Research Article

miRNA-191 and miRNA-548c-3p Expression in Embryo Culture Medium and Their Association with Chromosomal Status

Riyan Hari Kurniawan[1,3] Raden Muharam Natadisastra[1,3], Indah Suci Widyahening[2] Pritta Ameilia Iffanolida[3], Budi Wiweko[1,3]

[1]Department of Obstetrics and Gynecology
[2]Department of Community Medicine
[3]Human Reproductive, Infertility and Family Planning Research Centre
Indonesian Medical Education and Research Institute
Faculty of Medicine Universitas Indonesia
Dr. Cipto Mangunkusumo General Hospital
Jakarta

Abstract

Objective: To analyze the relationship between the expression levels of miRNA-191 and miRNA-548c-3p in embryo culture media and embryo chromosomal status.

Methods: This cross-sectional study was conducted on 30 embryos from 12 patients aged 28–40 years who underwent an in vitro fertilization (IVF) program in three clinics. Embryos were cultured until the blastocyst stage. PGT-A was performed after blastocyst biopsy on day 5 or 6, and chromosomal analysis was carried out using the NGS method. The expression levels of miRNA-191 and miRNA-548c-3p were analyzed from embryo culture media samples using the qPCR method. The relationship between the expression levels of these miRNAs and embryo chromosomal status was then assessed.

Results: There were no significant differences in subjects' characteristics between the euploid and aneuploid embryo groups ($p > 0.05$). There was no significant difference in the expression level of miRNA-191 between euploid and aneuploid embryos (median quantification 7.260 vs 1.039, $p = 0.497$). Similarly, there was no significant difference in the expression level of miRNA-548c-3p between the two groups (median quantification 1.919 vs 4.311, $p = 0.707$).

Conclusion: The expression levels of miRNA-191 and miRNA-548c-3p show no relationship with embryo chromosomal status. Their expression in embryo culture media does not appear to be a good biomarker candidate for predicting embryo chromosomal status.

Keywords: Chromosomal status, IVF, miRNA-191, miRNA-548c-3p.

Correspondence author. Riyan H. Kurniawan. Department of Obstetrics and Gynecology.
Faculty of Medicine Universitas Indonesia. Dr. Cipto Mangunkusumo General Hospital. Jakarta.
Email: riyanharikurniawan@yahoo.com

INTRODUCTION

In Vitro Fertilization (IVF) is a form of Assisted Reproductive Technology (ART) used to achieve pregnancy in infertile couples.[1,2] The success of IVF is closely related to the number and quality of embryos produced.[2] However, the methods currently used to assess embryo quality in IVF remain imperfect.[1] Embryo selection is still primarily based on morphological criteria. In the blastocyst stage, embryos with good morphological features are assumed to have normal chromosomal status and are selected for transfer. Nevertheless, it is now known that morphologically normal blastocysts may still carry chromosomal abnormalities.[3] Thus, embryo assessment based solely on morphology is considered imprecise, subjective, and has a low predictive value for implantation success.[4]

Preimplantation Genetic Testing for Aneuploidies (PGT-A) is a method used to determine embryo chromosomal status. PGT-A evaluates the number of chromosomes in an embryo through biopsy of blastomeres,

trophectoderm cells, or polar bodies.[5] However, this invasive technique has several drawbacks. it may interfere with embryonic development because cells are removed during biopsy,[6] it is relatively expensive, and it requires operators with specialized skills.

MicroRNAs (miRNAs) are small non-coding RNAs (~22 nucleotides) that regulate gene expression by binding to target mRNAs and inhibiting protein translation. miRNAs are present in almost all cells, including those involved in embryonic development and stem cell differentiation. They are highly expressed in rapidly dividing and undifferentiated cells, such as cancer cells and embryonic stem cells.[3]

Because miRNAs are highly expressed in rapidly growing cells, it is hypothesized that they are also highly expressed in embryos and may show distinct expression patterns in euploid versus aneuploid embryos. miRNAs are known to be secreted into the surrounding medium, leading to the hypothesis that embryos may release miRNAs into the culture medium. miRNAs are considered promising biomarker candidates because they are stable, resistant to degradation, correlated with pathological conditions, and easy to detect. Based on these characteristics, miRNAs have the potential to serve as biomarkers for embryo selection in IVF. The authors hypothesize that miRNAs secreted into IVF culture media could be used to assess embryo chromosomal status.

