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Possible miRNA coreguation of target genes in brain regions by both differential miRNA expression and miRNA-targeting-specific promoter methylation

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Abstract: We computationally reanalysed public domain data set deposited to gene expression omnibus, of mRNA expression, miRNA expression and promoter methylation pattern in four brain regions, i.e., frontal cortex, temporal cortex, pons and cerebellum. Then we found that more than hundreds of both miRNA regulation of target genes and miRNA-targeting-specific promoter methylation are significant on all six pairwise comparisons among the above mentioned four brain regions. We also showed that some of miRNA regulation of target genes are highly correlated with both or either of miRNA-targeting-specific promoter methylation and differential miRNA expression. We concluded that the combinatorial analysis of miRNA regulation of target genes, miRNA-targeting-specific promoter methylation and differential miRNA expression can figure out brain region specific contribution of miRNAs to brain functions and developments.

1. Introduction

miRNAs are short non-coding RNAs that are believed to suppress target gene expression through complementary sequence matching between "seed" region of miRNA and 3' untranslated region (UTR) of target genes[3]. Since biogenesis and functionality of miRNAs were relatively well-known compared with other non-coding RNAs, there were huge number of papers published about miRNAs. miRNAs are generally supposed to regulate cellular processes related to animal development[41], differentiation and several diseases/tumor formation. Thus, miRNAs are often regarded to be candidates of tumor suppressor[39] or cancer biomarkers [42]. miRNAs were also used for the reprogramming [1]. As such, miRNAs are considered to play critical roles over the wide range of biological processes.

Recently, miRNA expression in brain attracts the interest of many researchers [16], [20], [27], [30]. Although there are extensive researches about miRNA regulation of target genes [16], [27], it is generally believed that majorities of miRNA regulation of genes are indirect[28] and not all target genes are directly regulated by miRNAs. In this sence, in order to understand miRNA regulation of gene expression in brain regions, we also need to know other mechanisms that regulate miRNA target genes than miRNAs, in brain regions.

One of such well-known additional mechanisms that cooperatively regulate miRNA target genes together with miRNAs is transcription factor (TF) binding to promoter regions, although it is not only specifically in brain regions but also generally in gene expression regulation. TF is a protein complex that binds to promoter region of genome and initiate transcription processes. There were many papers that report cooperative regulation between miRNAs and TFs[5], [14], [15], [29], [45]. Since there are many TFs known to regulate many biological processes in brain regions[6], [7], [22], it is natural to investigate the combinatorial target gene regulation of miRNAs and TFs also in the brain [11], [33]. In contrast to the number of existing researches about cooperative regulation between miRNA and TF of genes, there are relatively limited number of researches about coregulation between miRNA and promoter methylayion, although promoter methylation induction by siRNA that targets CpG island was reported [12], [18], [23], [38]. Promoter methylation is generally thought to suppress gene expression[34]. Suppression of gene expression by promoter methylayion is often important. For example, aberrant promoter methylation is often related to cancers[17], [24]. Promoter methylation also plays a critical roles in reprogramming [9]. In spite of the importance of promoter methylation, correlation between promoter methylation and miRNA regulation of target genes was rarely discussed.

One of seldom researches about the coregulation between promoter methylation and miRNA regulation of target genes was recently conducted by Su *et al* [32]. They found that promoters of genes not targeted by miRNAs have tendencies to be methylated. Although there were no follow-up studies of it, we recently found that miRNA-targeting-specific promoter methylation takes place over many cell lines[35], [37]. In this paper, we report that miRNA-targeting-specific promoter methylaion also exists between distinct brain-regions in a brain-region specific manner. Considered brain regions are frontal cortex, temporal cortex, pons, and cerebellum [4]. Frontal cortex is located at the

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front of head in human. It is considered to be the hub of most higher functions and understanding and is believe to govern most behavioral traits, motor skills, and problem solving tactics. Actually, frontal cortex is the center of higher processing of information [31]. On the other hand, temporal cortex is located at lower right and left of brain [21]. Temporal cortex has various functions including hearing, understanding languages, face recognition, some kind of memories, and so on. Cerebellum is located at lower back of brain. The main function of cerebellum is believed to be motion control[19]. Finally, pons are located as the center of these three region and mediate information transfer between several brain regions including cortex and cerebellum [4]. Reflecting the functional varieties of these four regions, structures differ from regions to regions. Thus, it is natural that miRNA-targeting-specific promoter methylaion is also region specific.

