Cloning and characterization of the 5' flanking region of microRNA let-7a-1/let-7f-1 gene cluster in human lung cancer cell

Jian Zhao2#, Nana Ni1#, Chang Liu3, Pengju Zhang1, Yang Yu1, Zhaobo Chen1, Weiwen Chen1* and Anli Jiang1

1Institute of Biochemistry and Molecular Biology, School of Medicine, Shandong University, 44 Wenhuaixi Road, Jinan, Shandong 250012, China.
2Department of Thoracic surgery, Qilu Hospital, Shandong University, Jinan, Shandong 250012, China.
3R&D department, Shandong Freda Biopharm CO., LTD, Jinan, Shandong 250014, China.

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In order to elucidate the molecular basis of microRNA let-7a-1/let-7f-1 gene cluster, the transcription initiation site which was determined by 5' rapid amplification of cDNA ends (5'RACE) and 2.1 kb of the 5' flanking region proximal to the pre-let-7a-1 was isolated and characterized. The promoter activity of the 2.1 kb fragment was analyzed by a firefly luciferase-encoding gene expression vector (pGL3) transiently transfected into lung cancer cell line A549. The 2.1 kb promoter of let-7a-1/let-7f-1 displayed a lower activity and was significantly enhanced by ectopic expression of c/EBPα or p53 and treatment with dexamethasone. Despite the induction of other let-7 family members such as let-7a-3, let-7c and let-7d, all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9cRA) display little enhancement effect on 2.1 kb promoter of let-7a-1/let-7f-1, as well as 1,25-(OH)2D3.

Key words: let-7a-1; let-7f-1, 5' rapid amplification of cDNA ends (5'RACE), promoter, lung cancer.

INTRODUCTION

MicroRNAs (miRNAs) are non-coding small RNAs found in diverse organisms (Chen, 2005; Hammond, 2006). They are encoded in long primary forms in the nucleus (pri-miR). The pri-miRs will be transported into the cytoplasm after being processed to 70 to 80 nucleotide pre-miR with a characteristic hairpin structure. The pre-miR are then processed by Dicer into the 22 to 25 nt mature forms (Lee et al., 2002). miRNAs mediate gene down-regulation by targeting mRNAs to induce RNA degradation and/or interfering with translation and thus, play key roles in regulating a wide array of cell functions (Ambros, 2003). Concordant with this, aberrant expression of miRNA genes could lead to human disease. In recent years, many reports have shown that miRNAs regulate cell proliferation and apoptosis and play a role in cancer: microRNAs can act as oncogenes or tumor suppressors (Chen, 2005; Hammond, 2006).

There are accumulating evidences that let-7, the first discovered miRNA, is tumor suppressor. A number of studies have demonstrated that let-7 was implicated in various cancers, particularly in lung cancer (Eder and Scherr, 2005; Lu et al., 2007; Sampson et al., 2007; Motoyama et al., 2008). Takamizawa et al. (2004) first reported that the expression of the let-7 microRNA was reduced in human lung cancers and overexpression of let-7 in A549 lung adenocarcinoma cell line inhibited lung cancer cell growth in vitro. Next, Johnson et al. (2005) identified that ras is one of the target genes of let-7 family. Lee and Dutta (2007) also found that let-7 repressed the HMGA2 oncogene in human lung cancer cells. Therefore, let-7 is tumor suppressor miRNA and maybe a potential target for lung cancer therapy. Despite
the method of rapid isolation of mammalian DNA. The primer pair PF (5'-CCGCTGAGACCCACGCTATCTCAGTTTCT-3'; with an Xho I site at its 5' end) and PR (5'-CCCAGCTTACGTTGAGAGAAGACATCAGGG-3'; with a Hind III site at its 5' end) were used to amplify the 5' flanking region of the let-7a-1/let-7f-1 gene cluster from the extracted human genomic DNA by PCR. The PCR-amplified fragment was 2123 bp (~1999 to +124 bp) and it was excised with Xho I and Hind III (TakaRa) and ligated into the equivalent site of the pGL3-Basic vector (Promega, Madison WI, USA) to form the let-7a-1/let-7f-1 cluster promoter-luciferase reporter construct, designated pGL3-2123. The resulting construct was confirmed by restriction enzyme digestion and sequence analysis using the general primers Rp primer 3 and GL primer 2.

