Lung Cancer 132 (2019) 99–106

Contents lists available at ScienceDirect

Lung Cancer

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

The novel microRNA hsa-miR-CHA1 regulates cell proliferation and apoptosis in human lung cancer by targeting XIAP

Jung Ki Yoo[a,1], Ji Min Lee[a,1], Seung Hee Kang[a], Seong Ho Jeon[a], Chang Min Kim[a], Seung-Hun Oh[b], Chang-Hyun Kim[c], Nam Keun Kim[d], Jin Kyeoung Kim[a,*]

[a] Department of Pharmacy, College of Pharmacy, CHA University, 335 Pangyo-ro, Bundang-gu, Seongman-si, Gyeonggi-do, 463-400, Republic of Korea
[b] Department of Neurology, CHA Bundang Medical Center, CHA University, Seongnam-si, Gyeonggi-do, 463-954, Republic of Korea
[c] College of Medicine, Dongguk Ilsan Hospital, Gyeonggi-do, 410-773, Republic of Korea
[d] Department of Biomedical Science, College of Life Science, CHA University, 335 Pangyo-ro, Bundang-gu, Seongman-si, Gyeonggi-do, 463-400, Republic of Korea

ARTICLE INFO

Keyword:
Novel microRNA
Lung cancer
XIAP
Cell proliferation

ABSTRACT

Objectives: MicroRNAs have critical roles in cancer development by regulating the expression of oncogenes or tumor suppressor genes. We identified and characterized a novel miRNA, miR-CHA1, in human lung cancer cells. The aim of this study was to investigate its novel function in human lung cancer by targeting XIAP.
Material and methods: Novel miRNA cloning, Real-time qRT-PCR, western blotting, dual luciferase assay, miRNA transfection, proliferation and apoptosis assay were carried on human lung cancer cell line A549. Fifteen paired NSCLC tissues and noncancerous lung tissues were collected. *In vivo* xenograft assay was performed.
Results: Expression of miR-CHA1 was downregulated in human lung cancer cell lines and tissues compared with normal cells and tissues. We identified a putative target gene, XIAP, whose expression was regulated by miR-CHA1 overexpression. XIAP is an inhibitor of apoptosis that represses the activation of caspase 3 and 9. XIAP mRNA and protein levels were directly suppressed by miR-CHA1. XIAP has an important role in carcinogenesis, and previous studies suggest that it may regulate cell survival and proliferation by its anti-apoptotic ability.
Conclusion: Taken together, miR-CHA1 inhibited cell proliferation and induced apoptosis *in vitro* and *in vivo* by targeting XIAP. These data can be applied to identify novel therapeutic targets for lung cancer therapy.

1. Introduction

MicroRNAs (miRNAs) are small noncoding RNAs of approximately 22nts in length. They suppress target genes via binding to the 3′-untranslated region (UTR) [1,2]. miRNAs regulate cellular functions including metabolism, development, senescence, proliferation, and apoptosis [3]. These miRNAs regulate networks of specific target genes that are involved in specific biological processes. For example, miR-23a/b regulates glutamine metabolism by suppression of c-Myc [4], miR-124 directly inhibits the SCP1 expression that blockades the REST/SCP1 network during embryonic CNS development [5], and the miR-29 and -30 family represses B-mybshown to induce cellular senescence [6]. Additionally the overexpression of let-7a repressed cell proliferation by significantly targeting the *RAS* gene and by playing a tumor suppressor role in human lung cancer [7]. miR-34 induces apoptosis and prevents tumorigenesis by targeting Bcl-2 CCND1 or c-Myc [8].

Lung cancer causes irregular cell proliferation in the lung through primary genetic or epigenetic modification [9]. Abnormal cells can form a tumor mass, requiring nutrients and oxygen though blood vessel formation, including angiogenesis [10]. These cells overcome anticancer therapy and express oncogenes or tumor suppressor genes [11]. Likewise, expression of oncogenes and tumor suppressor genes is negatively regulated by specific miRNAs. Among them, let-7 negatively regulates oncogenes such as *RAS*, *MYC*, *HMGA2* [12] and is downregulated in non-small cell lung cancer (NSCLC) patients with a let-7 family chromosomal deficiency [13]. miR-9500 directly suppresses Akt1, which negatively regulates proliferation and metastasis in lung cancer [14]. On the other hand, the miR-17-92 cluster is overexpressed and modulates tumor formation in lung cancer [15]. miR-205 promotes cell proliferation through targeting PTEN and PHLPP2 in NSCLC [16].

Abbreviations: miRNA, microRNA; UTR, untranslated region; NSCLC, non small cell lung cancer; ASO, antisense oligonucleotide; NC, negative control; XIAP, X-linked inhibitor of apoptosis; IAP, inhibitor of apoptosis

* Corresponding author.
E-mail address: kyeoung66@hanmail.net (J.K. Kim).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.lungcan.2018.04.011
Received 28 October 2017; Received in revised form 4 April 2018; Accepted 12 April 2018

These miRNAs are involved in tumorigenesis in human lung cancer.

Apoptosis is the programmed cell death important for cell division and differentiation [17]. Dysregulation of apoptosis causes abnormal cell growth and various diseases [18]. Apoptosis is a multiple step process, with the expression of and interactions among pro-apoptotic proteins (including Bax, Bad, and Bak) or anti-apoptotic proteins (including Bcl-2, Bcl-xL, and Bcl-w) [17]. In cancer cells, pro-apoptotic proteins are significantly upregulated, whereas anti-apoptotic proteins are downregulated [18]. Additionally, specific miRNAs negatively regulate these genes [8]. miR-192 regulates cell proliferation and promotes apoptosis by targeting retinoblastoma 1 in lung cancer cells [19]. miR-409-3p directly regulates PHF10, inhibiting cell proliferation and increasing apoptosis in gastric cancer cells [20]. These miRNAs are involved in apoptosis and inhibit cell proliferation in cancer cells.

In this studywe identified and characterized a novel miRNA in lung cancer cell lines. We profile the expression pattern of miR-CHA1 in lung cancer cell lines and patient tissues. We discovered a miR-CHA1 target gene using various techniques. We find that miR-CHA1 might affect lung carcinogenesis including cell proliferation and apoptosis.

2. Materials and methods

2.1. Cell culture and lung tissue samples

The human lung cancer cell lines A549 (lung adenocarcinoma), HCC-1588 (lung squamous carcinoma,), and NCI-H596 (lung adenosquamous carcinoma), were cultivated in RPMI-1640 medium (Corning, Manassas, VA, USA). WI-38 (human diploid lung normal fibroblast) cells were maintained in Eagle's minimum essential medium (Corning). All media were supplemented with 10% fetal bovine serum (Corning) and 1% antibiotic (100 U/ml penicillin and 100 μg/ml streptomycin, Corning). All human cell lines were incubated at 37 °C in a 5% CO_2 atmosphere. WI-38 and NCI-H596 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A549 and HCC-1588 were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). All lung tissue samples (Supplementary Table 1) were obtained from the Korea Lung Tissue Bank, which is supported by the Korea Science and Engineering Foundation at the Ministry of Science and Technology.

