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Identification of methylation-dependent regulatory elements for intergenic miRNAs in human H4 cells

Kwang Hee Lee [a,c], Hyunyoung Kim [b], Byeong Jae Lee [c,d,*], Kiejung Park [a,*]

[a] Division of Bioinformatics and Bio-Medical Informatics, Center for Genome Science, National Institute of Health, Republic of Korea
[b] Division of Brain Disease, Center for Bio-Medical Science, National Institute of Health, Republic of Korea
[c] Laboratory of Molecular Genetics and Genomics, Interdisciplinary Program in Bioinformatics, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Republic of Korea
[d] School of Biological Sciences, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

MicroRNAs (miRNAs) are important post-transcriptional regulators of various biological processes. Although our knowledge of miRNA expression and regulation has been increased considerably in recent years, the regulatory elements for miRNA gene expression (especially for intergenic miRNAs) are not fully understood. In this study, we identified differentially methylated regions (DMRs) within 1000 bp upstream from the start site of intergenic miRNAs in human neuroglioma cells using microarrays. Then we identified a unique sequence pattern, $C[N]_6CT$, within the DMRs using motif analysis. Interestingly, treatment of cells with a methyl transferase inhibitor (5-aza-2-deoxycytidine, DAC) significantly increased expression of miRNA genes with a high frequency of the $C[N]_6CT$ motif in DMRs. Statistical analysis showed that the frequency of the $C[N]_6CT$ motif in DMRs is highly correlated with intergenic miRNA gene expression, suggesting that $C[N]_6CT$ motifs associated with DNA methylation regions play a role as regulatory elements for intergenic miRNA gene expression.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNA molecules that act as post-transcriptional regulators of gene expression by inhibiting translation or degrading mRNA genes through partial or complete base pairing with complementary sequences of target genes [1]. In addition, some miRNAs participate in the remodeling of chromatin structures [2]. miRNAs are initially transcribed as large precursor RNAs, or primary miRNAs (pri-miRNA), and sequentially processed by Drosha and Dicer to produce ~22-nucleotide-long active mature miRNAs [3–5]. miRNAs are highly conserved in multiple organisms and play crucial roles in development, cell differentiation, determination of cell fate, and cancer [6,7].

miRNA genes can be classified into two categories according to their genomic contexts: intronic and intergenic miRNAs. Intronic miRNAs are embedded within other genes. Therefore, they are thought to be transcribed by sharing promoters with host genes [8]. On the other hand, intergenic miRNAs are believed to have independent transcription units because they are positioned within flanking regions or in antisense orientation to annotated genes [9]. Intronic miRNAs are generally believed to be transcribed by RNA polymerase II (pol II); however, it remains unclear what type of RNA polymerase is responsible for intergenic miRNA transcription, although pol II and RNA polymerase III (pol III) are obvious candidates. For example, pri-miR-23a~27a~24-2 and pri-miR-21 are transcribed by pol II and have a 5′-7-methylguanosine cap structure and a 3′-polyadenylated [poly(A)] tail similar to the structure of mRNAs [10,11], while miR-517a and miR-517c, which are interspersed among Alu repeats in the human chromosome 19, are transcribed by pol III [12].

The transcriptional start site of intergenic miRNA genes usually occurs within 2 kb upstream from the start site of miRNAs [13]. Using computational methods, several conserved sequence patterns for intergenic miRNA genes, including putative promoters, have been proposed from various species [14,15]. Among these, CT repeats are most well known. They are highly conserved among four species, such as *Caenorhabditis elegans*, *Homo sapiens*, *Arabidopsis thaliana* and *Oryza sativa*, and are abundant within 1000 bp upstream sequences from miRNA hairpins [14]. Another sequence pattern, GANNNNGA, was identified within 1000 bp upstream of

Abbreviations: DMPs, differentially methylated probes; DMRs, differentially methylated regions; DAC, 5-aza-2-deoxycitidine; pol II, RNA polymerase II; pol III, RNA polymerase III; pri-miRNA, primary miRNA.