### Transient transfection

A549 cells in 70 to 80% of confluence were transfected with Fugene HD (Roche) in 24-well plates. Each well contained 0.5 µg pGL3-2123, 0.02 µg of the internal control vector pRL-TK, 1 µl Fugene HD and 500 µl F-12K medium without serum or antibiotics. For co-transfection experiments, 0.3 µg pGL3-2123 was transfected along with 0.2 µg of one of the eukaryotic expression plasmids pCMV-EGFP (Clontech, Palo Alto, CA), pcDNA3.1-Sp1 and pcDNA3.1-NFκBp65 (gifts from Dr. Charles Young, Mayo clinic, USA), pcDNA3.1-PPARγ2, pcDNA3.1-c-EBPα (gifts from Dr. Jianhua Shao, University of Kentucky, USA), pBABEpuromycin and pBABEhygro-myc (gifts from Dr. Weijing He, University of Texas, USA). All the cells underwent the dual-luciferase reporter assay 48 h after the completion of the transfection procedure, following the protocol recommended by Promega.

### Treatment of the transfected cells with hormones

The stocks of all-trans retinoic acid (ATRA), 9-cis-retinoic acid (9cRA) and 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) were prepared in dimethylsulfoxide (DMSO); the stock of dexamethasone (DEX) were prepared in ethanol. All of the hormones were purchased from Sigma (St. Louis, MO, USA). After the transfection of pGL3-2123 in 24-well plates, the cells were treated for 48 h with 10-6-10-8 M of ATRA, 9cRA, 1,25(OH)2D3 or DEX in 10% FBS-F-12K medium, respectively. The controls received the DMSO or ethanol vehicle at a concentration equal to that of the treated cells. All the cells underwent the dual-luciferase reporter assay 48 h after the completion of the treatment procedure.

### Dual-luciferase reporter assay

The activities of firefly luciferase in pGL3 and Renilla luciferase in pRL-TK (Promega) were determined following the dual-luciferase reporter assay protocol recommended by Promega. The cells were rinsed with PBS after harvest and cell lysates were prepared by manually scraping the cells from the culture plates in the presence of 1×PLB (passive lysis buffer). 20 µl of cell lysate was transferred into luminometer tubes containing 100 µl LAR. Firefly luciferase activity (M1) was measured first and then Renilla luciferase activity (M2) was measured after the addition of 100 µl of Stop and Glo Reagent.

### Statistical analysis

Data are expressed as mean ± SD of at least three independent experiments. Statistical significance of differences between groups...
Figure 1. Map the 5' end of primary transcript of the let-7a-1/let-7f-1 cluster. The transcription start site detected by 5' RACE is boxed and numbered +1. The primers used for 5'RACE (SP1-3) are shaded in gray. The lines and arrows indicate the pre-miR sequence of let-7a-1 (80nt) and let-7f-1 (87nt).

Figure 2. Activity assay of pGL3-2123 in A549 cells. pGL3-2123 was transfected into A549 cells along with a negative control pGL3-Basic and a positive control pGL3-Control. The promoter activities were determined via the dual-luciferase reporter assay. The results are expressed as the relative luciferase activities (M1/M2), that is, the ratio of firefly luciferase activity (M1) in the pGL3 plasmid and Renilla luciferase activity (M2) in the pRL-TK plasmid.
RESULTS AND DISCUSSION

