primary gallbladder adenocarcinoma stem cells. The inhibitory effect of *miR-34a* in xenograft tumor growth was associated with the

Fig. 5 *miR-34a* mRNA, *PNUTS* mRNA and protein expression, and relative T/S ratio in xenograft tumors. Mice were treated as described in “Materials and methods”. **a** Real-time PCR of *miR-34a* expression in xenograft tumor tissues. * $p < 0.001$, # $p < 0.05$ vs. Ad-NT controls. **b** Relative T/S ratio in xenograft tumors. * $p < 0.001$ vs. Ad-NT controls, # $p < 0.05$ vs. Ad-miR-34a and < 0.001 vs. Ad-NT controls. **c** Real-time PCR of *PNUTS* mRNA expression. * $p < 0.001$ vs. Ad-NT controls. **d** Western blot of *PNUTS* protein levels. * $p < 0.001$ vs. Ad-NT controls. $N = 10$



downregulation of *PNUTS* expression and a decrease in telomere length. Therefore, the *miR-34a* gene was identified as a tumor suppressor gene in gallbladder cancer and an ideal target for gene therapy.

The association of telomere length with carcinogenesis and progression of cancer has been widely studied with varied outcomes. Most studies indicated that shorter telomeres are more closely associated with increased prospective or cross-sectional risk of a variety of cancer types and cancer mortality although long telomeres are commonly thought to be a cause of cancer [24–26]. A number of studies have reported the prognostic role of telomere length in solid tumors. The majority of studies have found an association between altered tumor telomere length and poor outcomes. For example, reduced telomere content has been found to be associated with poorer survival in breast and prostate cancer [26], while in sarcoma, short telomere length was linked to poor survival [27]. In contrast, long telomeres have been associated with tumors of more advanced stages, and poor prognosis in hepatocellular carcinoma, colorectal carcinoma, Barrett carcinoma, and head and neck tumors [25]. In lung cancer, both telomere reduction and elongation have been associated with a worse outcome of therapy [28–30]. However, research on telomere length in gallbladder cancer specimens has not been reported. In this study, we found that longer telomere lengths correlated with shorter survival in gallbladder cancer. Importantly, we further revealed that the telomere length in gallbladder tumor cells is regulated by *miR-34a* level.

Many reports suggest that *miR-34a* functions as a tumor suppressor gene and contributes to the inhibition of invasion or metastasis in various tumor types [23]. Most recently, the epigenetic inactivation of *miR-34a* was identified in some of the most common cancers, such as breast, lung, colon, kidney, bladder, pancreatic cancer, and melanoma [15]. The low or undetectable expression of *miR-34a* has recently been found to be associated with poor prognosis of squamous cell carcinoma and lung cancer [21, 22]. In this study, we demonstrated that lower *miR-34a* levels were associated with shorter survival in gallbladder cancer. Furthermore, we produced an adenovirus to express *miR-34a* in CD44⁺CD133⁺ GBC tumor cells, which have been shown to have GBC stem-like cell characteristics [31]. Forced expression of *miR-34a* significantly inhibited Matrigel colony-forming ability and sensitized the inhibitory effect of radiation. Further study in xenograft animal model inoculated with CD44⁺CD133⁺ GBC tumor cells showed that forced expression of *miR-34a* significantly inhibited xenograft tumor growth and sensitized the effect of radiation. *miR-34a* exerted an inhibitory role through shortening telomere length by negatively regulating *PNUTS* gene expression. *PNUTS* has been demonstrated to regulate telomere length through direct interaction with the telomeric repeat binding factor (TRF2) through the [Y/F]XL motif [32]. *PNUTS* overexpression reduced telomere attrition in vitro

without affecting telomerase activity [16]. Therefore, telomere length may be one of the key downstream effective factors of *miR-34a*.

In conclusion, our study is the first to identify *miR-34a* as a tumor suppressor in gallbladder cancer through regulating telomere length. The loss of *miR-34a* gene expression is a prognostic indicator for poor prognosis of patients with gallbladder cancer. Inhibition of tumor growth in GBC can be established by forced expression of *miR-34a* with or without radiotherapy. Our study suggests that the *miR-34a* gene can be an ideal target for the targeting therapy of GBC.