2.2. Novel miRNA cloning

Total RNA was isolated from the A549 cell line using TRIzol reagent (Ambion, Foster City, CA, USA), according to the manufacturer's protocols. The miRNAs were cloned into a vector using a ^{DYNA}Express miRNA cloning kit (BioDynamics Laboratory Inc., Tokyo, Japan) according to the manufacturer's instructions, with modification. Details have been described previously [21].

2.3. Analysis of novel miRNA expression using an miScript miRNA assay

Total RNA was prepared and reverse transcribed according to the manufacturer's instructions (Qiagen, Hilden, Germany). Quantitative PCR was carried out in a total volume of 20 μl, using 1 μl of reverse transcribed samples under the following cycling conditions: 15 min at 94 °C for initial denaturation, followed by 35 cycles of 94 °C for 10 s, 65 °C for 10 s and 72 °C for 30 s, followed by a final extension step at 72 °C for 5 min. Data were generated using CFX Manger™ software (Bio-Rad, Hercules, CA, USA). The data were representative of three independent experiments performed on different days.

2.4. Transfection and mimic

The A549 cell lines transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The media was changed 4–6 h after transfection without washing with PBS. The miRNA mimics (miR-CHA1: sense 5′-UGG CGG AGC CCU GGG CUG GGG UU-3′ and antisense 5′-CCC CAG CCC AGG GCU CCG CCA UU-3′), miR-CHA1 inhibitor (antisense oligonucleotide (ASO)-miR-CHA1; 5′-CCC CAG CCC AGG GCU CCG CCA-3′), siRNA (si-XIAP; sense 5′-CAA GAA UAU AUA AAC AAU AUU-3′ and antisense 5′- UAU UGU UUA UAU AUU CUU GUU-3′), and negative control (NC) (sense 5′- CCU CGU GCC GUU CCA UCA GGU AGU U-3′ and antisense 5′- CUA CCU GAU GGA ACG GCA CGA GGU U-3′, non-homologous with human genome sequence) were designed and purchased from Genolution Pharmaceuticals, Inc. (Seoul, Korea).

2.5. Target prediction and dual-luciferase assay

Approximately 500 nts of the XIAP 3′-UTR, containing the predicted miR-CHA1 binding site, were inserted into the pmiRGLO Dual–Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The mutant construct was generated using a Site-Directed Mutagenesis Kit (iNtRON, Seoungman, Korea) that mutated seven base pairs in the predicted seed region that was targeted by miR-CHA1 in the target 3′-UTR. Next, 150 ng of plasmid and 100 nmol of either miR-CHA1, ASO-miR-CHA1 or NC were co-transfected for normalization. Luciferase activity was measured using a Dual-Luciferase Reporter Assay System Kit (Promega), and luminescence intensity was measured using a VICTOR^3 analyzer (PerkinElmer, Foster City, CA, USA). The data were representative of three independent experiments performed on different days.

2.6. Western blotting

The A549 cell line was transfected with mimics of NC, miR-CHA1, and ASO-miR-CHA1 for 48 h. Protein was extracted with RIPA cell lysis buffer (ELPIS-Biotech, Daejeon, Korea). Western blotting was performed using anti-XIAP (1:1000 dilution in 5% skim milk, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Caspase-3 (1:1000 dilution in 5% skim milk, Cell Signaling, Danvers, MA, USA), anti-PARP (1:1000 dilution in 5% skim milk, Cell Signaling), or anti-GAPDH (1:5000 dilution in 5% skim milk, glyceraldehyde-3-phosphate dehydrogenase; Santa Cruz Biotechnology). Anti-GAPDH was used as an internal control. The data are representative of three independent experiments performed on different days.

2.7. XTT proliferation assay

A549 cells were plated at a density of 1×10^4 cells per well in 96-well culture plates. The miR-CHA1 miRNA, NC, si-XIAP, and ASO-miR-CHA1 were transfected into the cells. The proliferation rates of the cells were determined using 50 μl of XTT test solution. Cell proliferation was assessed at 0, 24, 48 h after transfection using the Cell Proliferation Kit II (XTT; Roche, Mannheim, Germany). The absorbance was measured and recorded at a wavelength of 490 nm, which was determined using an ELISA reader. The data are representative of three independent experiments performed on different days.

2.8. Annexin V-FITC/PI flow cytometry

After the indicated treatments (mimic of miR-CHA1 and NC, si-XIAP, and ASO-miR-CHA1), cells at 90% confluence were harvested by trypsinization. Apoptosis was measured with the Annexin V-FITC/PI kit (BD Bioscience, San Diego, CA, USA). Flow cytometry was performed using BD FACSCalibur™ (BD Bioscience). The data are representative of three independent experiments performed on different days.

2.9. In vivo xenograft assay and immunohistochemistry

All animals used in the *in vivo* experiments were housed under specific pathogen-free conditions, and the experiments were approved

by the institutional animal care and use committee of CHA University. Nude mice (male BALB/c, 6 weeks old) were used in the xenograft experiments. Each mouse experiment was repeated three times. A total of 5×10^6 A549 cells were injected s.c. into a 6-week-old nude mouse. After 2 weeks, PBS, NC, miR-CHA1, or ASO-miR-CHA1 were s.c. injected twice a week. The formation of tumor width and length were measured every week. Every 2 weeks, 3 mice from each group were sacrificed. Xenograft tumors that formed from A549 cells after 4, 6, and 8 weeks were dissected and fixed in 4% paraformaldehyde (PFA). The sections were stained with hematoxylin and eosin (H&E), TUNEL Apoptosis Detection Kit (Millipore, Darmstadt, Germany), or anti-Ki-67 and anti-XIAP immunostaining (Santa Cruz Biotechnology).

2.10. Statistical analysis

The data are presented as the mean ± S.E.M. from at least three independent experiments. The significant differences were analyzed using Student's *t*-test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. The identification and characterization of a novel miRNA, hsa-miR-CHA1, in lung cancer cells

We identified a novel miRNA, hsa-miR-CHA1, in lung cancer cell lines. First, we performed to characterize the sequence using NCBI Basic Local Alignment Search Tool (BLAST), and confirmed the folding structure formation of precursor miRNA using the RNAfold program (Fig. 1A). miR-CHA1 is transcribed from genomic DNA on the chromosome of 11q12.3 (61909723-61909743) (Fig. 1B). It is located in the intergenic region between FADS2 and BEST1. The mature miRNA sequence is 5′-UGG CGG AGC CCU GGG CUG GGG-3′, and miR-CHA1 is partially conserved in other vertebrates (Fig. 1C).