Mapping the transcription start site of let-7a-1 let-7f-1 gene cluster

Many evidences have indicated that microRNA genes are transcribed by RNA polymerase II (Tam, 2001; Johnson et al., 2003; Xie et al., 2005) and a handful of miRNA promoters in Homo sapiens have been identified so far (Lee et al., 2004; Houbaiviy et al., 2005). According to MiRBase data, the hsa-let-7 family has 11 members that distribute on different chromosomes. Searching result from Entrez gene indicate that the let-7a-1 and let-7f-1 gene is an intergenic cluster located within chromosome 9q22.32 (Gene ID: 406881) (Figure 1). To determine the transcription initiation site of let-7a-1/let-7f-1 cluster, 5’RACE was performed using RNA isolated from A549 lung cancer cell line (Takamizawa et al., 2004), suggesting the inhibition of the reduced expression of endogenous let-7a-1/let-7f-1 (Li et al., 2001) or hormones (Lee et al., 2002). Computer-based analysis of this 2.1 kb 5’-flanking fragment sequence with MatInspector 2.0 software (http://www.genomatix.de), three putative TATA-boxes (-414/-398, -366/-344 and -110/-94) and two CCAAT boxes (-118/-104 and -314/-300) were searched within the -1000/+1 region. In addition, more than 700 other putative transcription factor binding sites (Matrix similarity > 0.75) were searched by MatInspector within the 2.1 kb fragment. Because let-7a-1/let-7f-1 cluster is tumor suppressor miRNAs, we are interested in the tumor-associated transcription factor binding sites. The computer search result displayed four c/EBPα sites (-1726/-1712, -1683/-1669, -722/-708 and -163/-149), two PPAR sites (-1434/-1412 and -533/-511), one NF-kB site (-1255/-1243), two Sp1 sites (-1953/-1933 and -1008/-988) and two P53 sites (-1170/-1148 and -1159/-1137). In order to investigate whether the putative cis-elements mentioned earlier have functions, the eukaryotic expression plasmids of c/EBPα, PPARγ2, NF-kB p50, Sp1 and p53, were co-transfected with pGL3-2123 into A549 cells and the corresponding empty eukaryotic expression vectors of themselves were co-transfected with pGL3-2123 into parallel wells as a control. In addition, because the oncogenes ras and myc are targets of let-7 (Boominathan, 2010), the co-transfection of ras/myc expression vector with pGL3-2123 were also performed to test whether the let-7 and ras/myc share a feedback loop. The relative luciferase activities from pGL3-2123 co-transfected with eukaryotic expression plasmids was divided by the relative luciferase activity from pGL3-2123 co-transfected with control vector from the same cell line and plotted in Figure 3. Our results indicate that ectopic expression of PPARγ2, NF-KappaB, Sp1, ras and myc have no significant effect on pGL3-2123 promoter activity, whereas c/EBPα and p53 increases the promoter activity of pGL3-2123 about 3.6-fold and 2.0-fold, respectively.

c/EBPα, one member of the c/EBF family, is a gene thought to be a tumor suppressor (Timchenko et al., 1996; Lekstrom-Himes and Xanthopoulos, 1998; Pabst et al., 2001). As a transcription factor, c/EBPα is discovered controlling the transcription of some tumor suppressor microRNA. Recently, the c/EBPα is reported to directly interacted with the miR-122 (hepatocyte-specific microRNA) promoter and transactivate it (Zeng et al., 2010). Studies in acute myeloid leukemia (AML) have shown that mir-34a and mir-223 were transcriptional targets of c/EBPα (Pulikkan et al., 2010, 2010). In A549 and H1299 lung cancer cells, the ectopic c/EBPα expression increases expression of mir-1 6.1-fold and 4.92-fold, respectively (Nasser et al., 2008). mir-661, which can inhibit metastatic tumor antigen 1, is another c/EBPα target (Reddy et al., 2009). In our experiment, the expression of ectopic c/EBPα significantly increase the promoter activity of let-7a-1/let-7f-1 cluster (3.6-fold) in A549 cells with little endogenous c/EBPα expression (Li