This study aims to analyze the relationship between the expression levels of miRNA-191 and miRNA-548c-3p in embryo culture media and embryo chromosomal status. It also investigates whether these miRNAs can serve as biomarkers for assessing chromosomal status in embryos. miRNA-191 was selected because previous studies have shown its association with embryo chromosomal status, while miRNA-548c-3p was included to strengthen the study and due to reagent availability in the laboratory. The demographic factors assessed include maternal age, number of blastocysts per patient, duration of ovarian stimulation, number of retrieved oocytes, number of mature oocytes, fertilization rate (%), presence of endometriosis, and ovulatory disorders.

METHODS

This research was a cross-sectional study, and subjects were recruited through consecutive sampling. The study included all patients who underwent IVF procedures in three IVF clinics between January 2021 and April 2021 and met the inclusion criteria: women undergoing an IVF program with ICSI fertilization who submitted embryo samples for PGT-A testing and agreed to participate. Patients who failed oocyte pick-up, embryos that did not develop to the blastocyst stage on day 5 or 6, embryos with abnormal pronuclear morphology (0PN, 1PN), or embryos contaminated during culture were excluded.

All patients underwent an IVF cycle using the GnRH antagonist stimulation protocol (short protocol). Choriogonadotropin alfa (250 mcg/0.5 mL) was used as the trigger. Embryos from participants with any infertility diagnosis were cultured to the blastocyst stage. Culture medium was replaced on day 3 and day 5. PGT-A was performed after blastocyst biopsy on day 5 or 6, and chromosomal analysis was carried out using the NGS method. After PGT-A, culture medium samples were collected. Expression levels of miRNA-191 and miRNA-548c-3p in culture media were then analyzed using qPCR. Based on PGT-A results, embryos were categorized as euploid or aneuploid. The relationship between the expression levels of the two miRNAs and embryo chromosomal status was subsequently analyzed.

Subjects' characteristics were also assessed, including maternal age, number of blastocysts per patient, stimulation duration, number of retrieved oocytes, number of mature oocytes, fertilization rate (%), parity, total rFSH dose (IU), presence of endometriosis, and presence of ovulatory disorders. Fertilization rate was calculated as the percentage of microinjected oocytes that developed into two pronuclei (2PN). Ovulatory disorders were defined as irregular menstrual cycles or midluteal progesterone < 10 ng/mL.

MicroRNA was isolated from embryo culture media using the mirVana miRNA Isolation Kit without phenol (Invitrogen, USA), following the manufacturer's instructions. MicroRNA quantity was measured using the Qubit microRNA Assay Kit (Thermo Fisher, USA). Expression levels were evaluated using the TaqMan MicroRNA

Assay with probe designs from the miRBase database for hsa-mir-191 and hsa-mir-548c-3p. Complementary DNA (cDNA) synthesis was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). The expression levels of miRNAs were quantified with TaqMan Fast Advanced Master Mix (Applied Biosystems) on the PCRmax Eco real-time PCR system. Relative expression was calculated using the U6 microRNA assay as the internal control.

Trophectoderm biopsies and embryo culture media, along with positive (male genomic DNA; Promega G1471) and negative controls (amplification mixture only), were lysed before genomic DNA fragmentation and amplification using the 24SurePlex DNA Amplification System (Illumina, San Diego, CA, USA), according to manufacturer protocols. All samples, including controls, were prepared for sequencing using the VeriSeq library preparation kit (Illumina).