Moreover, some miRNA regulation of target genes turned out to be controlled by not only differential miRNA expression itself but also miRNA-targeting-specific promoter methylaion.

2. Methods

2.1 miRNA exprerssion, mRNA expression and promoter methylation patterns

miRNA expression, mRNA expression and promoter methylation patterns used in this study were downloaded from Gene Expression Omnibus (GEO) under GEO ID GSE15745. Gibbs *et al*, who deposited these dataset to GEO, analysed this data set in detail in connection with genomic information, miRNA expression was not analyzed at all [10]. This data set includes data in the distinct four human brain regions (frontal cortex, temporal cortex, pons and cerebellum) from 150 subjects. Thus, in total, 600 tissue samples are included. Processed signals were used without any further normalization.

2.1.1 *P*-values computation for miRNA regulation of target genes and miRNA-targeting-specific promoter methylation using MiRaGE method

Although how we can attribute *P*-values of miRNA regulation of target genes to each miRNA [36], [44] and how we can attribute *P*-values of miRNA-targeting specific promoter methylation [35], [37] by MiRaGE method were reported in detail, we briefly explain them. First of all, we have to prepare the matrix $x_{ij\ell}$ that represents expression or promoter methylation of ith gene at ℓth region of jth sample. Then, compute differential expression/methylation as follows,

$$\Delta x_{ij}^{\ell\ell'} \equiv \log\left(\frac{x_{ij\ell'}}{x_{ij\ell}}\right).$$

When $x_{ij\ell}$ is not positive definite, one can simply ignore the cases that do not satisfy positive definite condition or alternatively

$$\Delta x_{ij}^{\ell\ell'} \equiv x_{ij\ell'} - x_{ij\ell}$$

can be used. Next, define a set of target genes G_m of mth miRNA and a set of genes G'_m not targeted by mth miRNA but targeted by any other miRNAs

$$G'_m \equiv \bigcup_{m' \neq m} G_{m'}$$

Then compute *P*-values to deny null hypothesis

$$\left\{\Delta x_{ij}^{\ell\ell'} \mid i \in G_m\right\} = \left\{\Delta x_{ij}^{\ell\ell'} \mid i \in G'_m\right\}$$

in favour of

$$\left\{\Delta x_{ij}^{\ell\ell'} \mid i \in G_m\right\} < \left\{\Delta x_{ij}^{\ell\ell'} \mid i \in G'_m\right\}$$

or

$$\left\{\Delta x_{ij}^{\ell\ell'} \mid i \in G_m\right\} > \left\{\Delta x_{ij}^{\ell\ell'} \mid i \in G'_m\right\}$$

using any statistical tests. The *P*-values computed based on the former (latter) is denoted as $P_{mj,<}^{\ell\ell'}$ ($P_{mj,>}^{\ell\ell'}$). The smaller $P_{mj,<}^{\ell\ell'}$ ($P_{mj,>}^{\ell\ell'}$) denotes that targets gene are more up(down)regulated in the ℓth tissue compared with the ℓ'th tissue. In our implementation, we employ t test, Wilcoxon rank sum test, and Kolmogorov-Smirnov test for the *P*-values computation. For more details, see Vignette in Bioconductor[*1] or the recent review article[37].

2.2 *P*-values for the comparison between distinct brain regions

P-values were attributed to each miRNA for the pairwise comparison among frontal cortex, temporal cortex, pons, and cerebellum. For each sample, six *P*-values are attributed to each miRNA for each of the six pairwise comparisons among four brain regions. Considered miRNAs are all human miRNAs registered in miRBase rel. 18 and Wilcoxon ranksum test was employed for *P*-value computation. The number of miRNAs M is equal to 1921.