Cloning and activity assay of the 2.1 kb promoter fragment of let-7a-1/let-7f-1 cluster

Based on the result of 5’RACE, starting 2.1 kb upstream of pre-let-7a-1, a genomic fragment including positions -1999 to +124 relative to the transcription start site was cloned into the pGL3-Basic to form pGL3-2123, which was confirmed by DNA sequencing. To evaluate the promoter activity, the pGL3-2123 was transfected into lung cancer cell line A549, pGL3-Control and pGL3-Basic were also introduced into parallel wells, respectively, as positive and negative controls. The luciferase activity was assayed and normalized to pRL-TK (Figure 2). After 48 h of transfection into A549 cells, pGL3-2123 yielded a result of 5.24 by dual-luciferase reporter assay (M1/M2). Comparing with respective 38.76 of pGL3-Control and 0.26 of pGL3-Basic, the 2.1 kb promoter of let-7a-1/let-7f-1 cluster display a lower activity in A549 cells, which was consistent with the fact that A549 cell line has the reduced expression of endogenous let-7a-1/let-7f-1 (Takamizawa et al., 2004), suggesting the inhibition of promoter may be one of the reasons for reduced expression of let-7a-1/let-7f-1 in lung cancer cells.

The effect of some tumor-associated transcription factors on promoter activity of pGL3-2123

As class-II genes, expressions of many microRNAs are also regulated by enhancers (Johnson et al., 2003; Lagos-Quintana et al., 2001). Computer-based analysis of this 2.1 kb 5’-flanking fragment sequence with MatInspector 2.0 software (http://www.genomatix.de), three putative TATA-boxes (-414/-398, -366/-344 and -110/-94) and two CCAAT boxes (-118/-104 and -314/-300) were searched within the -1000/+1 region. In addition, more than 700 other putative transcription factor binding sites (Matrix similarity > 0.75) were searched by MatInspector within the 2.1 kb fragment. Because let-7a-1/let-7f-1 cluster is tumor suppressor miRNAs, we are interested in the tumor-associated transcription factor binding sites. The computer search result displayed four c/EBPα sites (-1726/-1712, -1683/-1669, -722/-708 and -163/-149), two PPAR sites (-1434/-1412 and -533/-511), one NF-kB site (-1255/-1243), two Sp1 sites (-1953/-1933 and -1008/-988) and two P53 sites (-1170/-1148 and -1159/-1137). In order to investigate whether the putative cis-elements mentioned earlier have functions, the eukaryotic expression plasmids of c/EBPα, PPARγ2, NF-kB p50, Sp1 and p53, were co-transfected with pGL3-2123 into A549 cells and the corresponding empty eukaryotic expression vectors of themselves were co-transfected with pGL3-2123 into parallel wells as a control. In addition, because the oncogenes ras and myc are targets of let-7 (Boominathan, 2010), the co-transfection of ras/myc expression vector with pGL3-2123 were also performed to test whether the let-7 and ras/myc share a feedback loop. The relative luciferase activities from pGL3-2123 co-transfected with eukaryotic expression plasmids was divided by the relative luciferase activity from pGL3-2123 co-transfected with control vector from the same cell line and plotted in Figure 3. Our results indicate that ectopic expression of PPARγ2, NF-KappaB, Sp1, ras and myc have no significant effect on pGL3-2123 promoter activity, whereas c/EBPα and p53 increases the promoter activity of pGL3-2123 about 3.6-fold and 2.0-fold, respectively.