* Corresponding authors. Address: Division of Bioinformatics and Bio-Medical Informatics, Center for Genome Science, National Institute of Health, Building 2, Room 521, Osong 363-951, Republic of Korea. Fax: +82 43 719 8869 (K. Park).
E-mail address: kjpark63@gmail.com (K. Park).

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worm miRNAs [15]. However, there is no direct evidence that these conserved patterns play a role as promoter or regulatory elements. Currently, the transcriptional mechanisms of most intergenic miRNAs are largely unknown.

Epigenetic signatures such as DNA methylation and histone modification, and the regulation of expression of miRNA genes are tightly linked similarly to other genes [16–19]. Recently it was reported that hypermethylation of the human miR-124 loci, which is the most abundant miRNA in the adult brain and plays a key role in neurogenesis, inhibits miR124a expression and results in brain tumors [20–22]. Interestingly, some miRNAs control the expression of epigenetic regulators, including DNA methyltransferases and histone deacethylases [23,24]. The fact that miRNA gene expression can be regulated by DNA methylation indicates the feasibility of using methylated sequences to predict miRNA gene promoters or regulatory elements.

In this study, we found a novel sequence motif, $C[N]_6CT$, for intergenic miRNA gene expression by predicting sequence patterns in the differentially methylated regions (DMRs), and by examining the relationship between the occurrence of this motif and methylation dependence of gene expression.

2. Materials and methods

2.1. Cell lines and culture

H4 cells, a human neuroglioma cell line, were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% antibiotics-antimycotics (Invitrogen, Carlsbad, CA, USA) at 37 °C in a humidified incubator containing 5% CO_2.

2.2. Identification of miRNAs from sequence and annotation data

The genomic coordinates of 1049 human miRNAs were obtained from the miRBase (ver. 16.0) [25], and all sequences and the annotated data were from the UCSC genome browser (http://genome.ucsc.edu). A total of 1049 miRNAs were classified into 621 intronic and 428 intergenic miRNAs according their genome contexts.

2.3. Probe design

Sequences up to 1000 bp upstream from the start site of 428 intergenic miRNAs were retrieved and cleaved into 60-bp-long sequences overlapped by 40 bp of adjacent sequence (Fig. 1A). Chopped sequences were filtered based on sequence redundancy, low GC ratios (GC ratio < 0.6), and low melting temperatures (Tm < 85 °C). A total of 7646 sequences were selected as probes for printing on an Agilent 15K array platform to build a customized array chip (Chip No. 253347810001).

2.4. Microarray experiment

Genomic DNA was isolated from H4 cells cultured in the presence or absence of 5 μM DAC, an inhibitor of DNA methyltransferase. Briefly, after sonicating the genomic DNA (0.5 μg), the fragments were incubated with 2 μg recombinant methylation-specific binding protein (MBD2bt) at 4 °C for 4 h on a rocking platform. The enriched methylated DNA was amplified using a Whole Genome Amplification Kit (GenomePlex®, Sigma–Aldrich, St. Louis, MO, USA) as recommended by the manufacturer's instructions. The amplified DNA from DAC-treated and untreated cells were labeled with cyanine 5 (Cy5) and cyanine 3 (Cy3), respectively. The labeled DNA samples were purified using a PCR Purification Kit (QIAquick, Qiagen, Valencia, CA, USA) and co-hybridized to the customized microarrays according to the manufacturer's protocol. The microarrays contained a total of 7646 oligonucleotide probes, including control probes and those covering the sequences upstream of the miRNA genes.

2.5. Microarray data analysis

The hybridized images were analyzed using an Agilent DNA Microarray Scanner and data quantification was performed using Feature Extraction software version 10.7.3.1 (Agilent Technologies, Palo Alto, CA, USA). Preprocessing of raw data and normalization steps were performed using R software (http://www.r-project.org). Background-corrected intensity data were normalized using the intensity-dependent LOWESS method to remove the dye bias within each array. The *p*-values for each probe were calculated using linear fit models implemented in the Limma package (http://bioconductor.org/), and the probes within the threshold (p-value < 0.05) were selected as differentially methylated probes.