2.3 Estimation of number of miRNAs whose target genes are significantly up/downregulated or target genes promoters are significantly hypo/hyper methylated between two brain regions

M $P_{mj,<}^{\ell\ell'}$s or $P_{mj,>}^{\ell\ell'}$s each of which was attributed to each miRNA for one of six pairwise compariosns of one sample were adjusted by Benjamin-hohenberg (BH) ctiterion. Then miRNAs with adjusted *P*-values less than 0.05 are regarded as significant. This number is averaged over all samples and averaged value is regarded to express the estimation of number of miRNAs whose target genes are significantly up/downregulated or target genes promoters are significantly hypo/hyper methylated between two brain regions.

2.4 Rank correlation coefficients between *P*-values attributed to miRNA regulation of target genes and *P*-values attributed to the miRNA-targeting-specific promoter methylation

M $P_{mj,<}^{\ell\ell'}$s for one of six pairwise comparisons of one sample are transformed to rank, $r_{mj}^{\ell\ell'}$. The rank, $r_{mj}^{\ell\ell'}$, attributed to each miRNA is averaged over all samples,

$$r_m^{\ell\ell'} \equiv \left\langle r_{mj}^{\ell\ell'} \right\rangle_j$$
$$\langle Q_j \rangle_j \equiv \frac{1}{J}\sum_j Q_j$$

where J is the total number of samples. Q_j is some variable attributed to the jth sample. The Spearman correlation coefficient of averaged rank between miRNA regulation of target genes and

[*1] http://www.bioconductor.org/packages/release/bioc/html/MiRaGE.html

miRNA-targeting-specific promoter methylation is,

$$\rho^{mRNA,Methyl.}_{\ell\ell'} \equiv \frac{\left\langle \Delta r^{\ell\ell',mRNA}_{0m} \cdot \Delta r^{\ell\ell',Methyl.}_{0m} \right\rangle_m}{\sqrt{\left\langle \left[\Delta r^{\ell\ell',mRNA}_{0m}\right]^2 \right\rangle_m} \sqrt{\left\langle \left[\Delta r^{\ell\ell',Methyl.}_{0m}\right]^2 \right\rangle_m}}$$
$$\Delta r^{\ell\ell',s}_{0m} \equiv r^{\ell\ell',s}_{0m} - \langle r^{\ell\ell',s}_{0m} \rangle_m$$
$$\langle Q_m \rangle_m \equiv \frac{1}{M} \sum_m Q_m,$$

where M is number of miRNAs, Q_m is some variable attributed to the mth miRNA. $r^{\ell\ell',s}_{0m}$ is the rank order of $r^{\ell\ell',s}_{m}$ over $m = 1, \ldots, M$, where s is either mRNA or Methyl. which represents mRNA expression and promoter methylaton, respectively, P-values attributed to Spearman correlation coefficients are also computed.

Alternatively, we compute the Spearman correlation coefficient of averaged rank between miRNA regulation of target genes and miRNA-targeting-specific promoter methylation before everaging,

$$\rho^{mRNA,Methyl.}_{j\ell\ell'} \equiv \frac{\left\langle \Delta r^{\ell\ell',mRNA}_{mj} \cdot \Delta r^{\ell\ell',Methyl.}_{mj} \right\rangle_m}{\sqrt{\left\langle \left[\Delta r^{\ell\ell',mRNA}_{mj}\right]^2 \right\rangle_m} \sqrt{\left\langle \left[\Delta r^{\ell\ell',Methyl.}_{mj}\right]^2 \right\rangle_m}}$$
$$\Delta r^{\ell\ell',s}_{mj} \equiv r^{\ell\ell',s}_{mj} - \langle r^{\ell\ell',s}_{mj} \rangle_m$$