Biopsied cells and 2.5 µL embryo culture media were mixed with 2.5 µL 1× PBS, lysed with 2.5 µL cell extraction buffer and 5 µL extraction cocktail (SurePlex), and incubated at 75°C for 10 minutes followed by 95°C for 4 minutes. Pre-amplification was performed by adding 5 µL SurePlex pre-amplification cocktail and running the following thermal protocol: one cycle of 95°C for 2 minutes, followed by 12 cycles of 95°C for 15 seconds, 15°C for 50 seconds, 25°C for 40 seconds, 35°C for 30 seconds, 65°C for 40 seconds, and 75°C for 40 seconds, then holding at 4°C. Subsequently, 60 µL of SurePlex amplification cocktail was added to 15 µL of the synthesis product, and amplification proceeded for one cycle of 95°C for 2 minutes, followed by 14 cycles of 95°C for 15 seconds, 65°C for 1 minute, and 75°C for 1 minute. Amplification success was confirmed using a 1.5% agarose gel.

Unpurified products were quantified using the Qubit dsDNA HS Kit (ThermoFisher Scientific). DNA concentration was adjusted to 0.2 ng/µL. For tagmentation, 5 µL of DNA was mixed with the VeriSeq PGS transposome by adding TD buffer and ATM, incubated at 55°C for 5 minutes, then cooled to 10°C before adding NT buffer. Limited-cycle PCR was then performed by adding index adapters (i7 and i5) and NPM buffer. The thermal protocol included: 72°C for 3 minutes, 95°C for 30 seconds, followed by 12 cycles of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, then 72°C for 5 minutes and hold at 4°C.

PCR cleanup was performed using AMPure XP beads (Beckman Coulter). Beads were mixed with PCR products, incubated, and washed twice with 80% ethanol. Purified libraries were eluted in Nextera XT Resuspension Buffer, normalized using the VeriSeq bead-based kit, and pooled. Sequencing was conducted on the MiSeq NGS platform (Illumina) for 8 hours. Chromosomal analysis was performed using BlueFuse 2.0 software.

Data were analyzed using IBM SPSS Statistics version 25. Univariate analysis was conducted for demographic variables, including numeric variables (maternal age, miRNA-191 expression, miRNA-548c-3p expression) and categorical variables (presence of endometriosis, ovulatory disorders, embryo chromosomal status). Data distribution was assessed using the Kolmogorov–Smirnov test. For bivariate analysis, comparisons between two groups were performed using the independent t-test for normally distributed variables and the Mann–Whitney U-test for non-normally distributed variables. A p-value < 0.05 with a 95% confidence interval was considered statistically significant.

RESULTS

This study was conducted on 30 embryos obtained from 12 patients. The demographic characteristics of the subjects are presented as median (minimum–maximum) values in Table 1. The analysis began with the assessment of embryo chromosomal status, followed by evaluation based on miRNA expression levels.

Among the 30 blastocysts analyzed, the results were evenly distributed: 15 euploid and 15 aneuploid blastocysts (50% each). After assessing the relationship between patients' characteristics and embryo chromosomal status, no statistically significant differences were found between the two groups ($p > 0.05$), as shown in Table 1.

Table 1. Characteristics of 12 subjects and 30 blastocysts

Characteristics of 12 subjects	Median (min-max)	Euploidy (n=15)	Aneuploidy (n=15)	*P-value*	Test
Age	36.00 (28 – 40)	35.0 (28.0-37.0)	33.0 (28.0-40)	0.690	Mann-Whitney
Number of blastocysts per patient	2.00 (1-5)	1.25 (0-3)	1.25 (0-3)	0.819	Mann-Whitney
Stimulation days per patient	9.00 (7-13)	9.0 (7.0-11.0)	9.0 (7.0-13.0)	0.539	Mann-Whitney
Number of oocytes per patient	16.50 (4-23)	13.0 (4.0-23.0)	19.0 (7.0-23.0)	0.589	Mann-Whitney
The number of mature oocytes per patient	11.00 (4-18)	9.0 (4-18)	13.0 (6-17)	0.394	Mann-Whitney
Fertilization rate (%) per patient	72.50 (25-100)	73.00 (54.0-100)	66 (25-100)	0.089	Mann-Whitney
Endometriosis, n (%)					
yes	1 (8.3)	2 (6.7)	1 (3.3)	0.50	Fisher Test
no	11 (91.7)	13 (43.3)	14 (46.7)		
Ovulation disorders, n (%)					Chi Square Test
yes	6 (50)	5 (16.7)	7 (23.3)	0.71	
no	6 (50)	10 (33.3)	8 (26.7)		

