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Search of miRNAs critical for medulloblastoma formation using MiRaGE method

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MiRaGE method estimates critical miRNAs based upon gene expression profile of their target genes. Expression profile of mRNA/miRNA is measured for tissues from neonatal and adult mice (6 days and 30 days after birth: P6 and P30, respectively), and medulloblastomas (2-3 months old: MB) with Agilent microarray is analyzed by MiRaGE method. Comparison between P30 and MB gives us the list of significantly up(down)regulated miRNAs whose target genes are down(up)regulated. The obtained list is biologically reasonable, possibly due to accuracy of Agilent microarray measurement, thus we conclude that MiRaGE method is useful to investigate tissue formation processes, too.

1. Introduction

MicroRNAs (miRNAs) are the single strand RNAs with the length from 20 to 25 nucleotide in cells. It is a member of non-coding RNAs which are supposed to control gene expression. They are believed to suppress expression of target genes, by aligned to complimentary seed sequence with the length of eight nucleotide, which is typically located at 3' untranslated region (UTR) of target genes. Even possible, it is a very time/cost consuming process to validate target gene experimentally[1)]. Thus, usually, computer oriented predictions are employed to list target genes of each miRNA.

Recently[2)], we have proposed MiRNA Ranking by Gene Expression (MiRaGE) method which estimates amount of contribution of each miRNA to target gene regulation. We have validated our algorithm by analysis of gene expression profiles of miRNA-transfected cells and computer-predicted potential miRNA target lists; our method mostly can correctly predict the transfected miRNA as only one significant miRNA after multiple comparison correction.

In this paper, we have applied MiRaGE method to analyze mouse medulloblastoma tumorigenesis model (Yaginuma, et al. manuscript in preparation:see Materials and Methods) and we have found reciprocal relationship between miRNAs and their target genes' expression. Statistical analyses revealed that our analysis strongly depends on the high-quality gene expression profiling methods such as Agilent and Illumina microarrays. List of critical miRNAs based upon both MiRaGE method and miRNAs' gene expression profile is compatible with previous studies[3)].

2. Materials and Methods

2.1 Gene expression data of miRNA/mRNA for P6, P30 and MB murine cells

Ptc1 heterozygous mice with B6 background were generated by Dr. Tetsuo Noda 's group (The JFCR-Cancer Institute). The macroscopically normal cerebellar tissues from neonatal and adult mice (6 days and 30 days after birth: P6 and P30, respectively), and medulloblastomas (2-3 months old: MB) were obtained from two mice for each time point. Total RNAs including miRNAs were extracted with miRNA easy mini column kit (QIAGEN). The RNAs were subjected to in vitro amplification and labeling with Low input quick Amp labeling kit (Agilent). The labeled RNA was hybridized with Sureprint G3 mouse GE 8 x 60 K microarray (Agilent) with manufacturer 's protocols. One technical replicate was obtained for each sample.

2.2 Inference of miRNA which regulates target genes significantly

The way to detect miRNA whose target genes are significantly differently expressed between two distinct samples is as follows. First, in order to obtain target genes' table of each miRNA, we have downloaded both 3' UTR Exons sequences by UCSC genome's table browser[4)] (NCBI37/mm9) and miRNA sequences from miRBase release 16[5)] which lists 1122 miRNAs. Then we have picked up genes which have at least one seed match to any of miRNAs. As a result, there remains 28614 genes. Since this is knownGene base, after conversion to RefSeq DNA ID by bioMart[6)], some RefSeq DNA IDs have appeared multiple times but we leave them as it is. Hereafter, we denote this set of 28614 genes as G. Next, for each

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miRNA, m, we have listed target genes of it. We denote this set of genes as G_m. Also we define a set of genes, $G'_m \equiv G \setminus G_m$. After denoting expression of gene g at sample S_i as $x_g^{S_i}$, where S_i is one of P6, P30, and MB and i runs over all of technical/biological replicates, we compute gene expression difference between two samples S_i and S'_j as follows,

$$\Delta x_g^{S_i,S'_j} \equiv \log x_g^{S_i} - \log x_g^{S'_j}.$$

Then we apply one-sided t-test to check if $\{\Delta x_g^{S_i,S'_j} \mid g \in G_m\}$ is significantly less than $\{\Delta x_g^{S_i,S'_j} \mid g \in G'_m\}$ and P-value, $P_m^{gene}(S_i, S'_j)$, is computed for each miRNA, m. For this, we used t.test module in base package of R[7]. We also apply FDR correction (BH method[8]) to see if FDR corrected P-value is less than 0.05. Such miRNAs regulate target genes significantly. One should remember that miRNAs with smaller P_m^{gene} have more downregulated target genes during the process from S'_j to S_i.

