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ANALYSIS OF MICRORNA MICROARRAY (MM CHIP) DATA FOR AGING MICE
MODELS

By
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A Thesis
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School of Public Health and Information Sciences
University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of

Masters of Science

Department of Bioinformatics and Biostatistics
University of Louisville
Louisville, Kentucky

August 2012

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Analysis of MicroRNA Microarray (MM chip) data for Aging mice models

By

Vikranth Shetty

A Thesis Approved on

Aug. 2, 2012

Date

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ABSTRACT

ANALYSIS OF MICRORNA MICROARRAY (MM CHIP) DATA FOR AGING MICE MODELS

Vikranth Shetty

August 2nd, 2012

The present experimental designs have unique challenges in the analysis of microRNA expression intensity levels. In this project, various Normalization techniques were discussed with different statistical techniques and their application in an analysis performed at the Genes Centre of aging on mice models. The project involved repeated measures over time, and the intra-subject correlation caused by the repeated measurements should be incorporated into the analysis, resulting in additional significant miRNAs. Collection of multiple samples from the same individuals over time from an experiment may have issues with the commonly used methodologies-these issues are discussed here. Mixed model approach is used here to see the difference between different normalized data with the raw data with repeated measurements.

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1. INTRODUCTION

MicroRNA (miRNA) are small family of non-coding RNAs about 20 to 25 nucleotides in length that play a key role in regulating the gene expression in biological processes. miRNAs are coded by genes that are transformed into protein(1). They are processed from primary transcripts known as pri-miRNA to short stem loop structure called pre-miRNA and finally to function miRNA. In Simple words, miRNAs are small RNAs with a big role in gene regulation (1). The first miRNA family was discovered in *Caenorhabditis elegans* as genes that were required for the timed regulation of developmental events (1). All the miRNA genes have been documented in the MicroRNA Registry (<http://www.mirbase.org>).

miRNAs have various expression patterns and might regulate developmental and physiological processes. miRNAs are involved in post-transcriptional gene regulation in organisms ranging from plants to humans. Important roles are that comprise control of developmental timing, tissue growth, tumor suppression, cell differentiation and apoptosis (1). Many miRNAs are highly expressed in certain organs or cell types and some are only expressed in certain stages during development (1). These regulations in expression patterns coordinate with their functions; therefore, much research has concentrated on the expression studies of miRNAs. Several studies have examined the role of miRNAs in various diseases such as aging (2) and cancer. The clinical understanding of the role of miRNAs in aging is growing very rapidly. There are several different techniques used to obtain the expression levels of miRNAs and include microarray analysis and TaqMan PCR from Applied Biosystems.

This project was inspired by the research work regarding three key miRNAs that target Bcl2 protein, which causes the apoptosis of the cell in calorie restricted mice conducted at Gheens Centre of Aging, which is already published in Aging journal. The difference in this project is that we will use statistical methods to screen all miRNAs that may change with calorie restriction. The comparative expression of the miRNAs of interest was screened by miRNA microarrays (MM Chip) in brain samples of CR- and ad lib-fed mice. For comparative proteomic and miRNA expression analysis, 18 mice were divided equally into CR and Ad Lib populations across 3 age groups: 12, 24 and 28 months. All experiments were carried out in triplicate. The whole data structure is shown in the figure 1.

Figure 1. Whole data procedure

The three miRNAs, mmu-mir-181a-1*, mmu-mir-34a and mmu-mir-30e, exhibit the most significant down-regulation in brains of CR-fed mice, in an age-dependent manner, compared to *ad lib*-fed mice(2) Figure 2.

Figure 2.Down regulation of CR mice in three miRNAs in brain(2)

Initially MM chip method was used to find the miRNA expression levels for 361 miRNAs. Among them the three miRNAs mentioned above were found to be down regulated in CR brain and those three miRNAs targeting the Bcl2 protein (this was found using target prediction sites). In *ad lib*-fed mouse brain tissues Bcl-2 expression levels show an age-dependent decline. To confirm this qPCR analysis was done followed by the MM chip method.

The purpose of this study was to carry out different normalization techniques for the MM chip data for 361 miRNAs and to see which one is better among them. To consider the repeated measures within each age group without taking the average and see if we can find more significant miRNAs using repeated measurements along with the replicates in raw data and the normalized data.

2. MATERIALS AND METHODS

2.1.1 MicroRNA Microarray (MM chip) method

Most commercially available microarrays are designed with prefixed templates of genes of general interest, allowing investigators little freedom of choice in attempting to focus gene screening. This is where the designer microarray came into existence as next generation of DNA chips, allowing individual investigators with a user-friendly do-it-yourself approach gene screening from designing the probe templates to data mining(3).