Since we have found that averaged correlation coefficient $\langle \rho^{mRNA,Methyl.}_{j\ell\ell'} \rangle_j$ turns out to take almost zero (not shown here), in other words positive and negative correlation appears with almost same probability, we computed standard deviation of correlation coefficient instead of averaged value so as to express how much amount of values that differ from zero are observed for correlation coefficients,

$$\Delta\rho^{mRNA,Methyl.}_{\ell\ell'} \equiv \sqrt{\left\langle \left[\rho^{mRNA,Methyl.}_{j\ell\ell'} - \left\langle \rho^{mRNA,Methyl.}_{j\ell\ell'} \right\rangle_j\right]^2 \right\rangle_j}$$

2.5 Multiple regression model between miRNA regulation of target genes, miRNA-targeting-specific promoter methylation, and differential miRNA expression, with additionally considering both age and gender

The proposed multiple regression model is

$$\log P^{\ell\ell',mRNA}_{mj,>} = A^{\ell\ell'}_m \cdot \log P^{\ell\ell',Methyl.}_{mj,<} + B^{\ell\ell'}_m \cdot \log\left(\frac{x_{mj\ell}}{x_{mj\ell'}}\right) + C^{\ell\ell'}_m \cdot \mathrm{age}_j + D^{\ell\ell'}_m \cdot \mathrm{gender}_j + E^{\ell\ell'}_m$$

where $P^{\ell\ell',mRNA}_{mj,>}$ and $P^{\ell\ell',Methyl.}_{mj,<}$ are P-values attributed to miRNA regulation of target genes and miRNA-targeting-specific promoter methylation, respectively. $A^{\ell\ell'}_m, B^{\ell\ell'}_m, C^{\ell\ell'}_m, D^{\ell\ell'}_m$ and $E^{\ell\ell'}_m$ are constants. $x_{mj\ell}$ is mth miRNA expression of the ℓth region of the jth sample. age_j and gender_j are age and gender of jth sample respectively. gender_j =1 when jth sample was taken from male otherwise gender_j=0. After obtaining regression results, feature extraction based upon AIC was applied to choose valid terms in the right hand side. This feature extraction procedure specifies the optimal combination of the terms in the right hand side, with the minimum AIC values. Positive (negative) $A^{\ell\ell'}_m$ indicates reciprocal (nonreciprocsal) relationship between miRNA and target genes. The multiple regression and feature extraction were performed by lm function in base package and stepAIC function in MASS package, implemented in R[25], respectively.

2.6 The selection of miRNAs that significantly regulate target genes based on multiple regression model

P-values that were computed based on F test and were attributed to each regression model of miRNA were adjusted by BH criterion for each of six pairwise comparisons of brain regions. Then we selected miRNAs whose P-values attributed to the constant $B^{\ell\ell'}_m$, which is the multiplier of $\log\left(\frac{x_{mj\ell}}{x_{mj\ell'}}\right)$, are less than 0.05 among the regression model whose adjusted P-values are less than 0.05.

3. Results and Discussion

In this section, we will discuss the mutual relationships among miRNA-related features and their biological meanings.

3.1 Mutual relationships between miRNA regulation of genes, miRNA-targeting-specific promoter methylaion and differential miRNA expression

We investigated miRNA regulation of target genes and miRNA-targeting-specific promoter methylation among frontal cortex, temporal cortex, pons, and cerebellum, based on the P-values, $P^{\ell\ell',s}_{mj,<}$ or $P^{\ell\ell',s}_{mj,>}$, that estimate miRNA regulation of target genes and miRNA-targeting-specific promoter methylation. Fig. 1 illustrates the results of this analysis. It is clear that target genes of substantial number of miRNAs are up/downregulated between these four brain regions. It is also sure that substantial number of miRNAs' target genes' promoters are hyper/hypomethylated between these four brain regions. This strongly suggests that both miRNA regulation of target genes and miRNA-targeting-specific promoter methylation play critical roles to make these four brain regions develop and function differently from each other.

