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Inference of target gene regulation via miRNAs during cell senescence by MiRaGE Server

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MiRNAs are recently known to be critical players causing cell senescence, by regulating target genes. Thus inference of miRNAs critically regulating target genes is important. However, miRNAs critically regulating target genes are believed to have significant fold changes, typically upregulations, during cell senescence. In this study, we consider the target gene regulation by miRNAs together with miRNAs expression change during fibroblast IMR90 cell senescence. Then we found that the simultaneous consideration of two criterion lists more feasible miRNAs: i.e., miRNAs being more often reported to be down/upregulated and/or having biological backgrounds inducing cell senescence. Thus, the amount of target gene regulation, which can be inferred by the recently developed MiRaGE Server, is recommended to be considered together for the estimation of miRNAs critically contributing to cell senescence.

1. Introduction

miRNAs are small non-coding RNAs playing important roles in a variety of biological processes. miRNAs are negatively regulating expression at the post-transcriptional level of specific target genes[1]. Because a single miRNA has the potential to regulate hundreds of mRNAs, it is conceivable that miRNAs are important regulatory molecules in complex biological processes, such as aging and cancer. They were recently reported to be one of critical players controlling the senescent processes of cells[2]. Despite this need, there are not yet any effective experimental procedures that can evaluate directly the target gene regulation by miRNAs. Target genes themselves are often computationally predicted[3], thus they are believed to include not-so-small-number of false positives. Because of this limitation, critical miRNAs for cell senescence are selected among those expressive largely in senescent cells[4]. Target gene expression change was considered only after estimating critical miRNAs. No critical miRNAs were selected based upon the target gene expression changes during cell senescence.

On the other hand, Hackl *et al*[5] recently reported that some miRNAs, including the member of miRNA-17-92 cluster, are significantly downregulated during cell senescence. This was the first pointing out the biological importance of miRNAs not upregulated during cell senescence. More recently, Wang *et al*[6] shows that the suppression of miRNAs known to be downregulated during cell senescence can induce cell senescence. That is, miRNAs can contribute to the progress of cell senescence even if they are not expressive in senescent cells.

More recently, Dhahbi *et al*[7] by next generation sequencing (NGS) investigated miRNAs up/downregulated during cell senescence. Then they found many more up/downregulated miRNAs than using microarray. Although the large number of miRNAs up/downregulated is because of the ability of NGS, the other problem occurs. If the number of miRNAs up/downregulated is more than hundreds, which one is the most important? Must most of hundreds miRNAs be up/downregulated to induce cell senescence? Although Dhahbi *et al*[7] showed that a set of genes targeted by miRNAs up/downregulated are biologically informative, ranking the importance of individual miRNA during cell senescence has not yet been done.

In this study, we have proposed one way to rank miRNAs based upon the criticality during cell senescence: ranking miRNA based upon the amount of regulation of target genes by miRNAs. It enables us to find "important" miRNAs among hundreds of up/downregulated miRNAs.

2. Materials and Methods

2.1 mRNA expressions

mRNA expression profiles with accession numbers GSE19018 and GSE15919 are downloaded from gene expression omnibus (GEO). The former is for IMR90 cell lines and the later for MRC5 cell lines. For GSE19018, CEL files are treated by rma function in affy package in Bioconductor in R[8]. For young samples, IMR90 cell lines at PD 30 under 20% oxygen (GSM4870491, GSM470492 and GSM470493) are employed. Those at PD 48 (GSM4870494, GSM470495 and GSM470496) are regarded to be senescent samples. For GSE15919, raw files are

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downloaded. Since it is two color dye, we have treated each dye as an independent sample. That is, MRC5 fibroblasts at PDL 28 (F532 Median of GSM399555, and GSM399561, GSM399570, and F635 Median of GSM399560, GSM399569, and GSM399581) are taken to be young samples. Senescent samples consist of those at PDL 63 (F635 Median of GSM399555, and GSM399561, GSM399570, and F532 Median of GSM399560, GSM399569, and GSM399581).

2.2 Transformation of gene expression using both principal component analysis and linear discriminant analysis

In order to extract distinct components between young and senescent samples, we have transformed gene expression profiles as follows. At first, we have applied principal component analysis (PCA) to gene expression. Then principal component scores are attributed to each sample. Next, we try to discriminate young and senescent samples with optimal number of principal components by linear discriminant analysis and successfully discrminated between senescent and young cell lines. Finally, we have obtained transformed expression x_g of gene g as,

$$x_g = \sum_i a_{gi} b_i \tag{1}$$

$$L_s = \sum_i b_i PCS_{is} \tag{2}$$

$$PCS_{is} = \sum_g a_{gi} x_{gs}, \tag{3}$$

where PCS_{is} is the ith principal component score attributed to sth sample used for discriminations, x_{gs} is the expression of gene g at sth sample. L_s is discriminant function such that positive (negative) L_s values mean senescent (young) samples. All of these analyses are done by several packages in R.