In this method the samples are first filled in tubes and then using a robotic machine where these different sample tubes are converted into a single plate. This plate is placed in one of the racks in the robotic machine and all the names for the individual samples in the plate are labeled in the system with all the required settings. When the machine is started the robotic device will pick the sample plate from the racks and will place it on the red area as shown in the figure 3. The pointers used to print the blots will dip themselves into the samples and print those on the glass slide with nylon membrane on it (used to increase the signal). This printing process is repeated three times for each miRNA on the same plate. These dipping pointers are cleaned after printing each miRNA to avoid the technical error. Required number of glass slides are printed and then labeled accordingly.

After the printing is done the nylon membranes are removed from the glass plates and are kept in a bottle with 1-micro gram of RNA sample and run it overnight using cDNA coding. After this process, the nylon sheet with the printing spots

matches up with sample and gives microRNA expression. A high-resolution scanner is used to scan those miRNA expression levels into digital images. Dr. Wang's lab developed a software program, Gene Analyzer, which accomplishes background subtraction, array normalization and quantification. Net intensity was derived from the whole cell area measurement, and corrected using mean intensity of ring background of surrounding spots.

2.1.2 Real time RT-qPCR method

Real time PCR is one of the most sensitive and reliable quantitative methods for Gene expression analysis. One of the most important steps in relative quantitation experimental design is the selection of an appropriate endogenous control. Endogenous control is used for normalization. Any gene shown to be expressed at the same level in all the study samples can potentially be used as an endogenous control.

In terms of accuracy and specificity, RT-qPCR has become the method of choice for measuring gene expression levels. However, the accuracy of result is largely dependent on the proper data normalization. The real-time machine is connected to a computer and necessary software on the computer is needed to run the real time PCR machine in real-time mode as shown in figure 4.

- Insert the plate with the samples into the machine
- Enter names for all reactions
- Enter the volume(sample added in the plate)
- Select the number of cycles
- Select detector(choosing which miRNA you want to analyze)
- Setup amplification conditions(Heating)
- Click start
- Adjust the threshold after the Analysis based on your amplifications and Threshold Cycle.
- Standard Deviation will be higher if it is not well adjusted.

Figure 4. RT-qPCR Machine

2.2 Data Analysis Methods

2.2.1 Delta Ct method

The Delta Ct method subtracts the expression levels of endogenous controls from the remaining miRNA expression intensity values. Nine endogenous controls were used few among them are 18S rRNA1, bACTIN and GAPDH.

The formula representing this is:

$$\Delta CT = CT^{\text{target}} - CT^{\text{reference}}$$

Where CT^{target} is the individual value of all the miRNA expressions in the sample and $CT^{\text{reference}}$ is the endogenous control. The Delta Ct method is the most popular Normalization method due to the natural biologic motivation.

2.2.2 Quantile Normalization

The quantile normalization forces the distribution of Ct values to be the same across all the plates. The method takes the largest value and replaces it with the mean of the largest values, and then repeats for each subsequent data point.

The formula representing this is:

$$q_k^* = \text{proj}_{\mathbf{d}} \mathbf{q}_k = \frac{\mathbf{q}_k \cdot \mathbf{d}}{\mathbf{d} \cdot \mathbf{d}} \mathbf{d} = \frac{1}{\sqrt{M}} \sum_{m=1}^M q_{mk} \mathbf{d} = \left(\frac{1}{M} \sum_{m=1}^M q_{mk}, \dots, \sum_{m=1}^M q_{mk} \right)$$

where

$$\mathbf{d} = \left(\frac{1}{\sqrt{M}}, \dots, \frac{1}{\sqrt{M}} \right) \cdot \mathbf{d} = \left(\frac{1}{\sqrt{M}}, \dots, \frac{1}{\sqrt{M}} \right)$$

and q_k is the k th row of ordered intensity values. The intensity values are ordered for each plate independent of the other plates. The quantile normalization methodology is commonly used in the analysis of microarray expression values but the technique assumes that the distribution of the expression values is the same.

2.2.3 Rank invariant Normalization

The virtual reference sample is created by averaging the content of the reference group (in differential analysis) or the first group of the group set (in gene analysis). All probes ranked between Low Rank = 50th and High Rank =90th percentiles are considered. If the change of rank relative to the virtual reference is less than 0.05, the probe is considered to be “rank invariant.”

$$\frac{r_x - r_v}{r_v} \leq 0.05$$

Where, r_x is the individual miRNA expression miRNA and r_v is the endogenous control.