For example, from the miRNA-centric point of views (Fig. 1), pons have both genes with more hypermethylated promoters and genes more downregulated than genes in the other investigated three brain regions, i.e., frontal cortex, temporal cortex and cerebellum. This observation is coincident with the general belief that expression of genes with methylated promoter is suppressive. Although this also means that mRNA expression in pons are especially distinct from the other three brain regions investigated, it does not contradict with the previous study where Hawrylycz *et al* [13] reported that there are very few genes expressed differently between temporal cortex, frontal cortex and cerebellum while that of pons differs from those of temporal cortex, frontal cortex and cerebellum (see, e.g., their Fig. 4a). Thus, it is suggested that both miRNA regulation of target genes and miRNA-targeting-specific promoter methylation may cooperatively mediate previously observed[13] differential expression between pons and other three brain regions.

Then, it is important to understand how these two cooperatively regulate target genes. In order to understand the mutual relationship between miRNA regulation of target genes and miRNA-targeting-specific promoter methylation, we computed the cor-

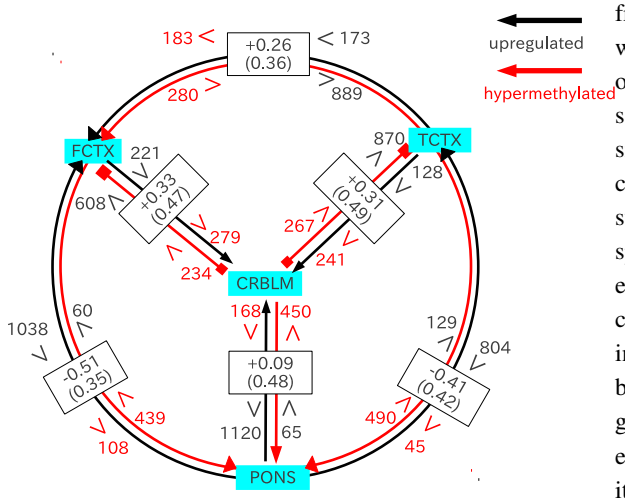

Fig. 1 Schematic illustration of the relationship between miRNA regulation of target genes and miRNA-targeting-specific promoter methyaltion. Arrows/segments indicate up/downregulation of miRNA target genes and miRNA-targeting-specific promoter methylation. Black (red) numbers next to inequality signs are the averaged number of miRNAs whose target genes are significantly up/downregulated (whose target genes promoters are hyper/hypomethylated). TCTX is temporal cortex, FCTX is frontal cortes, CRBLM is cerebellum, and PONS is pons. For example, between TCTX and FCTX, there are 280 (183) miRNAs whose target genes promoters are significantly hyper(hypo)methylated in FCFX compared with TCTX. Similarly, there are 889 (173) miRNAs whose target genes are significantly up(down)regulated in FCFX compared with TCTX. Since 280 is large enough than 183, promoters of genes in FCFX is regarded to be hypermethylated than TCTX in the miRNA-centric-view, thus red arrow directs from TCTX to FCTX. Similarly, since 889 is large enough than 173, genes in FCFX is regarded to be upregulated than TCTX in the miRNA-centric-view, thus black arrow directs from TCTX to FCTX. The numbers in rectangular indicate Spearman correlation coefficients between miRNA regulation of target genes and miRNA-targeting-specific promoter methylation, $\rho_{\ell\ell'}^{mRNA,Methyl.}$. Those in parentheses are the standard deviations of Spearman correlation coefficients, $\Delta\rho_{\ell\ell'}^{mRNA,Methyl.}$.