2.3 Inference of target gene expression via MiRaGE server

Target gene regulation by miRNAs is inferred by MiRaGE server[11]. Gene expression to be uploaded to MiRaGE Server of genes at young samples is taken to be unit and that at senescent cells is $\exp(x_g)$. All options other than "Select the conservation of miRNA" which is taken to be "all" are defaults.

2.4 miRNA expression extraction from sequencing data

Fastq files of GSE27404 are downloaded from ftp server[9]; accession numbers are SRR107296 for young IMR90 cell lines and SRR107297 for senescent IMR90 cell lines, respectively. The young (senescent) sample is the IMR90 cell line at PD 14 (34). Obtained fastq files are treated by miRDeep2[10]. Reads are aligned using mapper.pl to hg19 human genome with adapter sequence TCGTATGCCGTCTTCTGCTTGT being trimmed. Obtained arf and fastq files are analyzed by miRDeep2.pl. Obtained csv files of miRNA expression is used for further analysis.

2.5 Statistical Validation

In order to see if inference by MiRaGE is trustable, some statistical test is applied to obtained results.

2.5.1 Coincidence of miRNAs ranking based upon P-values between two distinct cell lines

We have checked if coincidence between the results for IMR90 cell lines and those for MRC5 cell lines is significant. P-values are computed via binominal distribution,

$$P(x, N, N/N_{all}),$$

where $P(x, N, N/N_{all})$ is binominal distribution function that the number of common miRNAs is greater than x when top ranked N miRNAs are considered for each of cell lines and N_{all} is total number of miRNAs considerd.

2.5.2 Correlation coefficients between the senescence-associated miRNA expression change and the rejection probability for target gene regulation

The miRNA expression in the young cell are subtracted from the miRNA expression in the senescent cell. Then, the resulting senescence-associated miRNA expression changes are compared with the rejection probability for target gene regulation obtained using MiRaGE server. The miRNA is excluded from the analysis when the miRDeep scores in both the young cell and the senescent cell are less than the threshold value. The miRNA is also ignored when the rejection probability for target gene regulation is not significant, i.e., not less than 0.05. Then, Pearson's correlation coefficients and associated P-values are computed between the miRNA expression changes and the rejection probabilities for target gene regulation, for several threshold values: none, 0, 1, 10, $\ldots, 10^4$.

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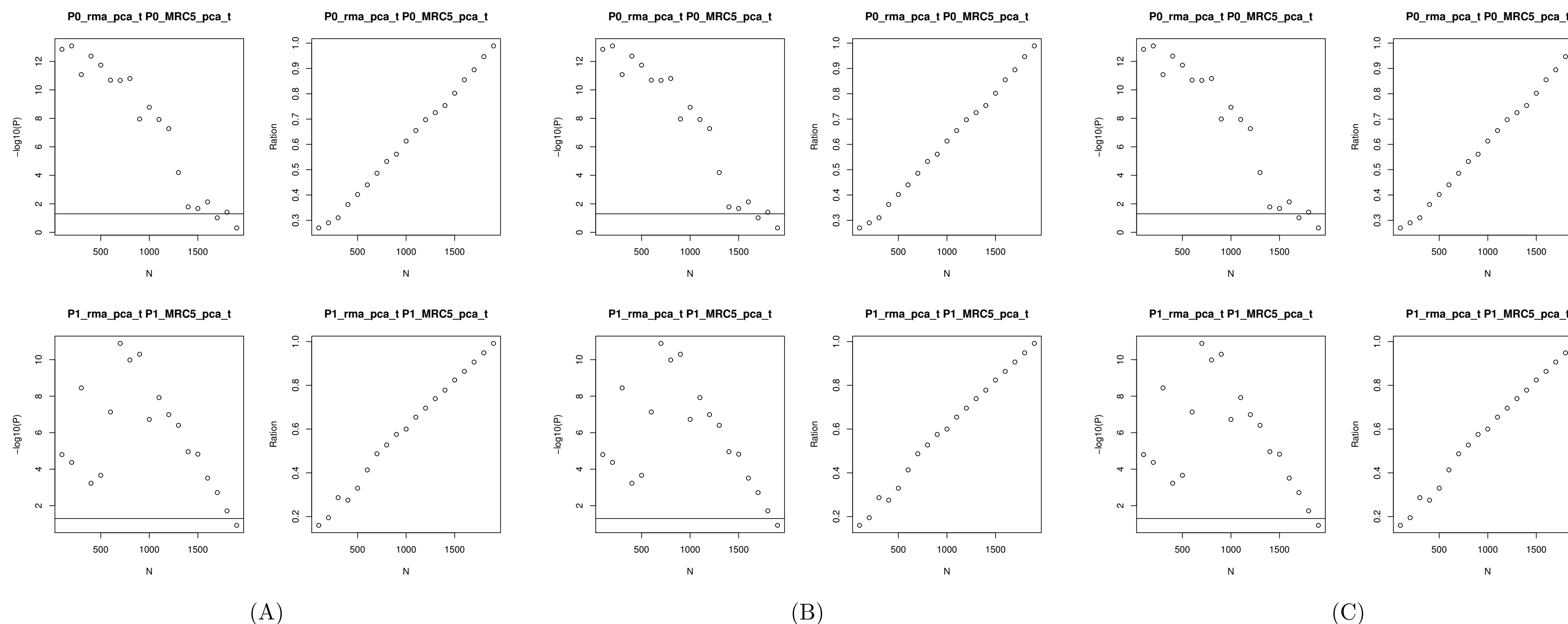