2.6. Reverse transcription PCR reaction

Total RNA was isolated using the RecoverAll, Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol. RNA quantity and purity was determined using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). All reactions were performed as per the manufacturer's protocol. *Rnu6b* was used as a negative control.

2.7. Quantitative real-time PCR

Quantitative real-time PCR was performed to amplify miRNAs with specific primer sets against target miRNAs (Applied Biosystems, Foster City, CA, USA) using the ABI-7500 Real Time PCR system according to the manufacturer's protocol. Template (10 ng) was amplified in 20 μl reaction volumes. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 50 cycles of 95 °C for 15 s, and 60 °C for 1 min. Experiments were performed in triplicate.

2.8. Motif analysis

MEME software [26] was used to search for top-ranking degenerate motifs within the probe sequences in each cluster, and its optional parameters were set as follows: optimum motif width was set to 8–12 bp, occurrence of motif in the input sequences was set to any number of repetitions in the input sequence, and other parameters were left as default. Alignment of DMR sequences was performed using ClustalX (ver. 2.0.12) software [27] with the default parameters.

3. Results

3.1. Identification of DMRs

To identify DMRs in the upstream of miRNA genes, microarray analysis was performed using our custom chips. A total of 7646 probes against the 5′-flanking region of 428 intergenic miRNA genes were designed (Fig. 1A) and implemented on the Agilent 15K array chip platform. Genomic DNA was isolated from H4 cells cultured in the absence or presence of 5-aza-2-deoxycytidine (DAC). Methylated sequences were enriched using MBD2bt

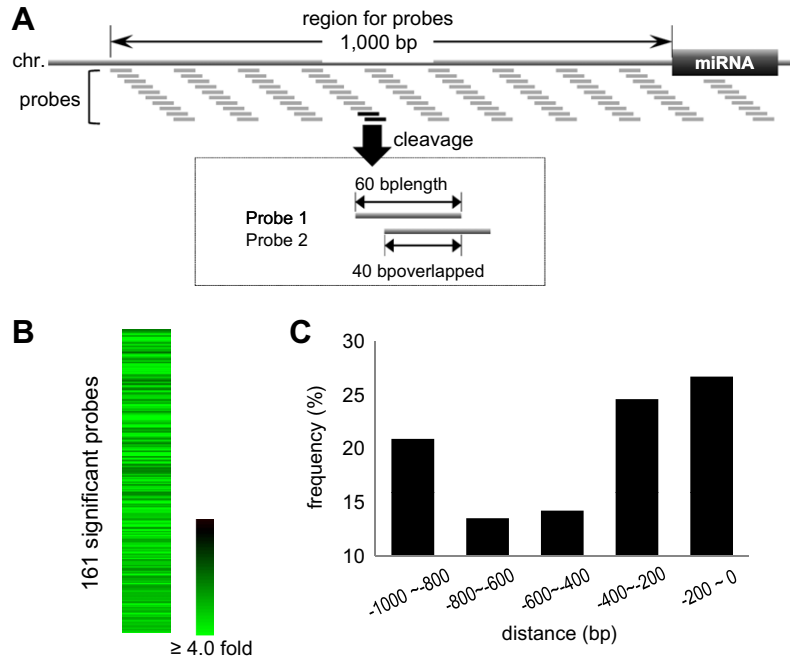


Fig. 1. Clustering and distribution of differentially methylated probes. (A) A schematic diagram of our custom designed probes. Probe sequences were retrieved from candidate regions and cleaved into 60-bp lengths with a 40-bp overlap with the adjacent probe. (B) Profiles of 161 differentially methylated probes (DMPs) identified from the 5′-flanking regions of 98 intergenic miRNAs. Green color designates hypomethylated probes, while black color represents non-methylated. (C) Distribution of DMPs in 200-bp intervals. The frequency is represented as a percentile of DMPs in each interval. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteins. The methyl group-enriched DNAs were labeled with fluorescent dyes and then hybridized with the probes on chips (see Section 2).