relation coefficient of mean rank of P-values, $\rho_{\ell\ell'}^{mRNA,Methyl.}$, for six pairwise comparisons among frontal cortex, temporal cortex, pons and cerebellum (see Fig. 1). Here the means were taken over all of samples in each brain region. Excluding a pair of cerebellum and pons, correlation coefficients for other five pairwise comparisons take values raging from 0.25 to 0.51. These values can be regarded to be large enough if we consider that the number of P-values attributed to each brain region is as large as M that is equal to the number of miRNAs. Acutally, the P-values attributed to each correlation coefficient are less than 2.2×10^{-16}. This means, the correlation between miRNA regulation of target genes and miRNA-targeting-specific promoter methylation is highly significant independent of pairs of brain regions. The remaining and the smallest correlation coefficient is that attributed to the pair of cerebellum and pons. However, its value is still as large as 0.09 and the attributed P-values to it is as small as 4×10^{-5}, thus is highly significant too, although the correlation itself cannot be said to be large enough. In order to confirm the firm correlation between miRNA regulation of target genes and miRNA-targeting-specific promoter methylation, the root mean squared averages of correlation coefficients of each sample, $\Delta\rho_{\ell\ell'}^{mRNA,Methyl.}$, were also computed. Excluding the pair of frontal cortex and pons where the absolute value of $\rho_{\ell\ell'}^{mRNA,Methyl.}$ was the maximum, $\Delta\rho_{\ell\ell'}^{mRNA,Methyl.}$ is larger than the absolute value of $\rho_{\ell\ell'}^{mRNA,Methyl.}$. This means, correlation coefficients within each sample are not small but only averaged value over samples is small because of the appearance of both positive and negative correlation with equal probabilities in each sample. As a result, miRNA regulation of target genes and miRNA-targeting-specific promoter methylation are significantly correlated with each other. One may notice that the signs of correlation coefficients, $\rho_{\ell\ell'}^{mRNA,Methyl.}$, are neither positive definite nor negative definite. One may think that they should be positive definite because both promoter methylation and miRNA targeting should suppress gene expression. However, since genes targeted by miRNAs are expected to be downregulated (upregulated) only when miRNA itself is upregulated (downregulated), there are no reasons to expect that the correlation coefficients between miRNA regulation of target genes and miRNA-targeting-specific promoter methlation always takes positive or negative values.

Then, in order to figure out the relationship between miRNA regulation of target gene, $P_{mj,<}^{\ell\ell'}$ or $P_{mj,>}^{\ell\ell'}$, and differential expression of miRNA itself, $\log\left(\frac{x_{mj\ell}}{x_{mj\ell'}}\right)$, the correlation coefficients were computed. However, these correlation coefficients took too small values to be significant enough (not shown here). This is apparently contradict with the above mentioned strong correlation between miRNA regulation of target genes and miRNA-targeting-specific promoter methylation.

In order to resolve this apparent discrepancy, we have introduced the multivariate regression models between miRNA regulation of target genes, miRNA-targeting-specific promoter methylation and differential miRNA expression, together with gender and age information (see Methods). In contrast to the above discrepancy, we have found some significant correlations between those variables. It was found that not all of features, i.e., miRNA regulation of target genes, miRNA-targeting-specific promoter methylation and differential miRNA expression together with age and gender, were always correlated but were selectively correlated. That is, dependent upon the miRNA considered, a combination of limited part of these features are correlated. In order to quantize this selective correlations, we picked up the combination of features significantly correlated with each other for each miRNA (see Methods). Table 1 lists the miRNAs selected for each pair of brain regions based on the criterion described in the subsection "The selection of miRNAs that significantly regulate target genes based on multiple regression model", i.e., miRNAs whose differential expression is significantly correlated to miRNA regulation of target genes. To our knowledge, for the first time, we showed that miRNA regulation of taget genes are mediated by both differential miRNA expression and miRNA-targeting-specific promoter methylation.

3.2 Possible biological reasons of coregulation by both miRNA and promoter methylation

One of the reasons why coregulations by miRNA and promoter methylation were not widely investigated is because there does

Table 1 miRNAs that significantly regulate target genes miRNAs supposed to regulate target genes for six pairwise comparisons among four brain regions, frontal cortex (FCTX), temporal cortex (TCTX), pons (PONS), and cerebellum (CRBLM). "Reciprocal" ("nonreciprocal") indicates relationship between miRNA expression and target gene mRNA is reciprocal (nonreciprocal). miRNAs in bold face appear more than once. Underlined miRNAs were previously reported to be related to brain development/diseases [2], [26], [43]. See subsection "The selection of miRNAs that significantly regulate target genes based on multiple regression model" for the detailed criterion of miRNAs selection.