3.2. Expression profiles of miR-CHA1 in cell lines and tissues derived from normal human lungs and lung cancer

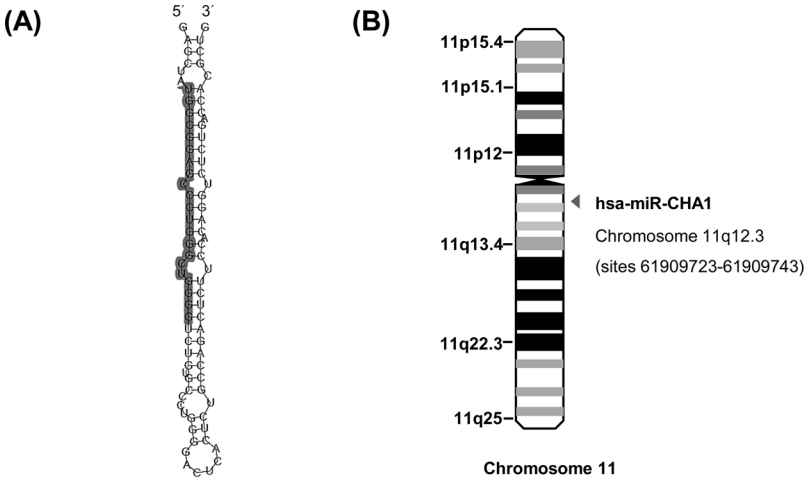
We assessed the miR-CHA1 expression levels, and found that miR-CHA1 was downregulated in human lung cancer cell lines, including A549, NCI-H596, and HCC-1588, compared with the WI-38 normal lung cell line (Fig. 2A). Additionally, we studied its expression pattern in human normal lung and cancer patient tissues. As shown in Fig. 2B, miR-CHA1 expression levels were downregulated in lung adenocarcinoma and squamous cell carcinoma tissues compared to normal lung tissues. These data suggest that miR-CHA1 is downregulated in human lung cancer cell lines and tissues compared with normal cell lines and tissues.

3.3. miR-CHA1 significantly suppressed XIAP expression in human lung cancer

In this study, miR-CHA1 was downregulated in human lung cancer, but target genes were upregulated. In our previous studies, we identified cancer related genes or oncogenes involved in lung carcinogenesis [14,22,23]. Among them, XIAP expression was significantly regulated by overexpression of miR-CHA1. As shown in Fig. 3A, miR-CHA1 binds the XIAP 3′-UTR (1930–1936). The seed region of miR-CHA1 was GGCGGAG (2–8). miR-CHA1 directly suppressed a wild type XIAP construct by approximately 60%, but did not affect the XIAP mutant construct in a dual-luciferase assay (Fig. 3B). The XIAP mutant construct was generated by modifying the miR-CHA1 binding site via PCR-based mutagenesis. miR-CHA1 inhibited XIAP mRNA and protein expression (Fig. 3C and D). Additionally, we silenced miR-CHA1 expression by the antisense oligonucleotide (ASO) miR-CHA1. As shown in Fig. 3E and F, ASO-miR-CHA1 did not regulate XIAP mRNA and protein expression levels. These data suggest that miR-CHA1 inhibits, via post-transcriptional modification, the translation of XIAP expression.

3.4. miR-CHA1 directly regulated the XIAP network in human lung cancer

As shown previously, miR-CHA1 is downregulated in human lung



(C)

Species	miR-CHA1
Human	5′ - U G G C G G A G C C C U G G G C U G G G G - 3′
Rat	5′ - C A G C G G A G C C C U G G G C U G G G G - 3′
Cattle	5′ - G A G C G G A G C C C U G G G C U G G G G - 3′
Pig	5′ - G A C C G G A G C C C U G G G C U G G G G - 3′
Mouse	5′ - G G C G G A G C C C U G G G C U G A A U - 3′

Fig. 1. Information on miR-CHA1. (A) The predicted precursor miRNA structure of miR-CHA1. Human genomic sequences were predicted using web-based programs (RNAfolds). The mature miR-CHA1 sequence is marked in grey. (B) miR-CHA1 is located on chromosome 11q.12.3. (C) The mature miR-CHA1 sequence is partially conserved in vertebrates.