The signal intensities of the 7646 probe spots on each chip were obtained after LOESS normalization. The Pearson's correlation of signal intensities between chips was 0.99 (Supplementary Fig. S1). By performing a linear fitting and Bayes function analysis, a total of 161 probes (adjusted p-value < 0.05) were found to have different methylation levels between DAC-treated and untreated samples (Fig. 1B) and these were defined as differentially methylated probes (DMPs). These DMPs were derived from 98 intergenic miRNAs. The sequences of the DMPs are shown in Supplementary Table S1.

To determine the distribution of the DMPs on the 5′-flanking region of each gene, we calculated the relative distance of DMPs from the 5′-end of each intergenic miRNA and constructed a frequency graph for DMPs in 200-bp intervals. As shown in Fig. 1C, the majority of DMPs were located within 400 bp upstream from the start site of each miRNA, suggesting that the major DMR, which is defined as the contig of DMPs, overlaps with the region containing the transcriptional regulatory elements such as promoter and proximal sequence elements. Interestingly, there is an additional DMR (20.9% of total DMPs) spanning from -800 to -1000 where enhancers are usually found.

3.2. Prediction of sequence motifs from DMPs

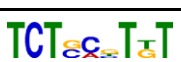
Because altering genome DNA methylation usually affects the efficiency of gene expression, it can be assumed that a specific sequence motif that regulates the transcription of its target miRNA gene is located within DMRs. Therefore, we analyzed the DMP sequences to predict a sequence motif using MEME software [26], which is used to predict statistically overrepresented sequence motifs.

After performing MEME with DMP sequences, we obtained six significant sequence patterns (p-value < 1.00e−05; Fig. 2A) which were 8–11 bp long. The most significantly overrepresented pattern was CNNNNNNCT (C[N]$_6$CT, p-value = 3.22e−18). N designates a non-conserved nucleotide. CTANCCTC, CTCTNCNC, TCTNNNTNT, GAGGTNTGATC, and CNNAGNGAC were also selected as significant patterns, the p-values of which were 2.15e−07, 5.26e−07, 2.34e−05, 2.80e−05, and 8.22e−05, respectively. It should be noted that there was a significant difference between the p-values for the C[N]$_6$CT pattern and CTANCCTC (11 logarithmic order), suggesting that C[N]$_6$CT is a major sequence motif in DMRs. Interestingly, CTANCCTC and CTCTNCNC patterns, as well as C[N]$_6$CT, contain two cytosine residues at positions 1 and 8, suggesting the cytosine residues at these positions are important for these patterns.

When performing multiple sequence alignment with 161 DMPs using the ClustalX program [27], only a single consensus sequence, CNNNNNNC (C[N]$_6$C), was found in all DMPs, which was also composed of conserved cytosine residues at position 1 and 8 (see Supplementary Fig. S2). It should be noted that this C[N]$_6$C motif is highly similar to the C[N]$_6$CT pattern predicted by MEME. These results suggest that the two conserved cytosine residues at positions 1 and 8 are likely embedded in the regulatory elements, and that the C[N]$_6$CT pattern may be a potential regulatory motif for intergenic miRNA gene expression. Thus, we selected the C[N]$_6$CT pattern as a candidate motif of regulatory element for intergenic miRNA expression.

We next examined the C[N]$_6$CT pattern within 1000 bp upstream of 98 intergenic miRNAs that contain DMPs, and identified a total of 1766 C[N]$_6$CT motifs. Among the 1766 C[N]$_6$CT motifs, 189 (10.7%) were located in DMPs (Supplementary Table S2). Overall, the number of C[N]$_6$CT motifs found in DMPs is small compared to that found in non-DMPs. However, further analysis of this motif showed that each gene contains a different frequency of C[N]$_6$CT motifs in its DMPs. For example, miR-200c has three DMRs, and 16 out of 22 C[N]$_6$CT motifs (72.7%) are concentrated in the DMRs (top line in Fig. 2B). In miR-124-1, there are four largely methylated regions that contain 11 out of 28 C[N]$_6$CT motifs (39.3%)