2.3 Estimation of miRNAs' up/downregulation

In this version of Agilent miRNA chip, each miRNA's expression is measured by multiple probes. Thus, instead of simply comparing between averaged value of probes' outputs, we have applied one-sided t-test to evaluate P-value to check if a set of probes' expression for miRNA m at sample $S"_k$, $\{x_m^{S"_k}\}$, is significantly less than $\{x_m^{S'''_l}\}$. These P-values are denoted as $P_m^{miRNA}(S"_k, S'''_l)$ in order to be distinguished from the above $P_m^{gene}(S_i, S'_j)$s. The same as above FDR correction procedure is applied to check if P-values are still significant after this correction. One should remember that miRNAs with smaller P_m^{miRNA} are more downregulated during the process from S'''_l to $S"_k$.

2.4 Detection of reciprocal relationship between miRNAs and their target genes' expression

In order to check if $P_m^{gene}(S_i, S'_j)$s and $P_m^{miRNA}(S"_k, S'''_l)$ satisfy reciprocal relationship, we have done the followings. First, we have divided miRNAs into two groups, $M(n)$ and $M(N-n)$. $M(n)$ is the n top-most up(down)regulated miRNAs based upon P_m^{miRNA} and the remaining is $M(N-n)$, where N is total number of miRNA. Then applying t-test to check if $\{P_m^{gene}(S_j, S'_j) \mid m \in M(n)\}$ is significantly smaller(larger) than $\{P_m^{gene}(S_i, S'_j) \mid m \in M(N-n)\}$, i.e., the test if target genes of $M(n)$ are more down(up)regulated than those of $M(N-n)$ or not. This P-value is denoted as $P^n(gene, S_i, S'_j \downarrow (\uparrow) \mid miRNA, S"_k, S'''_l \uparrow (\downarrow))$, where $P(\mathcal{A} \mid \mathcal{B})$ is the conditional probability of $\mathcal{A}$ under the condition of $\mathcal{B}$. We choose $n = 10, 20, \ldots 1000$ and check if there are n having $P < 0.05$ with this t-test.

This computation is repeated after replacing $P_m^{gene}(S_i, S'_j)$ with $P_m^{miRNA}(S"_k, S'''_l)$, i.e., we check whether miRNAs whose target genes are n top-most up(down)regulated are significantly down(up)regulated than remaining miRNAs. This P-value is denoted as $P^n(miRNA, S"_k, S'''_l \downarrow (\uparrow) \mid gene, S_i, S'_j \uparrow (\downarrow))$.

Thus, in total, four kinds of tests are performed towards each pair of $P_m^{gene}(S_i, S'_j)$s and $P_m^{miRNA}(S"_k, S'''_l)$s taken from all of technical/biological replicates $S_i, S'_j, S"_k, S'''_l$s.

3. Results

3.1 Comparison between MB and P30

First, we consider target genes' expression and S_i and S'_j are taken to be MB and P30 respectively. Between two technical and two biological replicates of P30 and MB, i.e., in total 4 samples $\times$ 4 samples = 16 combinations, from 977 to 1066 miRNAs' target genes sets are significantly downregulated with $P_m^{gene}(S_i, S'_j) < 0.05, (i, j = 1, \ldots, 4)$ even after multiple comparison correction. On the other hand no significant upregulation is observed.

Besides, when miRNAs' expression is considered and $S"_k$ and S'''_l are taken to be MB and P30 respectively, between two biological replicates of P30 and MB, i.e., in total 2 samples $\times$ 2 samples = 4 combinations, from 269 to 328 (from 158 to 259) miRNAs are significantly down(up)regulated. This means, $P_m^{miRNA}(S"_k, S'''_l) < 0.05, (k, l = 1, 2)$ even after multiple comparison correction.