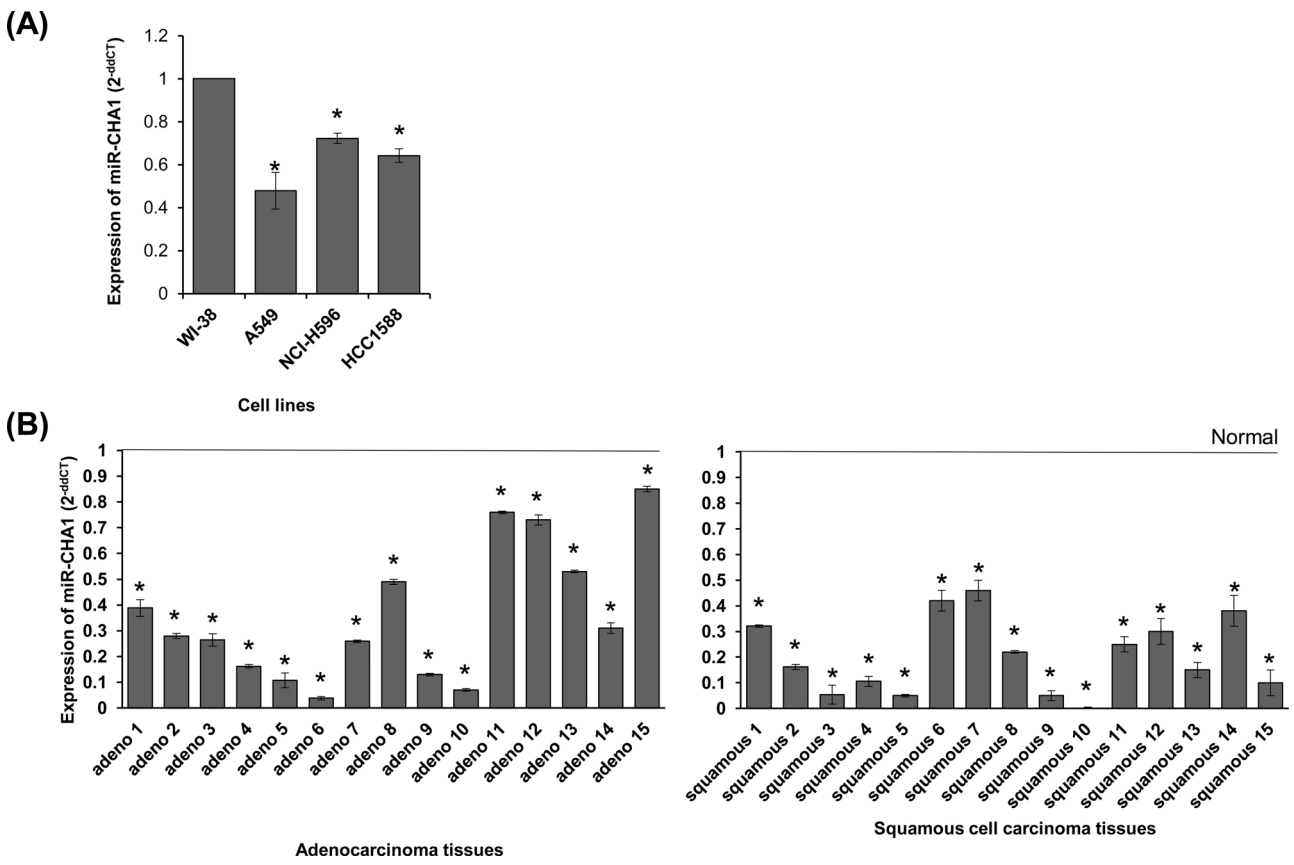


Fig. 2. miR-CHA1 is downregulated in human lung cancer cell lines and tissues. (A) miR-CHA1 expression was inhibited in A549, NCI-H596, and HCC1588 compared with WI-38 using miScript miRNA assay. (B) miR-CHA1 was suppressed in both adenocarcinoma and squamous cell carcinoma tissues compared with normal tissues. The expression was normalized to GAPDH. The data represent the average of three independent experiment and are shown as the mean ± S.E.M.; *P < 0.05. gr2

cell lines and human lung cancer patient tissues. It directly suppressed XIAP signaling. Overexpression of miR-CHA1 and silencing of XIAP approximately decreased threefold cell proliferation compared with treatment with mock or negative control (Fig. 4A). Likewise, inhibition of miR-CHA1 nearly increased threefold cell proliferation compared with miR-CHA1 transfected cells. After transfection of NC, miR-CHA1, ASO-miR-CHA1 or si-XIAP in A549 cells, we assessed the effect of miR-CHA1 on apoptosis or cell cycle using FACS analysis (Fig. 4B). Like XIAP silencing, miR-CHA1 arrests G1 phase by 10% that induces apoptosis in A549 cells (Supplementary Fig. 1). Additionally, using western blotting, we assessed the network downstream of XIAP after the overexpression of miR-CHA1 (Fig. 4C). miR-CHA1 slightly affected the cleavage of capsase-3 and downregulated the expression of pro-PARP. These data suggest that miR-CHA1 automatically transferred the signaling pathway of the XIAP network. However, miR-CHA1 slightly promoted apoptosis despite it directly suppresses to XIAP expression.

3.5. miR-CHA1 inhibits cell proliferation and promotes apoptosis in an in vivo xenograft model

As in our previous studies, miR-CHA1 suppressed the XIAP network that was downregulated in cell proliferation in human lung cancer. We assessed proliferation and tumorigenesis *in vivo* by subcutaneously (s.c.) injecting A549 cells into the thighs of nude mice. After 2 weeks, both thighs displayed tumor formation. We s.c. injected PBS in all left thighs and NC, miR-CHA1, or ASO-miR-CHA1 in each right thigh twice a week for 6 weeks. After 6 weeks, nude mice were generated to form tumor masses by NC, miR-CHA1 or ASO-miR-CHA1. miR-CHA1 suppressed tumor growth compared with PBS,N.C or ASO-miR-CHA1 injection (Fig. 5A and B). Histologically stained tumor sections from nude mice showed that miR-CHA1 overexpression repressed cell proliferation and increased apoptosis (Fig. 5C and Supplementary Fig. 2). miR-CHA1 inhibited XIAP expression in nude mice, reducing cell proliferation, as shown by XIAP and Ki67 immunohistochemistry. Likewise, miR-CHA1 promoted apoptosis in nude mice, as shown by a TUNEL apoptosis assay. These data suggest that miR-CHA1 suppressed cell proliferation and induced apoptosis in nude mice by targeting XIAP.

4. Discussion

In this study, we discovered and characterized a novel miRNA, hsa-miR-CHA1, in lung cancer cell lines. miR-CHA1 is transcribed in the intergenic region between FADS2 and BEST1 on chromosome 11q12.3. The miR-CHA1 modified mature form is generated from precursor miRNA by dicer dependent processing (Supplementary Fig. 3). FADS2 function is lost in various cancers [24]. BEST1 increased cell proliferation in colonic carcinoma cells [25]. These regions demonstrate copy number alteration in cervical cancer [26]. Additionally, one study described genomic instability that caused loss of function in NSCLC such as translocation, deletion or chromosome loss [27]. miR-CHA1 expression is downregulated in NSCLC cell lines and tissues because of transcription at the 11q12.3 locus and is associated with the repression of cancer development in NSCLC.

Following the functional analysis of miR-CHA, we identified upregulated expression of target genes in A549 cell lines. For novel miRNAs, we could not use target prediction programs (Target Scan, miRanda or miRDB) because they did not provide information about target genes [14,21]. Despite the problems with using target prediction programs, we discovered miR-CHA1 target genes using manual methods [28–30]. Using a luciferase assay, miR-CHA1 was verified to regulate X-linked inhibitor of apoptosis (XIAP) expression. XIAP is a member of the inhibitor of apoptosis (IAP) family. The IAP family is composed of eight