A

Consensus	motif logo	p-value[a]
CNNNNNNCT		3.22e-18
CTANCCTC		2.15e-07
CTCTNCNC		5.26e-07
TCTNNNTNT		2.34e-05
GAGGTNTGATC		2.80e-05
CNNAGNGAC		8.22e-05

B

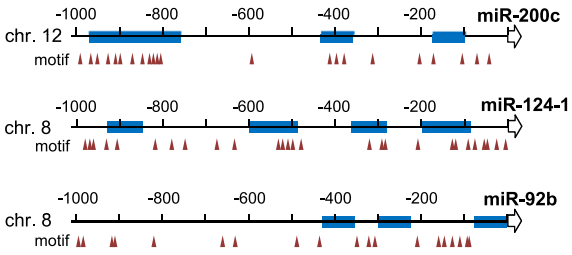


Fig. 2. Identification of significant sequence patterns and the distribution of C[N]$_6$CT motifs on the 5′-flank of intergenic miRNA genes. (A) Six highly significant motifs were identified in the DMRs using MEME software (see Section 2). Consensus designates the conserved sequence at each position. N represents non-conserved nucleotides. Motif logos are the graphically represented sequences showing homology at each position. The *p*-value designates significance of the sequences calculated against the random sequences. (B) Schematic representation of the genomic region encompassing miR-200c, miR-124-1, and miR-92b showing the distribution of C[N]$_6$CT motifs. The blue box represents DMR, which is the contig of DMPs. C[N]$_6$CT motifs are marked with triangles. All features were drawn based on the distance from the 5′-end of each miRNA.

(middle line in Fig. 2B). On the other hand, miR-92b contains three methylated regions, but there is no C[N]$_6$CT motif in these regions (bottom line in Fig. 2B).

3.3. Effect of demethylation on intergenic miRNA expression

It is important to determine whether the expression level of intergenic miRNA changes depending on the frequency of C[N]$_6$CT motifs in DMRs. Therefore, we first treated H4 cells with DAC to demethylate DNA. Then we isolated total RNA and measured the expression levels using quantitative PCR analysis against eight selected intergenic miRNA genes: one miRNA which had the highest frequency of C[N]$_6$CT motifs in DMRs (miR-200c), two with a 30–40% frequency (miR-124-1, miR-375), two with a 20–30% frequency (miR-34c, miR-210), one with a 10–20% frequency (miR-212), and two with less than 10% frequency (miR-3188 and miR-92b), all of which had more than three DMRs in their upstream regions but had no or only one C[N]$_6$CT motif in the DMRs (see Supplementary Table S2). *Rnu6b* was used as a negative control. As shown in Fig. 3, the expression levels of six of eight intergenic miRNAs (miR-200c, miR-124-1, miR-375, miR-34c, miR-210, and miR-212) increased significantly in DAC-treated cells compared to untreated normal cells. The expression level of miR-200c, which shows the highest frequency of C[N]$_6$CT motifs in DMRs (72.7%), was increased by 22.3-fold after DAC treatment. The expression levels of miR-124-1, miR-375, miR-34c, miR-210, and miR-212 were also increased by 11.3, 9.4, 8.4, 3.5, and 13.2-fold after DAC treatment, respectively. On the other hand, DAC treatment did not significantly change the expression levels of miR-92b or miR-3188. It should be noted that the frequencies of the C[N]$_6$CT motif in the DMR of miR-92b and miR-3188 are less than 10%, although they have more than three DMRs. These results strongly suggest that the frequency of C[N]$_6$CT motifs in DMRs is related to intergenic miRNA expression.