CRBLM vs FCTX		CRBLM vs PONS		CRBLM vs TCTX	
reciprocal	nonreciprocal	reciprocal	nonreciprocal	reciprocal	nonreciprocal
hsa-miR-181c-5p	hsa-miR-200a-5p	hsa-miR-20a-5p	hsa-let-7b-5p	hsa-miR-210	hsa-miR-99a-5p
hsa-miR-135a-5p	hsa-miR-381	hsa-miR-23a-3p	hsa-let-7e-5p		hsa-miR-191-5p
hsa-miR-137	**hsa-miR-202-3p**	hsa-miR-148a-3p	hsa-miR-197-3p		**hsa-miR-99b-5p**
hsa-miR-363-3p	hsa-miR-561-3p	hsa-miR-10a-5p	hsa-miR-181b-5p		hsa-miR-617
hsa-miR-369-3p	hsa-miR-568	hsa-miR-221-3p	hsa-let-7i-5p		
hsa-miR-487a	hsa-miR-618	hsa-miR-223-3p	hsa-miR-9-5p	FCFX vs PONS	
hsa-miR-514a-3p	**hsa-miR-630**	hsa-miR-1	hsa-miR-126-3p	hsa-miR-365a-3p	hsa-miR-302d-3p
hsa-miR-553		hsa-miR-133a	hsa-miR-134	hsa-miR-378a-5p	hsa-miR-432-5p
hsa-miR-554		**hsa-miR-137**	hsa-miR-154-3p		hsa-miR-595
hsa-miR-655		hsa-miR-146a-5p	hsa-miR-299-5p		
hsa-miR-421		hsa-miR-452-5p	**hsa-miR-99b-5p**	FCTX vs TCTX	
		hsa-miR-484	hsa-miR-377-3p	has-miR-373-3p	hsa-miR-24-3p
		hsa-miR-511	hsa-miR-383		hsa-miR-485-5p
		hsa-miR-515-5p	hsa-miR-431-5p		hsa-miR-766-3p
		hsa-miR-571	hsa-miR-329		
		hsa-miR-549	hsa-miR-485-3p	PONS VS TCTX	
			hsa-miR-487a	hsa-miR-9-3p	hsa-miR-222-3p
			hsa-miR-202-3p	hsa-miR-302a-3p	hsa-miR-125b-5p
			hsa-miR-432-3p	hsa-miR-410	hsa-miR-328
			hsa-miR-495	hsa-miR-487b	hsa-miR-581
			hsa-miR-504	**hsa-miR-630**	hsa-miR-661
			hsa-miR-505-3p		
			hsa-miR-563		
			hsa-miR-578		
			hsa-miR-630		
			hsa-miR-668		

not seem to exist any direct relationship between functionalities of miRNA and promoter methylation. miRNA regulation of target genes take place in cytoplasm while promoter methlation occurs in nucleus. These two processes are apart from each other temporally and spatially. However, it is not a rational attitude, because the relationship between TF and miRNAs is popular topics while TF works in nucleus and miRNA works in cytoplasm. Major difference is that there are few number of proteins that mediate promoter methylation while TF has many species. If promoter methylation cooperatively regulate gene expression with miRNAs which have many number of species, what is the bearer of information for promoter methylation?

For this problem, recently, Halytskiy [12] suggested that Ago protein binding miRNA may function in nucleus and induce promoter methylayion. Actually, Ago protein was suggested to function in nucleus [8] and Weinmann *et al* recently found that Ago protein localized to nucleus [40]. If Ago protein binding miRNA can directly methylate promoter in nucleus, this mechanism may mediate miRNA-targeting-specific promoter mathylation. The further studies along this line is waited.

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