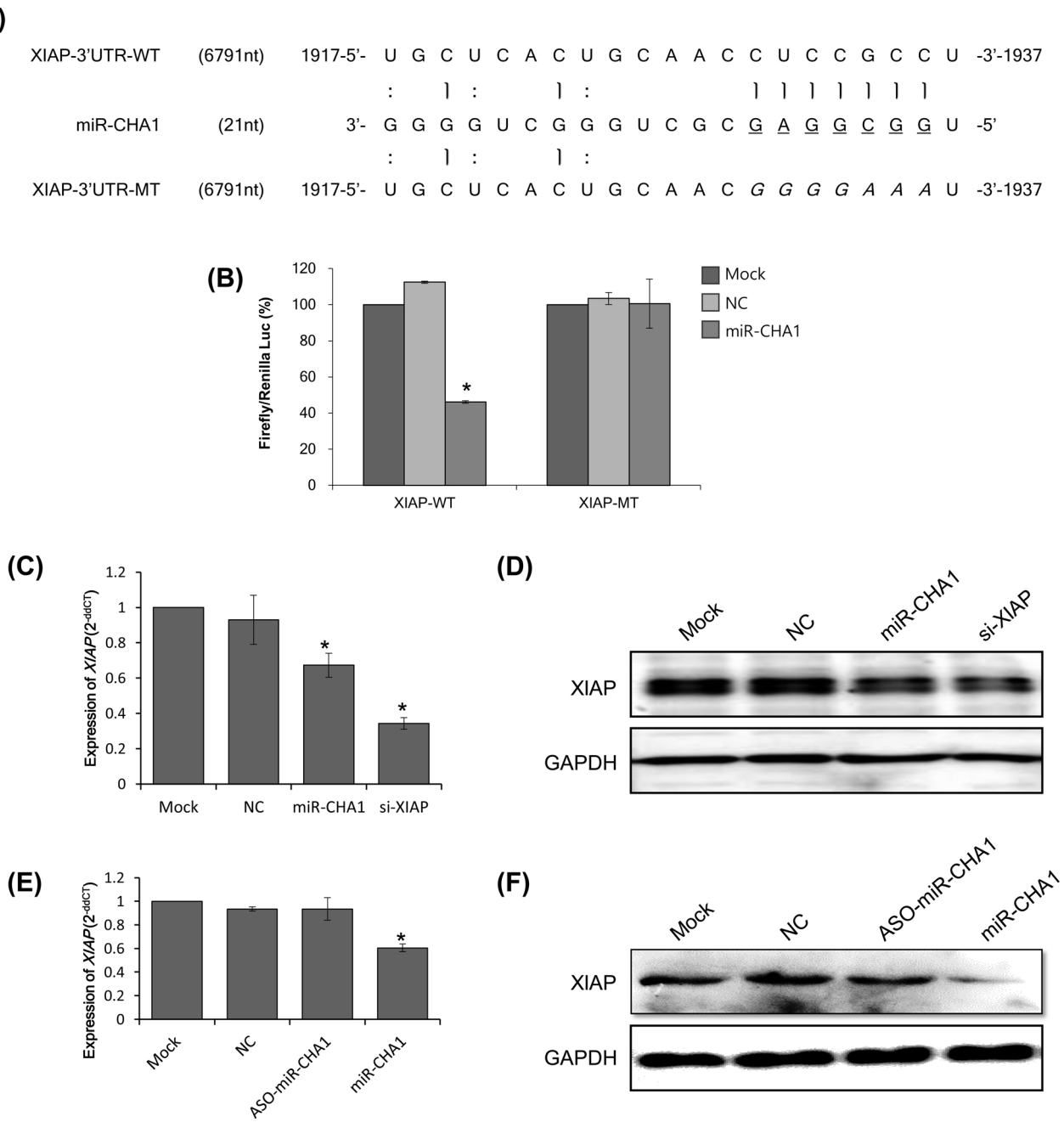


Fig. 3. miR-CHA1 directly repressed the XIAP networks by targeting the 3′-UTR of XIAP. (A) The binding site (underline) of miR-CHA1 in the XIAP 3′-UTR is displayed. The mutated miR-CHA1 (*italic*) binding site was generated using site-directed mutagenesis. (B) Luciferase activity was analyzed by co-transfecting the pmiR-Glo conjugated 3′-UTR of XIAP and NC or miR-CHA1. miR-CHA1 negatively regulated XIAP in the luciferase assay. (C and D) miR-CHA1 and si-XIAP downregulated the mRNA and protein levels. (E and F) ASO-miR-CHA1 inhibited miR-CHA1 expression but did not affect XIAP mRNA and protein levels. The data represent the average of three independent experiments and are shown as the mean ± S.E.M.; *P < 0.05.

members: NAIP, cIAP1, cIAP2, XIAP, ILP-2, ML-IAP, Survivin and Apollon [18]. These proteins affect various cellular mechanisms, including apoptosis [31]. XIAP activation prevented caspase-3 and caspase-7 activation of apoptosis by binding these two factors [32]. XIAP suppressed apoptosis networks and upregulated expression in malignant tissues compared with normal tissues (Supplementary Fig. 4) [33]. XIAP regulation by miRNAs was already reported. miR-7 inhibited cell proliferation and promoted apoptosis in cervical cancer by targeting BCL2 and XIAP [34,35]. Loss-of-function of miR-24 occurs in cancer cells, whereas XIAP is overexpressed [36]. miR-519d suppressed cell proliferation in ovarian cancer by targeting XIAP [37]. miR-CHA1 directly regulated the XIAP network, suppressing cell proliferation and promoting apoptosis in A549 cell lines.

Knockdown of XIAP repressed lung cancer development and promoted apoptosis [38]. XIAP silencing activated the cleavage cascade of caspase-3 or -9 that promoted PARP signaling in NSCLC [39]. Overexpression of miR-CHA1 directly suppressed XIAP expression, resulting in cleaved caspase-3 and PARP. miR-CHA1 inhibited cell proliferation and slightly increased apoptosis in this study. As shown in Fig. 4b, miR-CHA1 and si-XIAP weakly promoted apoptosis in A549 cell lines. Silencing of XIAP by transfection of miR-CHA1 or si-XIAP arrested cell cycle of G1 phase that slightly induced apoptosis. Despite lower increasing of apoptosis, we study to regulation for XIAP network by miR-CHA1. XIAP may be a useful prognostic tool due to the significantly