For all of 64 combinations, i.e., 16 $P_m^{gene}(S_i, S'_j)$ sets vs 4 $P_m^{miRNA}(S"_k, S'''_l)$ sets, there are at least one n with $P < 0.05$ for each $P^n(gene, S_i, S'_j \downarrow (\uparrow) \mid miRNA, S"_k, S'''_l \uparrow (\downarrow))$ and $P^{S_i,S'_j,S"_k,S'''_l,n}(miRNA \downarrow (\uparrow) \mid gene \uparrow (\downarrow))$. This means, at least statistically, reciprocal relationships stands between miRNAs and their target genes. In average, 23.7% of n has $P < 0.05$. This is clearly large enough portion to cover most of the biologically critical miRNAs in the medulloblastoma tumorigenesis.

In Fig. 1, we plot logarithmic P-values averaged over all 64 combinations,

$$\langle \log_{10} P_n^{MB\leftarrow P30}(gene\downarrow(\uparrow) \mid miRNA\uparrow(\downarrow))\rangle$$
$$\equiv \langle \log_{10} P^n(gene, S_i, S'_j\downarrow(\uparrow) \mid miRNA, S"_k, S'''_l\uparrow(\downarrow))\rangle_{S_i,S'_j,S"_k,S'''_l}$$

and

$$\langle \log_{10} P_n^{MB\leftarrow P30}(miRNA\downarrow(\uparrow) \mid gene\uparrow(\downarrow))\rangle$$
$$\equiv \langle \log_{10} P^n(miRNA, S"_k, S'''_l\downarrow(\uparrow) \mid gene, S_i, S'_j\uparrow(\downarrow))\rangle_{S_i,S'_j,S"_k,S'''_l}$$

as function of n, where $\langle\ldots\rangle_{S_i,S'_j,S"_k,S'''_l}$ is average over $S_i, S'_j, S"_k, S'''_l$.

$\langle \log_{10} P_n^{MB\leftarrow P30}(gene\downarrow\mid miRNA\uparrow)\rangle$ takes smallest value $P \simeq 10^{-3}$ at around $n \simeq 100$ while $\langle \log_{10} P_n^{MB\leftarrow P30}(gene\uparrow\mid miRNA\downarrow)\rangle$ takes smallest value at around $n \simeq 950$. This means, more or less, miRNAs are divided into two groups with 100 miRNAs and 1000 miRNAs respctiely. The former is a set of upregulated 100 miRNAs whose target genes are downregulated.

In contrast to this, inverse relationships are weaker. $\langle \log_{10} P_n^{MB\leftarrow P30}(miRNA\uparrow\mid gene\downarrow)\rangle$ takes smallest value $P \simeq 10^{-1.5} \simeq 0.03$ at around $n \simeq 150$ while $\langle \log_{10} P_n^{MB\leftarrow P30}(miRNA\downarrow\mid gene\uparrow)\rangle$ takes smallest value at around $n \simeq 850$. However, minimum P-vales are relatively larger than the above. This means, to predict miRNAs expression via their target genes' expression is more difficult than to predict miRNAs' target genes' expression via miRNAs' expression. This indicates that many miRNAs share their targets each other and the overlapped target mRNAs for multiple miRNAs are frequently downregulated in the biological process[9].

3.1.1 Upregulated miRNAs whose target genes are downregulated from P30 to MB

Next, in order to pick up critical miRNAs, we have done the followings. First, we have listed 100 miRNAs with larger $P_m^{miRNA}(S"_k, S'''_l)$ for each of 4 sets of $k, l = 1, 2$. That is, top 100 most upregulated miRNAs are listed. Then frequency of being listed as top 100 among four combinations of $S"_k, S'''_l$ is counted. The ratio to be selected as top 100 is computed for each m by dividing this frequency by four. Next, 100 miRNAs with smaller $P_m^{gene}(S_i, S'_j)$ for 16 sets of $i, j = 1, \ldots, 4$ are listed. This time, top 100 miRNAs whose target genes are downregulated are