If less than 2% of all probes are picked as rank invariant, Low Rank is gradually decreased until it reaches the 25th percentile. Normalization coefficients are computed using iteratively reweighted least squares. This method is applied after the background normalization method described above.

2.2.4 Coefficient of variation

The empirical distribution (EDF) of the coefficient of variation is used to compare the various normalization techniques. We calculated the coefficient of variation for each miRNA over all the plates. Let K be the total number of miRNAs then the coefficient of variation is

$$CV_k = \frac{sd(Ct)}{\text{mean}(Ct)}, k = 1, 2, \dots, K$$

The empirical distribution of the coefficient of variation is created by using

$$\tilde{F} = \frac{1}{K} \sum_{k=1}^K I\{CV_k \leq t\}$$

2.2.5 Mixed model

A mixed model was constructed to consider the repeated sampling. Let $j = 1, 2$ be the two different groups 1 corresponds to control group(AD) and 2 corresponds to the Treatment group(CR). Let $t = 1, 2, 3$ be three time points 1 corresponds to 12 months, 2 corresponds to 24 months and 3 corresponds to 28 months. Let $l = 1, 2, 3$ be the repeated measures for each individual(mice). Let $k = 1, 2, 3$ be the repeated measurements for each time point. The following represent the model for each individual miRNA($n=361$).

$$= \alpha + \beta_j + \gamma_{jt} + \eta_{jlt} + \epsilon_{jkl}$$

Where,

α is overall mean

β_j is the effect on group j

γ_{jt} is the effect of time point in group j

η_{jlt} is the residual term

$\alpha, \beta, \gamma, \eta$ are the unknown parameters that need to be estimated.

3. RESULTS AND DISCUSSION

Three Normalization techniques have been used for this project 1). Delta Ct method, 2). Rank invariant method and 3).Quantile normalization method. R software was used to carry out all the analysis.

The Delta Ct method: This method is very popular and is a widely used normalization technique for Real time qPCR experiments. In this method, endogenous control is subtracted from the target gene to get the normalized expression value, this works well because the endogenous control in qPCR experiments is always less than the target genes. However for the MM chip data used in this project the data was already kind of normalized with the ring back ground intensity using software. The endogenous controls are expressed almost in the same range as target miRNA genes. Therefore the normalized values using the Delta Ct method are negative. So this method is not applicable to this method.

Rank invariant normalization: This method is similar to Delta Ct method since it uses the endogenous control to normalize the data. When we use this method we again get negative values after normalization. So this method is not applicable for our data.

Quantile normalization: This method worked well because it forced the distribution of all the plates to be the same. For this method endogenous control is not used as discussed in the methods section. By looking at the density plots below(Figure 5 - 12) for the control and the treatment between raw data and the quantile normalization at each age point in all the plates it clearly shows that quantile normalization is

consistent in all plates without any variation within the age groups and plates compared to raw data.

From the cumulative distribution graph in Figure 14 & 13. It is clear that the quantile normalization is better than the raw data but there is no huge difference between them this is because the data which we are using as raw data in this project is already kind of normalized so we are not able to see much difference between these two methods. If actual raw data was used, quantile normalization would be much higher in cumulative distribution graph.

Figure 5. Density plots for each age point in each plate in Control group-No normalization

Figure 6. Density plots for each age point in each plate in Control group – Quantile normalization

Figure 7. Density plots for each age point in each plate in treatment group - No normalization

Figure 8. Density plots for each age point in each plate in treatment group - Quantile normalization

Density Plots For AD

Figure 9. Density plots for all age points in each plate in Control group - No normalization

Density Plots For AD

Figure 10. Density plots for all age points in each plate in Control group - Quantile normalization

Density Plots For CR

Figure 11. Density plots for all age points in each plate in Treatment group – No normalization

Density Plots For CR

Figure 12. Density plots for all age points in each plate in Treatment group – Quantile normalization

Figure 13. Cumulative Distribution associated with normalization technique and the raw data for Control group

Figure 14. Cumulative Distribution associated with normalization technique and the raw data for Treatment group

Mixed model for replicates:

Most of the biologists use the mean/average of the repeated measures for doing the data analysis. By doing this you may not find more miRNAs significant. So here we considered using all the replicates for the analysis. We found 8 miRNAs which are significant in all the coefficients and p-values are below < 0.001 which are highly significant using the quantile normalization (Table 1.) and we found 6 miRNAs which are significant in all the coefficients and p-values are below < 0.001 which are highly significant using raw data (Table 2.) that is 10% more miRNAs. We found 1 miRNAs which are significant in both the methods (Table 2. & 3) and p-values are below < 0.001 which are highly significant. Interestingly, We found only 3 miRNAs which are significant in treatment and interaction term using quantile normalization (Table 5.) but whereas we found 14 miRNAs which are significant in treatment and interaction term using raw data (Table 6.) and the p-value here are around 0.03, here we didn't adjust the p-values using multiple adhoc if we do it then you may not find these many miRNAs using raw data which would be our future research.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-miR-148a	4.98 (0.3556)	-2.08 (0.4236)	-0.06 (0.0134)	0.10 (0.0189)
mmu-miR-127	2.94 (0.3715)	-1.99 (0.523)	-0.064 (0.0165)	0.088 (0.0234)
mmu-miR-135a	3.63 (0.265)	-0.93 (0.368)	-0.037 (0.0116)	0.036 (0.0164)
mmu-miR-146	3.54 (0.2287)	-1.24 (0.3149)	-0.030 (0.0099)	0.053 (0.0141)
mmu-miR-293	2.36 (0.4113)	-1.57 (0.5172)	-0.038 (0.0163)	0.07 (0.0231)
mmu-miR-129-3p	4.43 (0.4207)	-1.42 (0.5724)	-0.04 (0.0181)	0.054 (0.0256)
mmu-miR-380-3p	0.76 (0.1758)	0.64 (0.2421)	0.018 (0.0076)	-0.022 (0.0108)
mmu-miR-294	0.92 (0.2306)	-0.58 (0.2419)	-0.016 (0.0076)	0.027 (0.0108)

Table 1. Significant miRNA in Treatment, Age and Interaction using Quantile Normalization. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-miR-148a	4.88 (0.3736)	-2.19 (0.4659)	-0.06 (0.0147)	0.10 (0.0208)
mmu-miR-127	2.95 (0.3833)	-1.95 (0.5309)	-0.06 (0.0168)	0.09 (0.0237)
mmu-miR-146	3.60 (0.2519)	-1.25 (0.3454)	-0.03 (0.0109)	0.05 (0.0154)
mmu-miR-294	0.94 (0.2473)	-0.55 (0.2483)	-0.02 (0.0078)	0.02 (0.0111)
mmu-miR-129-3p	4.42 (0.4143)	-1.35 (0.562)	-0.04 (0.0177)	0.05 (0.0251)
mmu-miR-380-3p	0.82 (0.1454)	0.56 (0.1988)	0.02 (0.0063)	-0.02 (0.0089)

Table 2. Significant miRNA in Treatment, Age and Interaction using Raw data. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-let-7c	0.10 (0.2349)	0.84 (0.3266)	0.021 (0.0103)	-0.039 (0.0146)

Table 3. Significant miRNA in Treatment, Age and Interaction except the intercept using Quantile Normalization. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-let-7c	0.11 (0.2132)	0.78 (0.2947)	0.02 (0.0093)	-0.04 (0.0132)

Table 4. Significant miRNA in Treatment, Age and Interaction except the intercept using Raw data. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-miR-669c	-0.074 (0.1174)	0.29 (0.166)	0.014 (0.0052)	-0.017 (0.0074)
mmu-miR-325	0.048 (0.1406)	0.36 (0.1967)	0.017 (0.0062)	-0.018 (0.0088)
mmu-miR-211	8.85 (0.614)	-1.61 (0.8683)	-0.059 (0.0274)	0.079 (0.0388)

Table 5. Significant miRNA in Treatment and Interaction using Quantile Normalization. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-miR-293	2.34 (0.4381)	-1.40 (0.5134)	-0.03 (0.0162)	0.06 (0.0229)
mmu-miR-27a	4.59 (0.5445)	-2.08 (0.7095)	-0.04 (0.0224)	0.09 (0.0317)
mmu-miR-223	0.59 (0.1329)	0.63 (0.1879)	0.00 (0.0059)	-0.02 (0.0084)
mmu-miR-17-3p	0.40 (0.1125)	0.43 (0.1582)	0.00 (0.005)	-0.02 (0.0071)
mmu-miR-216a	0.33 (0.1107)	0.40 (0.1509)	0.01 (0.0048)	-0.02 (0.0067)
mmu-miR-125a	0.31 (0.1072)	0.35 (0.1513)	0.00 (0.0048)	-0.02 (0.0068)
mmu-miR-21	0.35 (0.1216)	0.52 (0.1699)	0.00 (0.0054)	-0.02 (0.0076)
mmu-miR-705	0.65 (0.239)	1.11 (0.3322)	0.00 (0.0105)	-0.04 (0.0148)
mmu-miR-667	0.72 (0.3252)	1.71 (0.4379)	0.02 (0.0138)	-0.07 (0.0196)
mmu-miR-320	0.22 (0.1015)	0.39 (0.1429)	0.00 (0.0045)	-0.02 (0.0064)
mmu-miR-26a	0.52 (0.2726)	0.95 (0.3667)	0.01 (0.0116)	-0.04 (0.0164)
mmu-miR-296	0.16 (0.1484)	0.53 (0.2099)	0.01 (0.0066)	-0.02 (0.0094)
mmu-miR-330	0.14 (0.1657)	0.64 (0.2343)	0.01 (0.0074)	-0.03 (0.0105)
mmu-miR-193	0.16 (0.1961)	0.72 (0.273)	0.01 (0.0086)	-0.03 (0.0122)