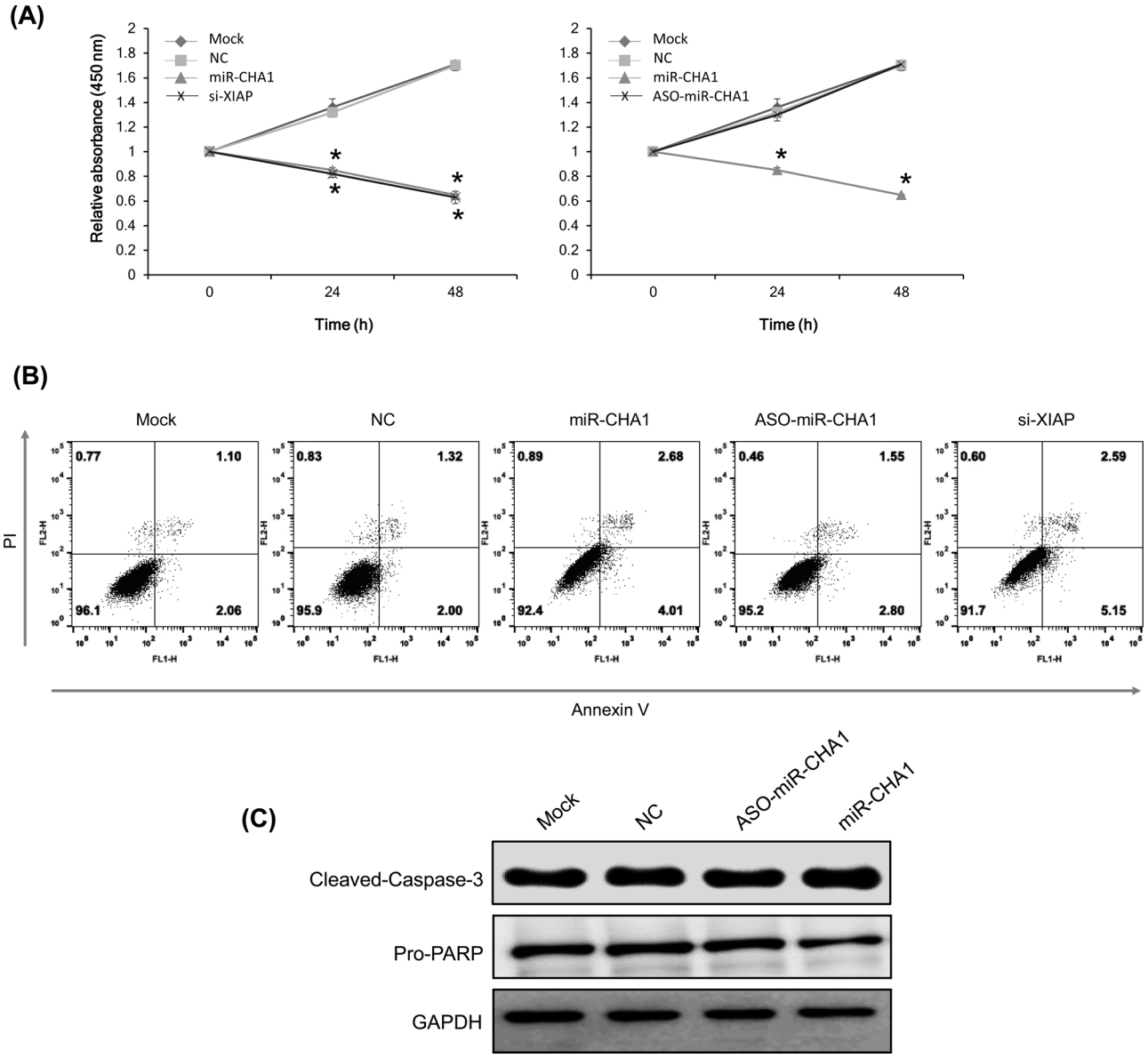


Fig. 4. miR-CHA1 suppressed cell proliferation, promoted apoptosis and regulated XIAP networks. (A) Cell proliferation was determined using XTT assay. (B) Annexin V/PI apoptosis detection using flow cytometry in NC, miR-CHA1, ASO-miR-CHA1, or si-XIAP transfected cells. (C) Western blot analysis of pathways downstream of XIAP associated with apoptosis signaling. The data represent the average of three independent experiments and are shown as the mean ± S.E.M.; *P < 0.05.

increased XIAP expression in NSCLC [40]. Likewise, Silencing XIAP alone did not strongly increase apoptosis but treatment with co-factors such as Apo2L, TRAIL, FasL or other specific compounds, significantly promoted apoptosis *in vitro* [39,41,42]. XIAP is a potential target for anticancer therapy, as its expression level is upregulated in various cancer including breast cancer, melanoma, and clear-cell renal cell carcinoma [43].

In tumorigenesis, XIAP has an anti-apoptotic ability that regulates cell survival and proliferation [44]. In chemotherapeutic treatment, XIAP blocked therapeutic effects that suppressed caspase activation in various malignant cells [45]. In spite of the resistance to chemotherapy by XIAP activation, multiple studies demonstrated potential therapeutics targeting XIAP in malignant cells, including small molecules or antisense oligonucleotides [5,10,44,45], as XIAP silencing does not affect normal cells [46]. Until now, an *in vivo* xenograft model of an miRNA targeting XIAP has not been reported. We performed that experiment, and assumed miR-CHA1 would regulate cell proliferation and apoptosis in mouse xenograft models through silencing XIAP. miR-CHA1 inhibited cell proliferation and induced apoptosis compared with PBS, NC or ASO-miR-CHA1 injection. In summary, miR-CHA1 negatively regulated tumorigenesis in NSCLCs by promoting apoptosis through direct suppression of XIAP networks.

In conclusion, we identified and characterized a novel miRNA, hsa-miR-CHA, that was downregulated in human lung cancer cell lines and tissues. miR-CHA1 significantly repressed XIAP mRNA and protein levels. The XIAP network modulated the apoptotic status by the presence or absence of miR-CHA1. Silencing the XIAP network suppressed cell proliferation and promoted apoptosis *in vitro* and *in vivo*. These results demonstrated that miR-CHA1 correlates with lung carcinogenesis and might provide diagnosis or therapeutic agents for lung cancer therapy.

Conflict of interest

The authors declare no conflict of interest.