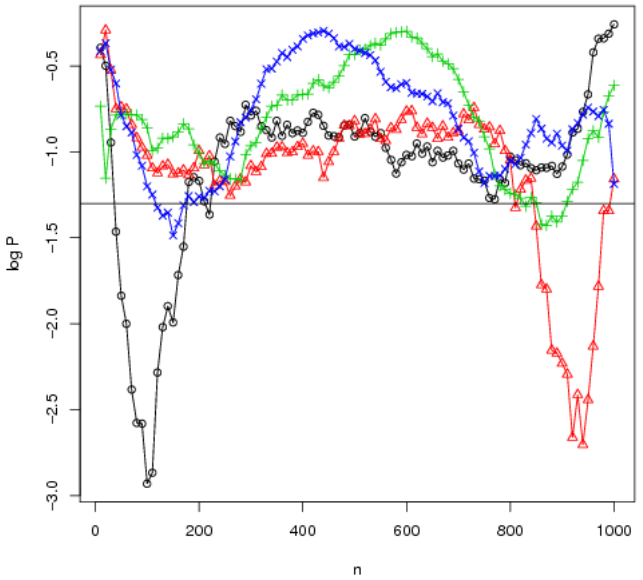

Fig. 1 P-values to check if reciprocal relationships stand between miRNAs and their target genes during the process from P30 to MB. A solid horizontal line indicates $P = 0.05$.
Black open circles: $\langle \log_{10} P_n^{MB\leftarrow P30}(gene\downarrow\mid miRNA\uparrow)\rangle$
Red open triangles: $\langle \log_{10} P_n^{MB\leftarrow P30}(gene\uparrow\mid miRNA\downarrow)\rangle$
Green crosses: $\langle \log_{10} P_n^{MB\leftarrow P30}(miRNA\downarrow\mid gene\uparrow)\rangle$
Blue X marks: $\langle \log_{10} P_n^{MB\leftarrow P30}(miRNA\uparrow\mid gene\downarrow)\rangle$,

listed. Again, we compute ratio of being listed as top 100. All miRNAs are ranked according to the sum of these two ratios (Table 1).

Table 1 Top 20 critical miRNAs during process from P30 to MB. Ratios for miRNA and mRNA are that of being listed as top 100 upregulated miRNA and miRNAs whose target genes are downregulated.

		Ratio	
	miRNAs	miRNA	mRNA
1	mmu-miR-25	1.00	1.00
2	mmu-miR-466i-5p	1.00	1.00
3	mmu-miR-92a	0.75	1.00
4	mmu-miR-19a	1.00	0.69
5	mmu-miR-19b	1.00	0.69
6	mmu-miR-3082-5p	1.00	0.56
7	mmu-miR-130a	1.00	0.50
8	mmu-miR-130b	1.00	0.50
9	mmu-miR-15b	1.00	0.50
10	mmu-miR-2861	1.00	0.50
11	mmu-miR-3096-5p	1.00	0.50
12	mmu-miR-32	0.50	1.00
13	mmu-miR-322	1.00	0.50
14	mmu-miR-721	1.00	0.50
15	mmu-miR-149*	0.50	0.88
16	mmu-miR-3081*	1.00	0.38
17	mmu-miR-574-5p	1.00	0.31
18	mmu-miR-669n	0.50	0.81
19	mmu-miR-1187	1.00	0.25
20	mmu-miR-182	0.50	0.75

It contains members of mir-17-92 cluster family (mir-92a, mir-19a, mir-19b, and mir-25). Since these clusters are expected to play important roles for tumor formation of medulloblastoma[3], this list is biologically reasonable.

3.1.2 Downregulated miRNAs whose target genes are upregulated from P30 to MB

Similar lists are generated for inverse relationship, i.e., downregulated miRNAs whose target genes are upregulated (Table 2). It contains let-7 family. It is reasonable since they are believed to be tumor suppressive miRNAs.

3.2 Comparison between P6 and P30

It is also interesting to see how these list changes if we replace MB with P6, since it is generally believed that these two are similar to each other.

Table 2 Top 20 critical miRNAs during process from P30 to MB. Ratios for miRNA and mRNA are that of being listed as top 100 downregulated miRNA and miRNAs whose target genes are upregulated.