After excluding three contaminated culture media samples, 27 embryo culture media samples were analyzed. The chromosomal status distribution remained nearly equal: 13 (48.15%) euploid embryos and 14 (51.85%) aneuploid embryos. The median expression level of miRNA-191 for all embryos was 4.056 (0.001–63.558). Using the Mann–Whitney test, the expression of miRNA-191 was found to be higher in euploid than in aneuploid embryos, although the difference was not statistically significant (median 8.690 vs. 1.064; p = 0.351). These results are shown in Table 2.

Table 2. The Relationship between miRNA-191 Relative Expression (RE) Values with Chromosome Status Including the Three Extreme Values

	Median (Minimum – Maximum)	*P-value*
Euploidy	8.69 (.001 – 2876.303)	0.351
Aneuploidy	1.064 (.006 – 1584.701)	

Analysis using the Mann–Whitney test showed that the expression of miRNA-548c-3p was lower in euploid embryos compared with aneuploid embryos; however, this difference was not statistically significant (median 1.919 vs. 4.311; p = 0.707). After identifying six extreme values, analysis of the remaining samples showed that miRNA-548c-3p expression was higher in euploid than in aneuploid embryos, although this difference was also not significant (median 9.06 vs. 6.635; p = 0.709). These results are presented in Table 3.

Table 3. The Relationship between miRNA-548c-3p Relative Expression (RE) Values with Chromosome Status Including the Six Extreme Values

	Median (Minimum – Maximum)	*P-value*
Euploidy	9.06 (.000 – 33923.561)	0.709
Aneuploidy	6.635 (.003 – 91405.921)	

DISCUSSION

We hypothesized that there is a correlation between the expression levels of miRNA-191 and miRNA-548c-3p in embryo culture media and embryo chromosomal status. From 12 subjects, we obtained balanced results from the 30 blastocysts analyzed, consisting of 15 euploid and 15 aneuploid embryos (50% each). However, we found no significant difference between the expression levels of miRNA-191 and miRNA-548c-3p in the spent culture media and the embryos' chromosomal status.

The most common genetic abnormality in humans is an abnormal number of chromosomes (aneuploidy) in gametes and embryos. Avoiding the transfer of aneuploid embryos and prioritizing euploid embryos has been shown to increase implantation rates and reduce the incidence of miscarriage. The absence of aneuploidy screening and reliance solely on embryo morphology during selection can increase the risk of transferring aneuploid embryos, which may lead to implantation failure, miscarriage, or the birth of children with chromosomal abnormalities.[4]

In this study, the chromosomal assessment of embryos at the blastocyst stage resulted in 15 (50%) euploid and 15 (50%) aneuploid embryos. Embryonic aneuploidy may occur due to several factors, including suboptimal ovarian stimulation

leading to poor-quality oocytes, suboptimal embryo culture conditions that trigger chromosomal segregation errors, and paternal or maternal chromosomal abnormalities.[7]

The examination of miRNA expression levels in spent embryo culture media to predict embryonic chromosomal status represents a novelty in this study. miRNAs are non-coding RNAs that regulate gene expression and influence various biological processes, including embryonic development and stem cell differentiation.[3] miRNAs may induce translational repression and/or mRNA degradation. A single mRNA can be targeted by multiple miRNAs, and one miRNA can regulate hundreds of mRNAs.

In our analysis of miRNA-191 expression from 30 spent culture media samples, we identified three extremely high values. These outliers came from three embryos belonging to three different patients from three IVF clinics two with male infertility and one with uterine pathology; one embryo was aneuploid and two were euploid. No specific patterns were observed based on patient characteristics or chromosomal status. These extreme values were likely due to contamination by cumulus cells or the presence of necrotic cells carried into the culture medium. This remains a limitation in metabolic studies using culture media.