3.4. Correlations between the C[N]$_6$CT motif in DMRs and intergenic miRNA expression

Because the expression of intergenic miRNA genes were changed after DAC treatment in a motif-frequency dependent manner,

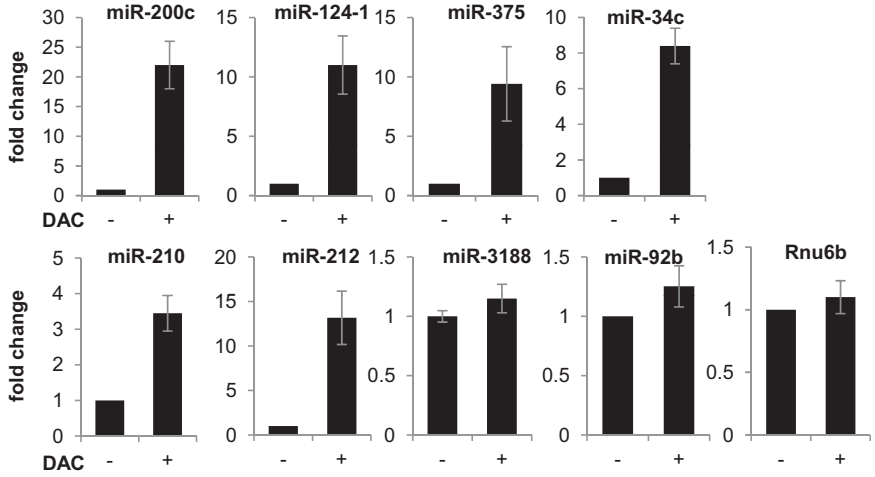


Fig. 3. Measurement of intergenic miRNA gene expression by qPCR. The intergenic miRNA levels were measured by qPCR. *Rnu6b* was used as a negative control. The *x*-axis shows the experimental condition treated with (+) and without (−) DAC, respectively. The *y*-axis represents the relative fold change of expression level of each intergenic miRNA after treating the cells with DAC. The fold change of non-treated cells was set to 1. The gene symbol is marked above each panel.

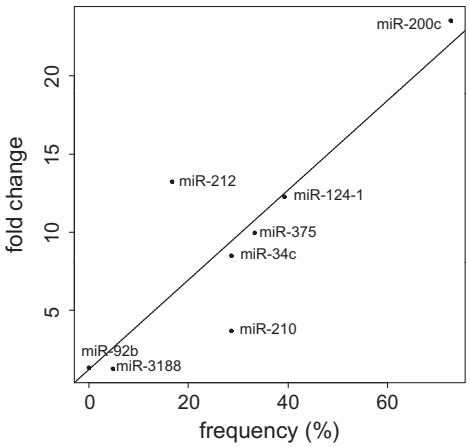


Fig. 4. Pearson's correlations between the expression levels and the frequency of the C[N]$_6$CT motif in DMRs of intergenic miRNAs. The *y*-axis represents fold change measured by RT-qPCR and the *x*-axis represents the frequency of the motifs within DMRs. The calculated Pearson's correlation (*R*) was 0.86.

we analyzed the relationship between the frequency of the C[N]$_6$CT motif in DMRs and the expression of intergenic miRNAs induced by demethylation. Correlation analysis between the fold changes of gene expression and the frequency of C[N]$_6$CT motifs in DMRs was performed with the eight miRNA genes described in the previous section. After performing Pearson's correlation test using those miRNAs, we obtained a high correlation value of 0.87 (p-value = 4.3e−03) between the frequency of C[N]$_6$CT motifs in DMRs and the fold changes in miRNA expression after demethylation (Fig. 4).

These results indicate that the frequency of C[N]$_6$CT motifs in DMRs plays a role in the expression efficiency of intergenic miRNAs in conjunction with the methylation status, and strongly suggests that the C[N]$_6$CT sequence pattern in DMRs is a methylation-dependent regulatory motif for intergenic miRNA expression.

4. Discussion

After identifying 161 DMPs within 1000 bp upstream of human intergenic miRNAs using microarray analysis, we searched for motifs within the DMRs and found a sequence motif, C[N]$_6$CT, which is conserved in the DMRs. Previous studies have reported that CT-repeat microsatellites are abundant within 1000 bp upstream of most intergenic miRNA ([14] and references therein). Some motifs containing CT-repeats, including (CCT)n, (CCTT)n, (CGCT)n, and (CCTCT)n, have previously been identified in plants [28,29]. Among these, the (CCT)n and (CCTCT)n motifs are very similar to the C[N]$_6$CT motif, which have two cytosine residues at positions 1 and 8. In other words, when n equals 3 in (CCT)n, the sequence of the motif will be CCTCCTCCT, which can be represented as C[N]$_6$CT. Similarly, (CCTCT)n can also be represented as CC[N]$_6$CT when n equals 2. Therefore, the C[N]$_6$CT motif is highly similar to (CCT)n and (CCTCT)n.