Fig. 1 The estimates of the number of commonly selected miRNAs between IMR90 and MRC5 and associated P-values by (A) t-test, (B) Wilcoxon test, and (C) Kolmogorov-Smirnov test. Left column : P-values of the number of commonly selected minRNAs based upon binominal distribution (see Materials and Methods in main text). If no points are plotted, this means that P-values are too small to plot within this scale. Horizontal solid lines indicate $P = 0.05$. Thus points above these lines are significant. Right column: the ratio of commonly selected miRNAs to N, which is the number of selected miRNAs from each of cell lines. Rows :Upper (lower) row corresponds to the ranking of miRNAs whose target genes are up(down)regulated in senescent cells based upon P-values computed by MiRaGE Server with t test.

3. Results

First of all, P-value is attributed to each miRNA via MiRaGE server[11]. The P-values, the rejection probabilities of target gene regulation, are measures of how likely target genes are up/downregulated if each miRNA does not regulate target genes. Thus, smaller P-value indicates more plausible target gene regulation by each miRNA. After statistical validation, we have found that top ranked miRNAs are often common between IMR90 cell lines and MRC5 cell lines ($P < 10^{-10}$, see Fig.1). If we remember that these two rankings are obtained from two different experiments for the distinct cell lines, it is remarkable coincidence and shows that our bioinformatic analysis are confident.

We have also calculated the correlation coefficients between the P-values ob-

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tained and the senescence-associated miRNA expression changes for IMR90 cell lines computed from Dhahbi *et al*'s[7] NGS results. Then we found that the P-values associated with the correlation coefficients are often significant (e.g., typically $P < 10^{-2}$, not shown here).

Thus, we concluded that the senescence-associated miRNA expression changes match the rejection probabilities of target gene regulation.

4. Discussion

Although in the previous section, we successfully showed that our inference of target gene regulation is biologically informative, here we will confirm the validity of our inference when selecting critical miRNAs during cell senescence.

Table 1 miRNAs downregulated during cell senescence. If the rejection probability for target gene upgulation is not significant, i.e., not less then 0.05, the miRNA is omitted. FC: Inversed fold change during cell senescence. NMRC: normalized mature read count such that summation over all over miRNAs is taken to be unity. SCORE: miRDeep socre. P-value : the rejection probabilities for target gene upregulation during cell senescence, computed with t-test implemented in MiRaGE Server.

	senescent		young			
miRNA	NMRC	SCORE	NMRC	SCORE	P-value	FC
hsa-miR-143-5p	1.41e-04	7.36e+03	5.87e-04	2.43e+04	1.91e-02	4.16e+00
hsa-miR-155-5p	1.30e-04	0.00e+00	1.32e-03	7.33e+03	2.29e-02	1.02e+01
hsa-miR-16-5p	3.53e-05	0.00e+00	3.57e-04	2.00e+03	1.65e-03	1.01e+01
hsa-miR-199a-3p	3.08e-02	1.57e+05	8.29e-02	4.61e+05	1.55e-02	2.69e+00
hsa-miR-199b-3p	3.08e-02	1.57e+05	8.29e-02	4.60e+05	1.55e-02	2.69e+00
hsa-miR-214-3p	1.55e-04	0.00e+00	2.02e-04	1.14e+03	6.89e-03	1.30e+00
hsa-miR-27a-5p	3.82e-06	5.15e+03	6.57e-06	2.76e+03	1.68e-04	1.72e+00
hsa-miR-423-3p	3.50e-04	3.68e+04	7.10e-04	3.64e+04	3.33e-03	2.03e+00
hsa-miR-424-5p	1.73e-04	2.24e+03	6.25e-04	7.02e+03	1.65e-03	3.62e+00
hsa-miR-503	9.74e-04	4.96e+03	2.00e-03	1.11e+04	1.67e-02	2.05e+00