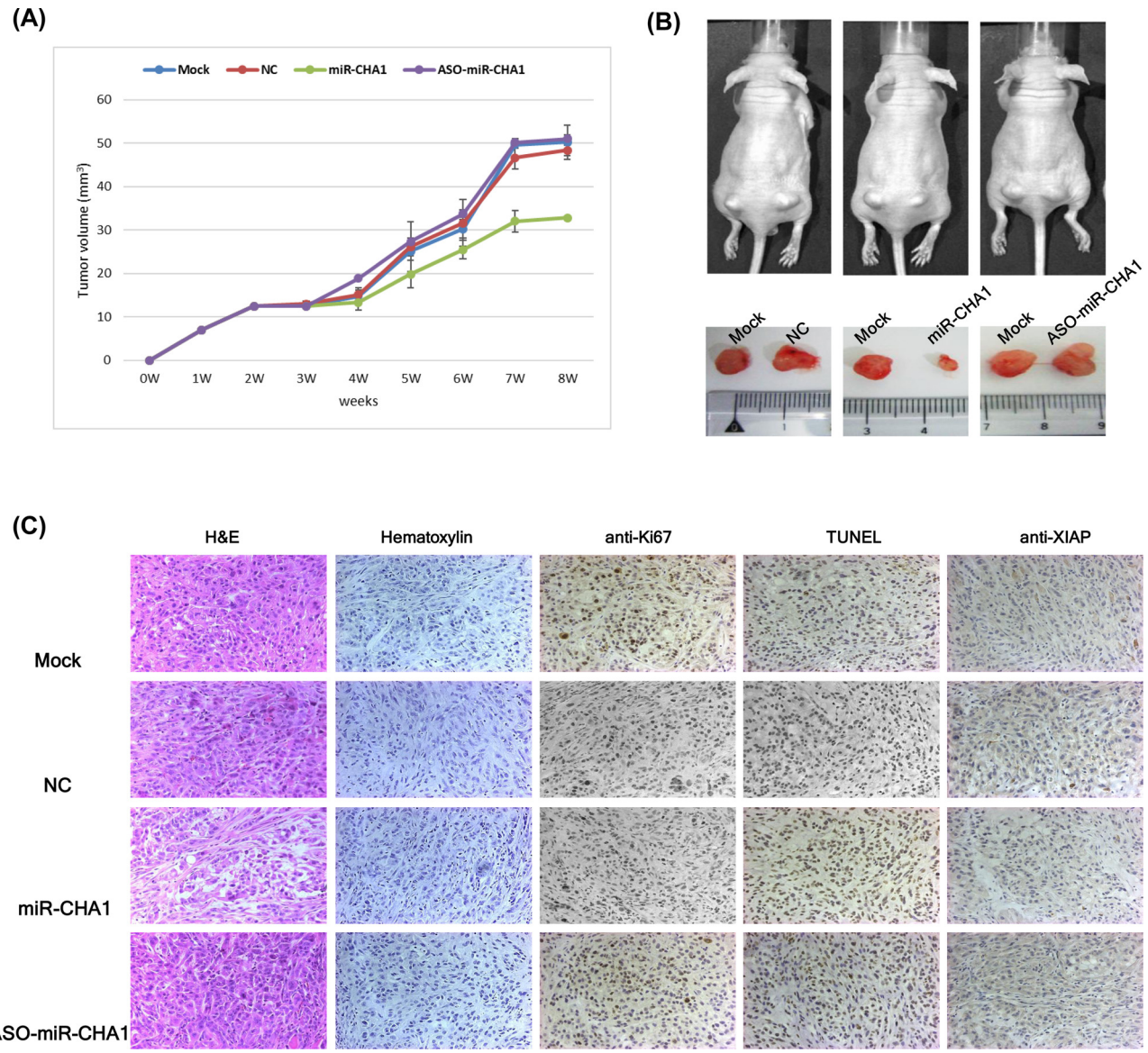


Fig. 5. miR-CHA1 affects tumor formation by inhibiting proliferation and inducing apoptosis *in vivo*. A549 cells were injected s.c. into both thighs of all mice. After 2 weeks, the left thigh was injected with PBS, whereas the right thigh was injected with either NC, miR-CHA1, or ASO-miR-CHA1. (A) Tumor size was measured every week. (B) After 6 weeks, the mice were photographed and sacrificed. (C) H&E staining, Ki67 and XIAP IHC, and TUNEL apoptosis assay in the tumor formed by A549 cells that were directly injected s.c. with PBS, NC, miR-CHA1, or ASO-miR-CHA1.

Acknowledgements

This research was supported by Basic Science Research Program, Mid-career Research Program and the Bio & Medical Technology Development Program through the National Research Foundation of Korea (NRF) grant funded by Korea government (MSIP) (No. 2016R1C1B1013723, No. 2016R1A2B4008291, No. 2015M3A9C6028961) of the Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.lungcan.2018.04.011.

References

[1] R.C. Lee, R.L. Feinbaum, V. Ambros, C. The, elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell 75 (1993) 843–854.

[2] A.M. Gurtan, P.A. Sharp, The role of miRNAs in regulating gene expression networks, J. Mol. Biol. 425 (2013) 3582–3600.

[3] V. Ambros, The functions of animal microRNAs, Nature 431 (2004) 350–355.

[4] P. Gao, I. Tchernyshyov, T.C. Chang, Y.S. Lee, K. Kita, T. Ochi, K.I. Zeller, A.M. De Marzo, J.E. Van Eyk, J.T. Mendell, C.V. Dang, c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism, Nature 458 (2009) 762–765.

[5] J. Visvanathan, S. Lee, B. Lee, J.W. Lee, S.K. Lee, The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development, Genes Dev. 21 (2007) 744–749.

[6] I. Martinez, D. Cazalla, L.L. Almstead, J.A. Steitz, D. DiMaio, miR-29 and miR-30 regulate B-Myb expression during cellular senescence, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 522–527.

[7] C.D. Johnson, A. Esquela-Kerscher, G. Stefani, M. Byrom, K. Kelnar, D. Ovcharenko, M. Wilson, X. Wang, J. Shelton, J. Shingara, L. Chin, D. Brown, F.J. Slack, The let-7 microRNA represses cell proliferation pathways in human cells, Cancer Res. 67 (2007) 7713–7722.

[8] H. Hermeking, The miR-34 family in cancer and apoptosis, Cell Death Differ. 17 (2010) 193–199.

[9] W.D. Travis, World Health Organization, International Agency for Research on Cancer, International Association for the Study of Lung Cancer, International Academy of Pathology, Pathology and Genetics of Tumours of the Lung, Pleura, Thymus and Heart, IARC Press Oxford University Press (distributor), Lyon Oxford, 2004.