		Ratio	
	miRNAname	miRNA	mRNA
1	mmu-miR-100	1.00	1.00
2	mmu-miR-126-3p	1.00	1.00
3	mmu-miR-29c	1.00	1.00
4	mmu-miR-376a	1.00	1.00
5	mmu-miR-451	1.00	1.00
6	mmu-miR-99b	1.00	1.00
7	mmu-miR-136*	1.00	0.94
8	mmu-miR-299*	0.75	1.00
9	mmu-miR-26a	1.00	0.50
10	mmu-miR-26b	1.00	0.50
11	mmu-miR-29a	0.50	1.00
12	mmu-miR-7a-1*	1.00	0.50
13	mmu-miR-3107	1.00	0.44
14	mmu-miR-340-5p	1.00	0.31
15	mmu-miR-369-5p	1.00	0.31
16	mmu-let-7a	1.00	0.25
17	mmu-let-7e	1.00	0.25
18	mmu-let-7g	1.00	0.25
19	mmu-let-7i	1.00	0.25

First we have checked if P_m^{miRNA}s and P_m^{gene}s are coincident with each other or not as above. Then, for all of 64 combinations, there are at least six ns with $P < 0.05$ for each of $P^n(gene, S_i, S'_j \downarrow (\uparrow) \mid miRNA, S"_k, S'''_l \uparrow (\downarrow))$ and $P^n(miRNA, S"_k, S'''_l \downarrow (\uparrow) \mid gene, S_i, S'_j, \uparrow (\downarrow))$.

This means, at least statistically, reciprocal relationships stands between miRNAs and their target genes. In average, 37.3% of n has $P < 0.05$ssssssss. This performance is even better than comparison between P30 and MB shown in the above. Thus, we conclude that between P30 and P6, miRNAs and their target genes keep reciprocal relationship as well. Actually speaking, this relationship is even stronger than that between P30 and MB. Our results suggested that miRNA suppresses its target mRNAs in a biological context-dependent manner.

In Fig. 2, we plot averaged logarithmic P-values over all 64 combinations as function of n. Again, we can notice generally P-values are smaller than those in Fig. 1. $\langle \log_{10} P_n^{P6 \leftarrow P30}(gene \downarrow \mid miRNA \uparrow) \rangle$ takes smallest value at around $n \simeq 10$, but are still smaller than 0.05 up to $n \simeq 400$. $\langle \log_{10} P_n^{P6 \leftarrow P30}(gene \uparrow \mid$

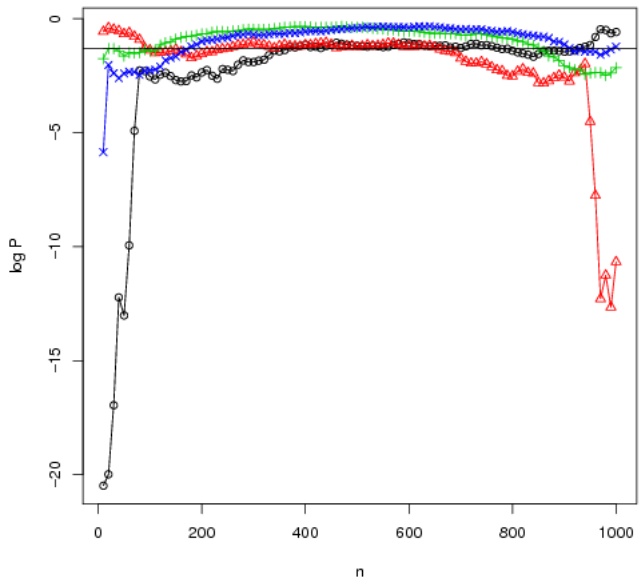

Fig. 2 P-values to check if reciprocal relationships stand between miRNAs and their target genes during the process from P30 to P6. A solid horizontal line indicates $P = 0.05$.
Black open circles: $\langle \log_{10} P_n^{P6 \leftarrow P30}(gene \downarrow | \ miRNA \uparrow) \rangle$
Red open triangles: $\langle \log_{10} P_n^{P6 \leftarrow P30}(gene \uparrow | \ miRNA \downarrow) \rangle$
Green crosses: $\langle \log_{10} P_n^{P6 \leftarrow P30}(miRNA \downarrow | \ gene \uparrow) \rangle$
Blue X marks: $\langle \log_{10} P_n^{P6 \leftarrow P30}(miRNA \uparrow | \ gene \downarrow) \rangle$,

$miRNA \downarrow) \rangle$ takes smallest value at around $n \simeq 1,000$ but are still smaller than 0.05 down to $n \simeq 600$. Thus, for wider range of n, reciprocal relationships are kept. In contrast to this, inverse relationships are weaker. However, $\langle \log_{10} P_n^{P6 \leftarrow P30}(miRNA \downarrow | \ gene \uparrow) \rangle$ and $\langle \log_{10} P_n^{P6 \leftarrow P30}(miRNA \uparrow | \ gene \downarrow) \rangle$ take P-values less than 0.05 for wider range of n than that between P30 and MB.