To demonstrate the validity of our inference, in Tables 1 and 2, we list the miRNAs that are down/upregulated and suppress target genes. That is, if the miRNA is upregulated during cell senescence, the rejection probability of the target genes downregulation should be small. The threshold miRDeep score is taken to be 10^4 such that the P-value associated with the correlation coefficient is the smallest.

In Table 1, the miRNAs downregulated during the cell senescence are listed.

Table 2 miRNAs upregulated during the cell senescence. FC: fold change during cell senescence. P-value : the rejection probabilities for target gene downregulation during cell senescence, computed with t-test implemented in MiRaGE Server. Other notations are the same as Table 1.

	senescent		young			
miRNA	NMRC	SCORE	NMRC	SCORE	P-value	FC
hsa-let-7a-5p	1.71e-01	4.53e+05	1.44e-01	4.36e+05	3.16e-05	1.19e+00
hsa-let-7c	8.82e-02	0.00e+00	8.68e-02	1.29e+04	3.16e-05	1.02e+00
hsa-let-7e-5p	9.18e-02	1.20e+05	7.33e-02	6.39e+04	3.16e-05	1.25e+00
hsa-let-7f-5p	1.47e-01	3.57e+05	1.29e-01	3.48e+05	3.16e-05	1.14e+00
hsa-let-7i-5p	1.72e-02	8.74e+04	1.17e-02	6.51e+04	3.16e-05	1.47e+00
hsa-miR-10a-3p	5.01e-05	1.86e+04	4.85e-05	2.04e+04	2.80e-09	1.03e+00
hsa-miR-125a-5p	6.03e-04	3.10e+03	3.12e-04	1.78e+03	2.45e-02	1.93e+00
hsa-miR-125b-5p	3.01e-03	1.59e+04	1.91e-03	1.13e+04	2.45e-02	1.58e+00
hsa-miR-136-5p	2.14e-04	1.26e+03	2.11e-04	1.30e+03	2.96e-06	1.01e+00
hsa-miR-154-3p	2.55e-04	1.46e+03	2.28e-04	1.41e+03	2.02e-02	1.12e+00
hsa-miR-154-5p	3.05e-05	1.46e+03	2.46e-05	1.41e+03	4.48e-02	1.24e+00
hsa-miR-181a-5p	5.08e-03	2.63e+04	4.17e-03	2.36e+04	7.51e-04	1.22e+00
hsa-miR-181b-5p	5.89e-03	2.85e+04	3.63e-03	1.62e+04	7.51e-04	1.62e+00
hsa-miR-181d	4.70e-03	0.00e+00	3.04e-03	5.38e+03	7.51e-04	1.55e+00
hsa-miR-193a-5p	5.05e-04	3.00e+03	3.07e-04	2.61e+03	4.81e-04	1.65e+00
hsa-miR-221-3p	7.69e-02	3.92e+05	4.75e-02	2.66e+05	7.59e-04	1.62e+00
hsa-miR-23a-3p	3.33e-03	1.61e+04	1.71e-03	9.02e+03	4.64e-02	1.95e+00
hsa-miR-23b-3p	3.09e-03	3.53e+03	1.59e-03	2.88e+03	4.64e-02	1.95e+00
hsa-miR-30a-3p	2.62e-03	5.14e+04	1.06e-03	1.83e+04	6.54e-04	2.47e+00
hsa-miR-30d-3p	7.04e-06	4.25e+03	4.17e-06	8.38e+03	6.54e-04	1.69e+00
hsa-miR-30e-3p	2.04e-03	2.54e+03	9.28e-04	3.23e+03	6.54e-04	2.19e+00
hsa-miR-323b-3p	1.98e-04	1.01e+03	4.93e-05	0.00e+00	7.06e-04	4.02e+00
hsa-miR-323b-5p	1.31e-06	1.01e+03	3.70e-07	0.00e+00	2.31e-02	3.53e+00
hsa-miR-369-3p	2.79e-03	1.47e+04	9.57e-04	5.62e+03	1.67e-02	2.92e+00
hsa-miR-369-5p	9.69e-05	1.47e+04	5.47e-05	5.62e+03	1.27e-02	1.77e+00
hsa-miR-382-5p	1.33e-03	7.30e+03	1.03e-03	6.48e+03	3.67e-03	1.29e+00
hsa-miR-409-3p	6.01e-04	0.00e+00	4.86e-04	3.72e+03	2.25e-02	1.24e+00
hsa-miR-485-3p	5.62e-04	7.02e+03	5.04e-04	5.28e+03	1.88e-02	1.12e+00
hsa-miR-493-5p	2.68e-04	2.60e+03	8.84e-05	1.05e+03	1.70e-03	3.04e+00
hsa-miR-494	4.35e-04	2.22e+03	2.09e-04	1.17e+03	2.78e-02	2.08e+00
hsa-miR-495	2.68e-03	1.36e+04	1.30e-03	7.25e+03	4.65e-04	2.06e+00
hsa-miR-98	3.76e-04	1.52e+03	1.87e-04	0.00e+00	3.16e-05	2.01e+00