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Conflicts of interest None

References

- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics 2008. *CA Cancer J Clin*. 2008;58:71–96.
- Jayaraman S, Jarnagin WR. Management of gallbladder cancer. *Gastroenterol Clin North Am*. 2010;39:331–42.
- Hawkins WG, DeMatteo RP, Jarnagin WR, Ben-Porat L, Blumgart LH, Fong Y. Jaundice predicts advanced disease and early mortality in patients with gallbladder cancer. *Ann Surg Oncol*. 2004;11:310–5.
- Ootani T, Shirai Y, Tsukada K, Muto T. Relationship between gallbladder carcinoma and the segmental type of adenomyomatosis of the gallbladder. *Cancer*. 1992;69:2647–52.
- Roa JC, Tapia O, Cakir A, Basturk O, Dursun N, Akdemir D, et al. Squamous cell and adenosquamous carcinomas of the gallbladder: clinicopathological analysis of 34 cases identified in 606 carcinomas. *Mod Pathol*. 2011;24:1069–78.
- Park SB, Kim YH, Rho HL, Chae GB, Hong SK. Primary carcinosarcoma of the gallbladder. *J Korean Surg Soc*. 2012;82:54–8.
- Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev*. 2006;20:515–24.
- Bagga S, Pasquinelli AE. Identification and analysis of microRNAs. *Genet Eng (N Y)*. 2006;27:1–20.
- Xiong J, Du Q, Liang Z. Tumor-suppressive microRNA-22 inhibits the transcription of E-box-containing c-Myc target genes by silencing c-Myc binding protein. *Oncogene*. 2010;29:4980–8.
- Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell*. 2007;26:731–43.
- Tarasov V, Jung P, Verdoodt B, Lodygin D, Epanchintsev A, Menssen A, et al. 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*. 2007;6:1586–93.
- Corney DC, Flesken-Nikitin A, Godwin AK, Wang W, Nikitin AY. MicroRNA-34b and microRNA-34c are targets of p53 and cooperate in control of cell proliferation and adhesion-independent growth. *Cancer Res*. 2007;67:8433–8.
- Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, et al. p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol*. 2007;17:1298–307.

14. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell*. 2007;26:745–52.
15. Lodygin D, Tarasov V, Epanchintsev A, Berking C, Knyazeva T, Korner H, et al. Inactivation of miR-34a by aberrant CpG methylation in multiple types of cancer. *Cell Cycle*. 2008;7:2591–600.
16. Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, et al. MicroRNA-34a regulates cardiac ageing and function. *Nature*. 2013;495:107–10.
17. Sahin E, DePinho RA. Axis of ageing: telomeres, p53 and mitochondria. *Nat Rev Mol Cell Biol*. 2012;13:397–404.
18. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res*. 2002;30:e47.
19. Hu Y, Ou Y, Wu K, Chen Y, Sun W. miR-143 inhibits the metastasis of pancreatic cancer and an associated signaling pathway. *Tumour Biol*. 2012;33:1863–70.
20. Zhang X, Kon T, Wang H, Li F, Huang Q, Rabbani ZN, et al. Enhancement of hypoxia-induced tumor cell death in vitro and radiation therapy in vivo by use of small interfering RNA targeted to hypoxia-inducible factor-1alpha. *Cancer Res*. 2004;64:8139–42.
21. Ogawa T, Saiki Y, Shiga K, Chen N, Fukushige S, Sunamura M, et al. miR-34a is downregulated in cis-diamminedichloroplatinum treated sinonasal squamous cell carcinoma patients with poor prognosis. *Cancer Sci*. 2012;103:1737–43.
22. Gallardo E, Navarro A, Viñolas N, Marrades RM, Diaz T, Gel B, et al. miR-34a as a prognostic marker of relapse in surgically resected non-small-cell lung cancer. *Carcinogenesis*. 2009;30:1903–9.
23. Hermeking H. The miR-34 family in cancer and apoptosis. *Cell Death Differ*. 2010;17:193–9.
24. Eisenberg DTA. An evolutionary review of human telomere biology: The thirty telomere hypothesis and notes on potential adaptive paternal effects. *Am J Hum Biol*. 2011;23:149–67.
25. Svenson U, Roos G. Telomere length as a biological marker in malignancy. *Biochim Biophys Acta*. 2009;1792:317–23.
26. Bisofti M, Heaphy CM, Griffith JK. Telomeres: Prognostic markers for solid tumors. *Int J Cancer*. 2006;119:2255–60.
27. Avigad S, Naumov I, Ohali A, Jeison M, Berco GH, Mardoukh J, et al. Short telomeres: a novel potential predictor of relapse in Ewing sarcoma. *Clin Cancer Res*. 2007;13:5777–83.
28. Shirotani Y, Hiyama K, Ishioka S, Inyaku K, Awaya Y, Yonehara S, et al. Alteration in length of telomeric repeats in lung cancer. *Lung Cancer*. 1994;11:29–41.
29. Hirashima T, Komiya T, Nitta T, Takada Y, Kobayashi M, Masuda N, et al. Prognostic significance of telomeric repeat length alterations in pathological stage I–IIIA non-small cell lung cancer. *Anticancer Res*. 2000;20:2181–7.
30. Frias C, Garcia-Aranda C, De Juan C, Morán A, Ortega P, Gómez A, et al. Telomere shortening is associated with poor prognosis and telomerase activity correlates with DNA repair impairment in non-small cell lung cancer. *Lung Cancer*. 2008;60:416–25.
31. Shi C, Tian R, Wang M, Wang X, Jiang J, Zhang Z, et al. CD44+CD133+ population exhibits cancer stem cell-like characteristics in human gallbladder carcinoma. *Cancer Biol Ther*. 2010;11:1182–90.
32. Kim H, Lee OH, Xin H, Chen LY, Qin J, Chae HK, et al. TRF2 functions as a protein hub and regulates telomere maintenance by recognizing specific peptide motifs. *Nat Struct Mol Biol*. 2009;16:372–9.