3.2.1 Upregulated miRNAs whose target genes are downregulated from P30 to P6

In the following, we have listed critical miRNAs as done for the comparison between P30 and MB. Table 3 denotes the results during process from P30 to P6, which is analogous to the results shown in Table 1. First of all, as in Table 1, Table 3 lists many miRNAs which belong to miRNA clusters; mir-106b and mir-93 from mir-106b-25 cluster, mir-17, mir-20a, mir-19a, mir-19b from mir-17-92 cluster, mir-20b and mir-19b from mir-106a-363 cluster. Thus this is biologically reliable and similar to Table 1. This coincidence confirms our above results are reasonable.

3.2.2 Downregulated miRNAs whose target genes are upregulated from P30 to P6

Table 4 which should correspond to Table 2 also gives us the confirmation. Let-7 family again appears in the list.

Table 3 Top 20 critical miRNAs during process from P30 to P6. Ratios for miRNA and mRNA are that of being listed as top 100 upregulated miRNA and miRNAs whose target genes are downregulated.

		Ratio	
	miRNAs	miRNA	mRNA
1	mmu-miR-106b	1.00	1.00
2	mmu-miR-130a	1.00	1.00
3	mmu-miR-130b	1.00	1.00
4	mmu-miR-15b	1.00	1.00
5	mmu-miR-17	1.00	1.00
6	mmu-miR-20a	1.00	1.00
7	mmu-miR-20b	1.00	1.00
8	mmu-miR-301b	1.00	1.00
9	mmu-miR-322	1.00	1.00
10	mmu-miR-721	1.00	1.00
11	mmu-miR-93	1.00	1.00
12	mmu-miR-542-3p	1.00	0.94
13	mmu-miR-3081*	1.00	0.88
14	mmu-miR-335-3p	1.00	0.88
15	mmu-miR-199a-5p	1.00	0.81
16	mmu-miR-199b*	1.00	0.81
17	mmu-miR-19a	1.00	0.81
18	mmu-miR-19b	1.00	0.81
19	mmu-miR-148a	0.75	0.94
20	mmu-miR-214	1.00	0.62

Besides expression ratio of miRNAs between two samples, we have checked if they are expressive larger than mean expression levels of miRNAs at the later samples. Almost all of miRNAs listed here satisfy this requirement. Only excep-

Table 4 Top 20 critical miRNAs during process from P30 to P6. Ratios for miRNA and mRNA are that of being listed as top 100 downregulated miRNA and miRNAs whose target genes are upregulated.

		Ratio	
	miRNAname	miRNA	mRNA
1	mmu-miR-29c	1.00	1.00
2	mmu-miR-376a	1.00	1.00
3	mmu-miR-451	1.00	1.00
4	mmu-let-7b	1.00	0.94
5	mmu-let-7e	1.00	0.94
6	mmu-let-7g	1.00	0.94
7	mmu-let-7i	1.00	0.94
8	mmu-miR-98	1.00	0.94
9	mmu-miR-126-3p	0.75	1.00
10	mmu-miR-299*	0.75	1.00
11	mmu-miR-29a	0.75	1.00
12	mmu-let-7a	0.75	0.94
13	mmu-miR-3070b-3p	1.00	0.69
14	mmu-miR-138	1.00	0.62
15	mmu-miR-3107	1.00	0.56
16	mmu-miR-181a-1*	0.50	1.00
17	mmu-let-7d	0.50	0.94
18	mmu-miR-1937b	0.25	1.00
19	mmu-miR-1937c	0.25	1.00
20	mmu-miR-337-5p	1.00	0.25

tion is mmu-miR-214, which is at the bottom of list of Table 3.

Considering the results presented here, we conclude that our method lists candidates of critical miRNAs for the formation of medulloblastoma.

4. Conclusion

In this paper, we have investigated mRNA and mRNA expression by Agilent microarrays and MiRaGE method for murine medulloblastoma tumorigenesis model. Obtained list of critical miRNAs whose expression are up/downregulated and target genes ' expression difference between two distinct samples are significant turns out to be biologically reasonable.

5. Acknowledgement

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