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Figure 3. The effects of some transcription factors on promoter activities of let-7a-1/let-7f-1 cluster. A549 cells were co-transfected with pGL3-2123 and eukaryotic expression plasmids of c/EBPα, PPARγ, NF-κB p50, Sp1, p53, ras or myc. The control cells were co-transfected with pGL3-2123 and corresponding empty eukaryotic expression plasmids. All the cells were harvested for the dual-luciferase reporter assay after 48 h of transfection. The results were expressed as ratio of relative activity, that is, the M1/M2 value from pGL3-2123 co-transfected with eukaryotic expression plasmids was divided by the M1/M2 value from controls. **P < 0.01, compared with the control.

et al., 1995), suggesting that the let-7a-1/let-7f-1 cluster is a new transcriptional target of c/EBPα.

p53 is a broad-spectrum tumor suppressor gene that plays very important roles in various cancers. The P53 protein functions as transcription factor to transactivate expression of a number of target genes to promote tumor suppression and genome integrity. Recently, a number of studies have shown that many miRNAs are also members of p53-network. For example, mir-34, mir-192/215, mir-107 and mir-145, all of which function as tumor suppressors and play a key role in control of tumor progression, angiogenesis and metastasis, are shown to be direct transcriptional targets of p53 (Hermeking, 2007; Tarasov et al., 2007; Boominathan, 2010). Besides mir-34a, Tarasov et al. (2007) also observed that activation of p53 in lung cancer cells results in increased expression of mature-let-7c (2.7-fold), mature-let-7e (2.1-fold) and mature-let-7a (1.9-fold). Our results further suggest that p53 increase the expression of let-7a-1/let-7f-1 at transcriptional level, but maybe due to the endogenous expression of wt p53 by A549 cells (Jia et al., 1997), the ectopic expression of p53 had a weaker increase effect on let-7a-1/let-7f-1 promoter than that of c/EBPα.

The effects of hormones on promoter activity of let-7a-1/let-7f-1 cluster

Besides the transcription factors mentioned earlier, two RAR-RXR heterodimer binding sites (-46/-22 and +90/+114), four VDR-RXR heterodimer binding sites (-1955/-1931, -1691/-1667, -1600/-1576 and -1592/-1568), three glucocorticoid responsive and related elements (GRE) (-1877/-1859, -1588/-1561 and -1416/-1398) were detected by MatInspector 2.0 software. The RAR (retinoic acid receptor), RXR (retinoid X receptor) and VDR (Vitamin D3 receptor) belong to the nuclear receptor superfamily of ligand-inducible transcription (Stunnenberg, 1993). The RAR and RXR mediate the effect of retinoids signals such as all-trans-RA (ATRA) and 9-cis-RA (9cRA), which influence processes such as growth and differentiation by regulation of target gene expression at the cellular level (Gudas, 1994). Retinoids suppress carcinogenesis in diverse epithelial tissues including lung. Clinical trials have demonstrated the efficacy of retinoids in suppressing lung cancer (Aapro, 1995). Several lines of evidence have shown that treatment with retinoic acid not only down-regulate miRNAs that function as oncogenes, but also up-regulate tumor suppressor miRNAs including members of let-7 family (Garzon et al., 2007; Careccia et al., 2009; Weiss et al., 2009). For example, acute promyelocytic leukemia (APL) successfully treated with ATRA showed upregulation of let-7c (Careccia et al., 2009), as well as Garzon et al. (2007) found up-regulation of let-7a-3, let-7c and let-7d by miRNA microarrays and qRT-PCR in APL patients and cell lines during ATRA treatment. To explore whether the retinoic acids are implicated in
controlling expression of let-7a-1/let-7f-1 cluster, the A549 cells transfected with pGL3-2123 were treated with $10^{-8}$ to $10^{-6}$ M of 9cRA or ATRA for 48 h. As shown in Figure 4, the 2.1 kb promoter activity of let-7a-1/let-7f-1 cluster did not display significant increase after treatment with 9cRA as well as ATRA.