Although not statistically significant, we observed higher levels of miRNA-191 expression in euploid embryos. All embryos included in the analysis were at the blastocyst stage, and previous studies have also shown that euploid blastocysts express and release miRNA-191 into culture media.[8]

The exact role of miRNAs in embryonic development remains unclear. miRNA-191 and miRNA-372 are thought to regulate mitogen-activated protein kinase 1 (MAP3K1) and cyclin-dependent kinase 6 (CDK6), which are critical for cell cycle regulation, signalling, and apoptosis. miRNA-372 and miRNA-191 levels were found to be higher in embryo culture media following ICSI compared with standard insemination, possibly due to zona pellucida and oolemma disruption causing miRNA leakage.[3] miRNAs are also upregulated under cellular stress. In our study, this potential bias was minimized by including only ICSI-fertilized embryos.

Consistent with our findings, the study by Sánchez-Ribas identified miR-181b-5p and miR-191-5p as highly expressed miRNAs. They reported higher miR-191-5p expression in euploid embryos and similar miR-181b-5p expression in both groups.[9] Their study also failed to find significant differences between the miRNA expression of euploid and aneuploid embryos in spent media. They suggested that residual miRNAs in commercial media may contribute to this variability. Our study minimized this effect by measuring miRNA expression in empty culture medium as a baseline. miR-191-5p has 45 predicted target genes, six of which have strong biological relevance to development, cell cycle regulation, signalling, and enzymatic activity including MDM4 (apoptosis), interleukin-1A, SOX4, CDK6, and SATB1.[10] miRNA expression changes dynamically during embryonic differentiation, which may also influence the findings. Although miRNA-191 expression tended to be higher in euploid embryos, it was not statistically significant, suggesting that miRNA-191 may not be a suitable biomarker for determining chromosomal status.

In our analysis of miRNA-548c-3p, we identified six extremely high values (319.573–91,405.921), whereas the remaining values ranged from 0.000 to 107.635. These outliers came from six embryos from four patients across three clinics. All six originated from couples with male infertility, with one also having ovulation disorders; three embryos were euploid and three were aneuploid. No consistent patterns were observed. As with miRNA-191, possible causes include cumulus cell contamination or necrotic cell carryover, which remain challenges in metabolic studies of culture media.

The median expression levels of miRNA-548c-3p were 1.919 (0.000–107.635) in euploid embryos and 4.311 (0.003–263.197) in aneuploid embryos. Although the expression tended to be lower in euploid embryos, the difference was not statistically significant ($p = 0.707$). Previous studies indicate that miRNA-548c-3p is not associated with embryo chromosomal status or IVF failure; instead, it is known to be upregulated in human embryonic stem cells, castration-resistant prostate cancer, and blood samples from patients with gastric cancer, indicating a greater role in tumor progression.[11]

Across studies evaluating miRNAs in blastocysts, there is minimal overlap in the specific miRNAs identified. This is likely due to methodological differences in RNA extraction, miRNA detection panels, and blastocyst culture media. miRNA expression is highly dynamic and varies according to the genes required at specific

developmental stages, making interpretation challenging.[12] Another limitation is the variation in culture media used by embryology laboratories in different clinics.

We believe this study provides preliminary evidence that miRNA expression levels in embryo culture media may serve as a comparative measure for chromosomal status. However, limitations include the likelihood of contamination in spent culture media and the relatively small sample size. Variation in miRNA extraction techniques and culture media composition also contributes to inconsistent findings across studies. Additionally, the dynamic nature of miRNA expression, which changes according to developmental needs, complicates interpretation.[12] Larger studies are needed to evaluate other non-invasive biomarkers for assessing embryonic chromosomal status and to explore additional miRNA candidates in embryo culture media to improve chromosomal status prediction.