Although the number of the listed miRNAs is small, they are frequently reported to be downregulated during the cell senescence. For example, miR-155-5p is downregulated in RPTEC, T-Cells (CA), and HDF . miR-16 is downregulated in HDF, HUVEC, RPTEC, and MSC. miR-199a/b-3p and miR-214 are downregulated in Foreskin. miR-27a is downregulated in T-cells (CA/RA). miR-423-3p is

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downregulated in Foreskin and T-cells(RA). miR-424 is downregulated in MSC, Foreskin, and RPTEC (Table S2 in 5)). Although the cell senescence-associated downregultion of miR-143 was not reported, Khach Lai *et al*[12] reported that the miR-143 downregulation induces the cell cycle arrest in MSC. Since the cell senescence is caused by the cell cycle arrest, the cell cycle arrest induced by the miR-143 downregulation matchs the miR-143 downregulation during the cell senescence. Although miR-503 was not reported to be downregulated during the cell senescence, the miR-503 downregulation induced apoptosis in MSC[13]. Apoptosis is often related to the cell senescence. For example, apoptosis or cell senescence is often caused by the same protein, e.g., TGF-β[14)–15)]. Thus induction of apoptosis by miR-503 downregulation matches the miR-503 downregulation during the cell senescence. miR-155 is reported to be downregulated in senescent human fibroblast[16] and senescent human foreskin (BJ) fibroblasts[17]. miR-27b and miR-199a are downregulated in the aged patients' MSC[18].

Table 2 lists the miRNAs upregulated during the cell senescence. Many of them have the previous publications related to downregulation during the cell senescence. Let-7c and 7f are upregulated in the senescent fibroids[19]. miR-136 is upregulated in senescent WI-38 fibroblast[20]. Both miR-409-3p and miR-495 are up-regulated in senescent WI-38 cells[6]. miR-30 is up-regulated during induced and replicative senescence[21]. miR-23a/b are upregulated in T-cells(CA/RA) (Table S2 in 5)). Although miR-221 was not reported to be upregulated during the cell senescence, Chen *et al*[22] reported that miR-221 upregulates GAX. GAX is known to cause cell senescence. Overexpression of miR-125 and miR-181 induces senescence through downregulation of Cbx7[23]. Mir-125b also induces senescence in human melanoma cells[24].

Although not all miRNAs listed in Tables 1 and 2 have the previous reports supporting the findings here, the most of miRNAs have. In addition to this, we can expect that the miRNAs yet without supporting reports will soon have them, since new findings are reported every week.

Before closing this section, it is important to recognize that not all miRNAs have more than two fold changes. This suggest that not only fold changes but also target gene regulations are better to be considered.

5. Conclusion

We have proposed the usage of the information of inference of target gene regulation by miRNAs together with miRNA expression in order to estimate critical miRNAs during cell senescence. In fact, recently, Liang *et al*[25] reported that miRNA expression does not always reflect miRNA activities. Their reports also support our strategy; consideration of target gene regulation together with miRNA expression. We have shown that this strategy enable us to list miRNAs not detected solely by miRNAs expressions. Considering target gene regulation and miRNA expression together is highly recommended to estimate critical miRNAs, especially when NGS is used to measure miRNAs expression which allows us to detect more subtle changes during cell senescence and lists more candidates for critical miRNAs than microarray analysis.

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