The VDR, upon activation by 1,25(OH)$_2$D$_3$, forms a heterodimer with the RXR and binds to corresponding hormone response elements on DNA resulting in expression of specific gene products (Stunnenberg, 1993). 1,25(OH)$_2$D$_3$ exerts important effects on cellular proliferation and differentiation and has been shown to decrease the growth of many cancers (Walters, 1992; Campbell et al., 1997). To date, a few studies have revealed the interaction between 1,25(OH)$_2$D$_3$ signal pathway and miRNAs (Mohri et al., 2009; Essa et al., 2010; Xi et al., 2010). In our study, 1,25(OH)$_2$D$_3$, like retinoic acids, have no significant effect on 2.1-kb promoter of let-7a-1/let-7f-1 cluster (Figure 5), suggesting the little possibility that the expression of let-
Figure 6. The effects of DEX on promoter activity of let-7a-1/let-7f-1 cluster. A549 cells transfected with pGL3-2123 were treated with $10^{-8}$M–$10^{-6}$ M of DEX for 48 h, as well as parallel wells transfected with pGL3-Basic as negative control. The cells were harvested for the dual-luciferase reporter assay to detect the effects of hormones on promoter activity of the let-7a-1/let-7f-1 cluster. The results were expressed as ratio of relative luciferase activities (M1/M2). **P < 0.01, compared with the control.

The let-7a-1/let-7f-1 cluster is regulated by retinoic acid or 1,25-(OH)$_2$D$_3$ at transcriptional level. Despite the 1,25-(OH)$_2$D$_3$ and retinoic acids, the glucocorticoid signal dexamethasone enhanced the promoter activity of let-7a-1/let-7f-1 1.91-fold, 2.38-fold and 2.54-fold at concentration of $10^{-8}$M, $10^{-7}$M and $10^{-6}$M, respectively (Figure 6). Dexamethasone, as a confirmed induction agent for apoptosis, has broad application in treating cancers (De Bosscher et al., 2000; Riccardi et al., 2000; Almawi et al., 2002). Dexamethasone act transcriptionally by binding the glucocorticoid receptor (GR) and subsequent binding to the promoter region of target genes on sites compatible with GRE motifs, which in turn directly or indirectly regulated gene expression (Almawi et al., 2002). Now the relationship between the glucocorticoid signal pathway and miRNAs has aroused the interest of investigators. For example, Liao and Lonnerdal (2010) found increase of mir-30e in dexamethasone-induced IEC-6 cells. Smith et al. (2010) studied the microRNA expression and processing during lymphocyte apoptosis induced by dexamethasone and found that mir-17-92 was repressed significantly (Smith et al., 2010). But so far, there is no evidence to support that dexamethasone directly activates the transcription through GRE within the promoter of microRNA. Thus, in our experiment, whether the dexamethasone enhance the promoter activity of let-7a-1/let-7f-1 by activate the GRE within its promoter need further work to confirm.

In summary, we determined the transcription start site of let-7a-1/let-7f-1 cluster and cloned its 2.1 kb 5' flanking fragment into the pGL3-Basic vector to form the let-7a-1/let-7f-1 promoter-luciferase reporter construct pGL3-2123, which represented lower promoter activity that consistent with the expression level of let-7a-1/let-7f-1 in A549 cells. Preliminary function analysis on pGL3-2123 revealed that this 2.1 kb promoter of let-7a-1/let-7f-1 was transactivated by ectopic expression of c/EBPα or p53. Treatment with 9-cis-retinoic acid, all-trans retinoic acid or 1,25-(OH)$_2$D$_3$ resulted in little induction of let-7a-1/let-7f-1 promoter, whereas $10^{-8}$–$10^{-6}$ M of dexamethasone significantly increase the promoter activity of let-7a-1/let-7f-1, further research should be done to identify the responsive functional cis-elements within the let-7a-1/let-7f-1 promoter and elucidate their regulatory mechanisms, thus, providing a foundation for understanding the transcription pattern of let-7a-1/let-7f-1 and their application in target therapy for lung cancer.

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