The C[N]$_6$CT motif must be located in DMRs to play a role as a regulatory element because the expression was increased in the genes with high frequency of the C[N]$_6$CT motif in DMRs after DAC treatment. It is possible that the cytosine residues at positions 1 and 8 of the C[N]$_6$CT motif in DMRs are methylated because they are found in methylated regions. The fact that this motif is associated with the expression of intergenic miRNA genes in a motif-frequency dependent manner suggests that the C[N]$_6$CT motif regulates gene expression by methylation/demethylation of cytosine.

Our findings indicate that the regulation of gene expression by C[N]$_6$CT motif is closely associated with DNA methylation status and the frequency of C[N]$_6$CT motif occurrence in DMRs of intergenic miRNA gene. This motif may be a regulatory factor binding site for transcription factors or demethylase. Combining the DNA methylation signature with the C[N]$_6$CT motif may be useful for computationally predicting novel intergenic miRNAs.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.03.005.

References

[1] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116 (2004) 281–297.
[2] A.S. Yoo, B.T. Staahl, L. Chen, G.R. Crabtree, MicroRNA-mediated switching of chromatin-remodelling complexes in neural development, Nature 460 (2009) 642–646.
[3] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, V.N. Kim, The nuclear RNase III Drosha initiates microRNA processing, Nature 425 (2003) 415–419.
[4] G. Hutvagner, J. McLachlan, A.E. Pasquinelli, E. Balint, T. Tuschl, P.D. Zamore, A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA, Science 293 (2001) 834–838.
[5] R.F. Ketting, S.E. Fischer, E. Bernstein, T. Sijen, G.J. Hannon, R.H. Plasterk, Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*, Genes Dev. 15 (2001) 2654–2659.
[6] I. Alvarez-Garcia, E.A. Miska, MicroRNA functions in animal development and human disease, Development 132 (2005) 4653–4662.
[7] C.M. Croce, G.A. Calin, MiRNAs, cancer, and stem cell division, Cell 122 (2005) 6–7.
[8] A. Rodriguez, S. Griffiths-Jones, J.L. Ashurst, A. Bradley, Identification of mammalian microRNA host genes and transcription units, Genome Res. 14 (2004) 1902–1910.
[9] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, T. Tuschl, Identification of novel genes coding for small expressed RNAs, Science 294 (2001) 853–858.
[10] Y. Lee, M. Kim, J. Han, K.H. Yeom, S. Lee, S.H. Baek, V.N. Kim, MicroRNA genes are transcribed by RNA polymerase II, EMBO J. 23 (2004) 4051–4060.
[11] X. Cai, C.H. Hagedorn, B.R. Cullen, Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs, RNA 10 (2004) 1957–1966.
[12] G.M. Borchert, W. Lanier, B.L. Davidson, RNA polymerase III transcribes human microRNAs, Nat. Struct. Mol. Biol. 13 (2006) 1097–1101.
[13] H.K. Saini, S. Griffiths-Jones, A.J. Enright, Genomic analysis of human microRNA transcripts, Proc. Natl. Acad. Sci. USA 104 (2007) 17719–17724.
[14] X. Zhou, J. Ruan, G. Wang, W. Zhang, Characterization and identification of microRNA core promoters in four model species, PLoS Comput. Biol. 3 (2007) e37.
[15] L. Heikkinen, S. Asikainen, G. Wong, Identification of phylogenetically conserved sequence motifs in microRNA 5′ flanking sites from *C. elegans* and *C. briggsae*, BMC Mol. Biol. 9 (2008) 105.
[16] N.D. Heintzman, R.K. Stuart, G. Hon, Y. Fu, C.W. Ching, R.D. Hawkins, L.O. Barrera, S. Van Calcar, C. Qu, K.A. Ching, W. Wang, Z. Weng, R.D. Green, G.E. Crawford, B. Ren, Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome, Nat. Genet. 39 (2007) 311–318.
[17] F. Ozsolak, L.L. Poling, Z. Wang, H. Liu, X.S. Liu, R.G. Roeder, X. Zhang, J.S. Song, D.E. Fisher, Chromatin structure analyses identify miRNA promoters, Genes Dev. 22 (2008) 3172–3183.
[18] H. Suzuki, S. Takatsuka, H. Akashi, E. Yamamoto, M. Nojima, R. Maruyama, M. Kai, H.O. Yamano, Y. Sasaki, T. Tokino, Y. Shinomura, K. Imai, M. Toyota, Genome-wide profiling of chromatin signatures reveals epigenetic regulation of MicroRNA genes in colorectal cancer, Cancer Res. 71 (2011) 5646–5658.
[19] M. Toyota, H. Suzuki, Y. Sasaki, R. Maruyama, K. Imai, Y. Shinomura, T. Tokino, Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer, Cancer Res. 68 (2008) 4123–4132.
[20] X. Agirre, A. Vilas-Zornoza, A. Jimenez-Velasco, J.I. Martin-Subero, L. Cordeu, L. Garate, E. San Jose-Eneriz, G. Abizanda, P. Rodriguez-Otero, P. Fortes, J. Rifon, E.