CONCLUSION

The expression levels of miRNA-191 and miRNA-548c-3p in spent embryo culture media did not show statistically significant differences between euploid and aneuploid embryos. These findings should be interpreted as preliminary. The absence of significant differences may indicate that these specific miRNAs are not directly involved in chromosomal segregation pathways or that their extracellular expression levels do not reliably reflect the embryo's ploidy status. Additionally, the results may have been influenced by several limitations, including the small sample size, potential confounding factors, and technical variability across the multicenter sample collection. Therefore, miRNA-191 and miRNA-548c-3p do not appear to be reliable stand-alone non-invasive biomarkers for determining chromosomal status in this cohort. Future research should involve larger, standardized multicenter cohorts. We also recommend exploring broader miRNA panels, potentially in combination with metabolic or proteomic profiling, to develop a more robust multimarker prediction model for non-invasive embryo assessment.

ACKNOWLEDGEMENT

The author would like to thank the embryologist and staff at IVF Yasmin Clinic, Dr. Cipto Mangunkusumo General Hospital, Bocah Indonesia Fertility Clinic, Human Reproduction Infertility Cluster and Family Planning (HRIFP) IMERI Faculty of Medicine Universitas Indonesia for their assistance in sample collection and embryo culture.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

COMPETING INTERESTS

The authors declare no competing interests

ADDITIONAL INFORMATION

This study was approved by Health Research Ethics Committee of the Dr. Cipto Mangunkusumo General Hospital Approval Number: 21-01-0060. All participants provided written informed consent.

REFERENCES

1. Hoffman, Schorge, Bradshaw. Williams gynecology. 3rd ed. Mc Graw Hill; 20164: 63–8.
2. Fritz M, Speroff L. Assisted reproductive technologies. 8th ed. Philadelpia. Lippicott Williams & Wilkins. 2011:1330–40.
3. Rosenbluth EM, Shelton DN, Wells LM, Sparks AET, Van Voorhis BJ. Human embryos secrete microRNAs into culture media - A potential biomarker for implantation. Fertil Steril. 2014;101(5):1493–500. http://dx.doi.org/10.1016/j.fertnstert.2014.01.058
4. Klitzman R. Deciding how many embryos to transfer: ongoing challenges and dilemmas. Reprod Biomed Soc Online. 2016;3:1–15. http://dx.doi.org/10.1016/j.rbms.2016.07.001
5. Munné S. Preimplantation Genetic Diagnosis for Aneuploidy and Translocations Using Array Comparative Genomic Hybridization. Curr Genomics. 2012;13:463–70.
6. Cimadomo D, Capalbo A, Ubaldi FM, Scarica C, Palagiano A, Canipari R, et al. The Impact of Biopsy on Human Embryo Developmental Potential during Preimplantation Genetic Diagnosis. Biomed Res Int. 2016;2016.
7. Gutierrez-Mateo C, Sanchez-Garcia J, Escudero T, Prates R, Ketterson K, Wells D, et al. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. Fertil Steril. 2011;3:953–8.
8. Ata B, Kaplan B, Danzer B, Glassner M, Opsahl M, Tan S, et al. Array CGH analysis shows that aneuploidy is not related to the number of embryos generated. Reprod Biomed Online. 2012;24(614).

9. Lu J, Zhang M, Yang X, Cui T, Dai J. MicroRNA-548c-3p inhibits T98G glioma cell proliferation and migration by downregulating c-Myb. Oncol Lett. 2017;13(5):3866–72.
10. Sauer M, Paulson R, Lobo R. Reversing the natural decline in human fertility. An extended clinical trial of oocyte donation to women of advanced reproductive age. JAMA. 1992;24(614).
11. Paul ABM, Sadek ST, Mahesan AM. The role of microRNAs in human embryo implantation: a review. J Assist Reprod Genet. 2019;36(2):179–87.
12. Sanchez-Ribas I, Diaz-Gimeno P, Quinonero A, et al. NGS Analysis of Human Embryo Culture Media Reveals miRNAs of Extra Embryonic Origin. Reprod Sci. 2019 Feb;26(2):214-22. doi: 10.1177/1933719118766252. Epub 2018 Mar 27.