[10] S. Singhal, A. Vachani, D. Antin-Ozerkis, L.R. Kaiser, S.M. Albelda, Prognostic implications of cell cycle, apoptosis, and angiogenesis biomarkers in non-small cell lung cancer: a review, Clin. Cancer Res. 11 (2005) 3974–3986.
[11] E.Y. Lee, W.J. Muller, Oncogenes and tumor suppressor genes, Cold Spring Harb. Perspect. Biol. 2 (2010) a003236.
[12] P.Y. Lin, S.L. Yu, P.C. Yang, MicroRNA in lung cancer, Br. J. Cancer 103 (2010) 1144–1148.
[13] 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. U. S. A. 101 (2004) 2999–3004.
[14] J.K. Yoo, H.Y. Jung, J.M. Lee, H. Yi, S.H. Oh, H.Y. Ko, H. Yoo, H.R. Kim, H. Song, S. Kim, J.K. Kim, The novel miR-9500 regulates the proliferation and migration of human lung cancer cells by targeting Akt1, Cell Death Differ. 21 (2014) 1150–1159.
[15] H. Matsubara, T. Takeuchi, E. Nishikawa, K. Yanagisawa, Y. Hayashita, H. Ebi, H. Yamada, M. Suzuki, M. Nagino, Y. Nimura, H. Osada, T. Takahashi, Apoptosis induction by antisense oligonucleotides against miR-17-5p and miR-20a in lung cancers overexpressing miR-17-92, Oncogene 26 (2007) 6099–6105.
[16] J. Cai, L. Fang, Y. Huang, R. Li, J. Yuan, Y. Yang, X. Zhu, B. Chen, J. Wu, M. Li, miR-205 targets PTEN and PHLPP2 to augment AKT signaling and drive malignant phenotypes in non-small cell lung cancer, Cancer Res. 73 (2013) 5402–5415.
[17] S. Elmore, Apoptosis: a review of programmed cell death, Toxicol. Pathol. 35 (2007) 495–516.
[18] A.M. Hunter, E.C. LaCasse, R.G. Korneluk, The inhibitors of apoptosis (IAPs) as cancer targets, Apoptosis 12 (2007) 1543–1568.
[19] S. Feng, S. Cong, X. Zhang, X. Bao, W. Wang, H. Li, Z. Wang, G. Wang, J. Xu, B. Du, D. Qu, W. Xiong, M. Yin, X. Ren, F. Wang, J. He, B. Zhang, MicroRNA-192 targeting retinoblastoma 1 inhibits cell proliferation and induces cell apoptosis in lung cancer cells, Nucleic Acids Res. 39 (2011) 6669–6678.
[20] C. Li, H. Nie, M. Wang, L. Su, J. Li, B. Yu, M. Wei, J. Ju, Y. Yu, M. Yan, Q. Gu, Z. Zhu, B. Liu, MicroRNA-409-3p regulates cell proliferation and apoptosis by targeting PHF10 in gastric cancer, Cancer Lett. 320 (2012) 189–197.
[21] J.K. Yoo, J. Kim, S.J. Choi, H.M. Noh, Y.D. Kwon, H. Yoo, H.S. Yi, H.M. Chung, J.K. Kim, Discovery and characterization of novel microRNAs during endothelial differentiation of human embryonic stem cells, Stem Cells Dev. 21 (2012) 2049–2057.
[22] J.M. Lee, J.K. Yoo, H. Yoo, H.Y. Jung, D.R. Lee, H.C. Jeong, S.H. Oh, H.M. Chung, J.K. Kim, The novel miR-7515 decreases the proliferation and migration of human lung cancer cells by targeting c-Met, Mol. Cancer Res. 11 (2013) 43–53.
[23] H.M. Jung, S.J. Choi, J.K. Kim, Expression profiles of SV40-immortalization-associated genes upregulated in various human cancers, J. Cell. Biochem. 106 (2009) 703–713.
[24] M. Azrad, K. Zhang, R.T. Vollmer, J. Madden, T.J. Polascik, D.C. Snyder, M.T. Ruffin, J.W. Moul, D. Brenner, R.W. Hardy, W. Demark-Wahnefried, Prostatic alpha-linolenic acid (ALA) is positively associated with aggressive prostate cancer: a relationship which may depend on genetic variation in ALA metabolism, PLoS One 7 (2012) e53104.
[25] M. Spitzner, J.R. Martins, R.B. Soria, J. Ousingsawat, K. Scheidt, R. Schreiber, K. Kunzelmann, Eag1 and Bestrophin 1 are up-regulated in fast-growing colonic cancer cells, J. Biol. Chem. 283 (2008) 7421–7428.
[26] F. Micci, I. Panagopoulos, L. Haugom, H.S. Dahlback, M.E. Pretorius, B. Davidson, V.M. Abeler, C.G. Trope, H.E. Danielsen, S. Heim, Genomic aberration patterns and expression profiles of squamous cell carcinomas of the vulva, Genes Chromosomes Cancer 52 (2013) 551–563.
[27] E. Dehan, A. Ben-Dor, W. Liao, D. Lipson, H. Frimer, S. Rienstein, D. Simansky, M. Krupsky, P. Yaron, E. Friedman, G. Rechavi, M. Perlman, A. Aviram-Goldring, S. Izraeli, M. Bittner, Z. Yakhini, N. Kaminski, Chromosomal aberrations and gene expression profiles in non-small cell lung cancer, Lung Cancer 56 (2007) 175–184.
[28] N. Rajewsky, microRNA target predictions in animals, Nat. Genet. 38 (Suppl) (2006) S8–S13.
[29] H. Yoo, J.K. Yoo, J. Lee, D.R. Lee, J.J. Ko, S.H. Oh, Y.K. Choo, J.K. Kim, The hsa-miR-5787 represses cellular growth by targeting eukaryotic translation initiation factor 5 (eIF5) in fibroblasts, Biochem. Biophys. Res. Commun. 415 (2011) 567–572.
[30] S. Vatolin, K. Navaratne, R.J. Weil, A novel method to detect functional microRNA targets, J. Mol. Biol. 358 (2006) 983–996.
[31] S.M. Srinivasula, J.D. Ashwell, IAPs: what's in a name, Mol. Cell 30 (2008) 123–135.
[32] E. Krepela, P. Dankova, E. Moravcikova, A. Krepelova, J. Prochazka, J. Cermak, J. Schutzner, P. Zatloukal, K. Benkova, Increased expression of inhibitor of apoptosis proteins, survivin and XIAP, in non-small cell lung carcinoma, Int. J. Oncol. 35 (2009) 1449–1462.
[33] G. Ambrosini, C. Adida, D.C. Altieri, A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma, Nat. Med. 3 (1997) 917–921.
[34] S. Liu, P. Zhang, Z. Chen, M. Liu, X. Li, H. Tang, MicroRNA-7 downregulates XIAP expression to suppress cell growth and promote apoptosis in cervical cancer cells, FEBS Lett. 587 (2013) 2247–2253.
[35] S. Xiong, Y. Zheng, P. Jiang, R. Liu, X. Liu, Y. Chu, MicroRNA-7 inhibits the growth of human non-small cell lung cancer A549 cells through targeting BCL-2, Int. J. Biol. Sci. 7 (2011) 805–814.
[36] Y. Xie, L.A. Tobin, J. Camps, D. Wangsa, J. Yang, M. Rao, E. Witasp, K.S. Awad, N. Yoo, T. Ried, K.F. Kwong, MicroRNA-24 regulates XIAP to reduce the apoptosis threshold in cancer cells, Oncogene 32 (2013) 2442–2451.
[37] Y. Pang, H. Mao, L. Shen, Z. Zhao, R. Liu, P. Liu, MiR-519d represses ovarian cancer cell proliferation and enhances cisplatin-mediated cytotoxicity in vitro by targeting XIAP, Onco Targets Ther. 7 (2014) 587–597.
[38] M. Ye, J. Zhang, Q. Miao, L. Yao, Curcumin promotes apoptosis by activating the p53-miR-192-5p/215-XIAP pathway in non-small cell lung cancer, Cancer Lett ***357***. (2014, 196-205).
[39] M. Chawla-Sarkar, S.I. Bae, F.J. Reu, B.S. Jacobs, D.J. Lindner, E.C. Borden, Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis, Cell Death Differ. 11 (2004) 915–923.
[40] C.G. Ferreira, P. van der Valk, S.W. Span, I. Ludwig, E.F. Smit, F.A. Kruyt, H.M. Pinedo, H. van Tinteren, G. Giaccone, Expression of X-linked inhibitor of apoptosis as a novel prognostic marker in radically resected non-small cell lung cancer patients, Clin. Cancer Res. 7 (2001) 2468–2474.
[41] A.V. Paschall, M.A. Zimmerman, C.M. Torres, D. Yang, M.R. Chen, X. Li, E. Bieberich, A. Bai, J. Bielawski, A. Bielawska, K. Liu, Ceramide targets xIAP and cIAP1 to sensitize metastatic colon and breast cancer cells to apoptosis induction to suppress tumor progression, BMC Cancer 14 (2014) 24.
[42] E.J. Dean, T. Ward, C. Pinilla, R. Houghten, K. Welsh, G. Makin, M. Ranson, C. Dive, A small molecule inhibitor of XIAP induces apoptosis and synergises with vinorelbine and cisplatin in NSCLC, Br. J. Cancer 102 (2010) 97–103.
[43] M.C. de Almagro, D. Vucic, The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy, Exp. Oncol. 34 (2012) 200–211.
[44] A.J. Schile, M. Garcia-Fernandez, H. Steller, Regulation of apoptosis by XIAP ubiquitin-ligase activity, Genes Dev. 22 (2008) 2256–2266.
[45] A.D. Schimmer, S. Dalili, R.A. Batey, S.J. Riedl, Targeting XIAP for the treatment of malignancy, Cell Death Differ. 13 (2006) 179–188.
[46] H. Harlin, S.B. Reffey, C.S. Duckett, T. Lindsten, C.B. Thompson, Characterization of XIAP-deficient mice, Mol. Cell. Biol. 21 (2001) 3604–3608.