Bandres, M.J. Calasanz, V. Martin, A. Heiniger, A. Torres, R. Siebert, J. Roman-Gomez, F. Prosper, Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia, Cancer Res. 69 (2009) 4443–4453.

[21] X. Cao, S.L. Pfaff, F.H. Gage, A functional study of miR-124 in the developing neural tube, Genes Dev. 21 (2007) 531–536.

[22] J. Silber, D.A. Lim, C. Petritsch, A.I. Persson, A.K. Maunakea, M. Yu, S.R. Vandenberg, D.G. Ginzinger, C.D. James, J.F. Costello, G. Bergers, W.A. Weiss, A. Alvarez-Buylla, J.G. Hodgson, MiR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells, BMC Med. 6 (2008) 14.

[23] M. Fabbri, R. Garzon, A. Cimmino, Z. Liu, N. Zanesi, E. Callegari, S. Liu, H. Alder, S. Costinean, C. Fernandez-Cymering, S. Volinia, G. Guler, C.D. Morrison, K.K. Chan, G. Marcucci, G.A. Calin, K. Huebner, C.M. Croce, MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B, Proc. Natl. Acad. Sci. USA 104 (2007) 15805–15810.

[24] E.J. Noonan, R.F. Place, D. Pookot, S. Basak, J.M. Whitson, H. Hirata, C. Giardina, R. Dahiya, MiR-449a targets HDAC-1 and induces growth arrest in prostate cancer, Oncogene 28 (2009) 1714–1724.

[25] S. Griffiths-Jones, R.J. Grocock, S. van Dongen, A. Bateman, A.J. Enright, MiRBase: microRNA sequences, targets and gene nomenclature, Nucleic Acids Res. 34 (2006) D140–D144.

[26] T.L. Bailey, N. Williams, C. Misleh, W.W. Li, MEME: discovering and analyzing DNA and protein sequence motifs, Nucleic Acids Res. 34 (2006) W369–W373.

[27] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. McGettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J. Gibson, D.G. Higgins, Clustal W and Clustal X version 2.0, Bioinformatics 23 (2007) 2947–2948.

[28] S. Fujimori, T. Washio, K. Higo, Y. Ohtomo, K. Murakami, K. Matsubara, J. Kawai, P. Carninci, Y. Hayashizaki, S. Kikuchi, M. Tomita, A novel feature of microsatellites in plants: a distribution gradient along the direction of transcription, FEBS Lett. 554 (2003) 17–22.

[29] C. Molina, E. Grotewold, Genome wide analysis of Arabidopsis core promoters, BMC Genom. 6 (2005) 25.