Table 6. Significant miRNA in Treatment and Interaction using Raw data. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-miR-34c	3.81 (0.2716)	-1.01 (0.3722)	-0.018 (0.0118)	0.035 (0.0166)
mmu-miR-27a	4.43 (0.4893)	-1.66 (0.6079)	-0.024 (0.0192)	0.074 (0.0272)
mmu-miR-223	0.56 (0.1299)	0.62 (0.1837)	0.001 (0.0058)	-0.022 (0.0082)
mmu-miR-17-3p	0.38 (0.1021)	0.43 (0.1426)	0.001 (0.0045)	-0.017 (0.0064)
mmu-miR-125a	0.31 (0.0963)	0.32 (0.1351)	0.002 (0.0043)	-0.015 (0.006)
mmu-miR-705	0.67 (0.2236)	0.94 (0.3103)	0.001 (0.0098)	-0.035 (0.0139)
mmu-miR-21	0.31 (0.1141)	0.56 (0.1583)	0.005 (0.005)	-0.020 (0.0071)
mmu-miR-216a	0.31 (0.1219)	0.49 (0.1701)	0.009 (0.0054)	-0.019 (0.0076)
mmu-miR-467b	0.31 (0.1213)	0.42 (0.17)	0.006 (0.0054)	-0.017 (0.0076)
mmu-miR-667	0.73 (0.3686)	1.89 (0.4879)	0.025 (0.0154)	-0.079 (0.0218)
mmu-miR-26a	0.51 (0.2647)	0.89 (0.3572)	0.008 (0.0113)	-0.037 (0.016)
mmu-miR-137	0.21 (0.1213)	0.37 (0.1715)	0.008 (0.0054)	-0.019 (0.0077)
mmu-miR-701	0.20 (0.1455)	0.57 (0.2006)	0.004 (0.0063)	-0.021 (0.009)
mmu-miR-296	0.15 (0.1527)	0.54 (0.216)	0.008 (0.0068)	-0.020 (0.0096)
mmu-miR-330	0.13 (0.169)	0.69 (0.2388)	0.011 (0.0075)	-0.028 (0.0107)
mmu-miR-193	0.15 (0.2076)	0.79 (0.289)	0.012 (0.0091)	-0.029 (0.0129)

Table 7. Significant miRNA in Age and Interaction using Quantile Normalization. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

NAME	$\hat{\alpha}$ (SE)	$\hat{\beta}$ (SE)	$\hat{\gamma}$ (SE)	$\hat{\eta}$ (SE)
mmu-miR-669c	-0.09 (0.1228)	0.32 (0.1737)	0.02 (0.0055)	-0.02 (0.0078)
mmu-miR-19b	0.08 (0.1412)	0.35 (0.1937)	0.01 (0.0061)	-0.02 (0.0087)
mmu-miR-325	0.03 (0.1438)	0.39 (0.2015)	0.02 (0.0064)	-0.02 (0.009)

Table 8. Significant miRNA in Age and Interaction using Raw data. $\hat{\alpha}$, $\hat{\beta}$, $\hat{\gamma}$ and $\hat{\eta}$ are the coefficient values of intercept, treatment, age, and age*treatment respectively. The values in parenthesis are the standard error values for the given coefficient.

4. CONCLUSIONS

Three Normalization methods were used in this study: 1). Delta Ct method, 2). Rank invariant method and 3). Quatile normalization method. Among these three normalization methods for MicroRNA Microarray (MMchip) analysis, Delta Ct method and Rank invariant method does not work/apply because reference gene is used to normalize. Clearly we can see that Quantile normalization is the best normalization method for MM chip analysis because it does not use reference gene to normalize the raw data. This is the only method which can be used in both qPCR analysis and the MM chip analysis. Using the whole date we can figure that more miRNA's are significant. In the studies like this one it's always better to use all the repeated samples rather than just taking the averages of the repeated samples to avoid the error within the replicates. The study shows how to do the sample size calculation with repeated replicates.

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