UNIVERSITY OF MEDICINE AND PHARMACIE "VICTOR BABEȘ" TIMIȘOARA
FACULTY OF MEDICINE
OBSTETRICS-GYNECOLOGY DEPARTMENT

VIZITIU ANDA-CORNELIA

SUMMARY

PHD THESIS

EVALUATION OF CHROMOSOME 21 MAPPED MICRORNAS AS POSSIBLE BIOMARKERS FOR DOWN SYNDROME PRENATAL DIAGNOSIS

Scientific Advisor

PROF. UNIV. DR. ANASTASIU DORU-MIHAI

Timișoara 2019

Contents

Keywords: microRNA, Down syndrome, pregnancy, prenatal diagnosis

Introduction

Down syndrome (DS) is not only the most common autosomal chromosomal abnormality but also the most viable trisomy, being determined in 95% of cases by trisomy 21. For the moment, the understanding of the pathogenic mechanisms of SD is based on two theories: the ‘gene dosage theory’, which postulates the disproportionate expression of genes from the duplicated regions of chromosome 21 and the theory of 'development instability', which takes into account the non-specific alteration of gene expression (18, 24, 25). The prenatal diagnosis of DS is primarily based on invasive procedures such as puncture of the chorionic villus sampling (CVS) and amniocentesis (AF), with a risk of pregnancy loss of 1-2% (1, 4). Several clinical studies have validated the analysis of free circulating fetal DNA as a method with high sensitivity and specificity for the screening of aneuploidies, which has led to the clinical implementation of NIPT since 2011 (62). However, 9 years after its introduction into the clinical practice, NIPT remains a costly test, whose results are expressed in degrees of risk and is accessible only to a small percentage of the population. The main disadvantage is the probability of not achieving a conclusive result due to the low amount of fetal DNA in the maternal blood, which explains its utility only after the 10_{th} week of pregnancy (18, 67).

The associated risks, high costs and rather late diagnosis during pregnancy of the current testing methods fully justify the efforts to discover a (non-invasive, if possible) cost-effective method for early prenatal diagnosis of Down syndrome. To this end, due to their high stability in biological fluids, microRNAs are ideal candidates.

MicroRNAs are small non-coding RNA molecules (22-25 nucleotides), whose main function is the posttranscriptional regulation of gene expression. MicroRNAs’ use as biomarkers for non-invasive diagnosis of various pathologies has been intensively studied, especially due to the increased stability in biological fluids (98,131-137).

Postulating that the presence of three copies of chromosome 21 causes an increase of at least 50% in the expression of the corresponding genes (including those that encode microRNAs), the aim of this study is to quantify by qRT-PCR the expression of microRNAs mapped on chromosome 21 (of which only five were confirmed experimentally: miR-99a, let-7c, miR-125b-2, miR-155 and miR-802). In addition, we aimed to analyze the suitability of CVS/AF miRNAs as DS biomarkers, the mechanisms leading to an alteration in mature microRNA expression (transcriptional or post-transcriptional) and to evaluate the impact that differentially expressed microRNAs on DS transcriptome.

To the best of our knowledge, here we provide the very first data indicating the alterations of posttranscriptional regulatory mechanisms of microRNA expression in a trisomic context. It should be noted that, at the time this study was initiated, (2014), there was one single published study analyzing the suitability of mi(21) as biomarkers for prenatal diagnosis (168).

Purpose and objectives

The purpose of this study is 1) to evaluate the utility of microRNAs mapped on chromosome 21 as biomarkers for prenatal diagnosis of DS, 2) to analyze the mechanism of alteration of expression of these mature microRNAs and 3) to evaluate the impact of the differentially expressed microRNAs on the DS amniocytes and cardiomyocytes.

In this regard, the following objectives have been achieved:

1. Establishment of the study group;
2. Quantification of microRNAs mapped on chromosome 21 and of the specific placental cluster miR-371/3 (used as biological control) in chorionic villi and amniotic fluid samples from DS and euploid pregnancies;
3. Quantification of pri-microRNAs corresponding to the mature microRNAs identified as having significantly altered expression in chorionic villi and amniotic fluid samples from Down syndrome and euploid pregnancies;
4. Quantification of let-7c expression in placenta samples collected from Dp (16) 1Yey / + transgenic mice (Down syndrome experimental model)
5. Statistical analysis of microRNA expression;
6. Bioinformatic analysis of the impact of microRNA expression placental and cardiomyocyte fetal transcriptome.

Results

This study included pregnant women which were recommended by an obstetrician-gynecology doctor to undergo prenatal screening for aneuploidy. The patients' recruitment took place between 2014-2016 and included 59 patients aged 21 to 42, of whom 23 were diagnosed with Down syndrome and 36 had normal, euploid pregnancies. There was no statistically significant difference ($p=0.07$) between the age of the patients with trisomic pregnancies (34.9) and the patients with normal, euploid pregnancies (35.45 yo). In total, 34 patients had pregnancies with female fetuses (F), and 25 patients had pregnancies with male fetuses (M); there are no statistcially significant differences between the two fetal sex groups.

Studiul s-a desfășurat conform principiilor de etică din cadrul Declarației de la Helsinki, după ce a fost obținută aprobarea comisiei locale de etică a Spitalului Clinic Județean de Urgență Timișoara (nr. 73/ 20 decembrie 2014).

Materialul biologic a fost obținut de la paciente cu risc crescut de aneuploidie în urma screeningului prenatal prin biopsia vilozităților coriale (CVS) și amniocenteză (AC) de către obstetricieni cu experiență, ca parte a procedurii de diagnostic a unei eventuale aneuploidii.

The study was carried out in accordance with the ethical principles of the Helsinki Declaration, with the approval of the Ethics Committee of the County Emergency Clinical Hospital of Timisoara (no. 73/20 December, 2014).

Biological material was obtained by biopsy of chorionic villi (CVS) or amniocentesis (CA) carried by experienced obstetricians, from patients diagnosed at increased risk of aneuploidy following prenatal screening, part of the diagnostic procedure for eventual aneuploidy.

Study 1. Quantification of mature miR(21) expression in biological samples of amniotic fluid and chorionic villi

The evaluation of mature miR(21) expression in biological samples from DS and euploid pregnancies was performed by qRT-PCR, using the TaqMan MicroRNA Cells-to-Ct kit (Ambion) and specific TaqMan primers.

Contrary to our expectations, none of the quantifiable (miR-99a, miR-125b-2, miR-155, let-7c) miR(21) had significantly differentiated expression in Down vs euploid samples. It is worth noting that, nevertheless, miR-99a, miR-125b-2 and miR-155 are increased while let-7c is decreased in both AF and CVS samples, tendencies that need wider, well structured lots of patients for confirmation (Fig. 1). miR-802 was amplified very late, after the threshold detection limit (Ct = 40) in more than 70% of the samples, thus being considered undetermined.

miR(21) expression analysis did not identify significant changes in trisomic samples from male fetuses compared with same-sex euploid samples, while an opposite expression pattern was observed when chorionic villi samples are compared to those of amniotic fluid.

In amniotic fluid samples from pregnancies with female fetuses, miR-99a was found to be significantly overexpressed (FC=3,769; p=0.0005) in pregnancies with DS compared to euploid ones (Fig. 2). It should be noted that in the case of samples of chorionic villi arising from trisomic pregnancies with female fetuses, all miR (21) were over-expressed, although not statistically significant (Fig. 2).

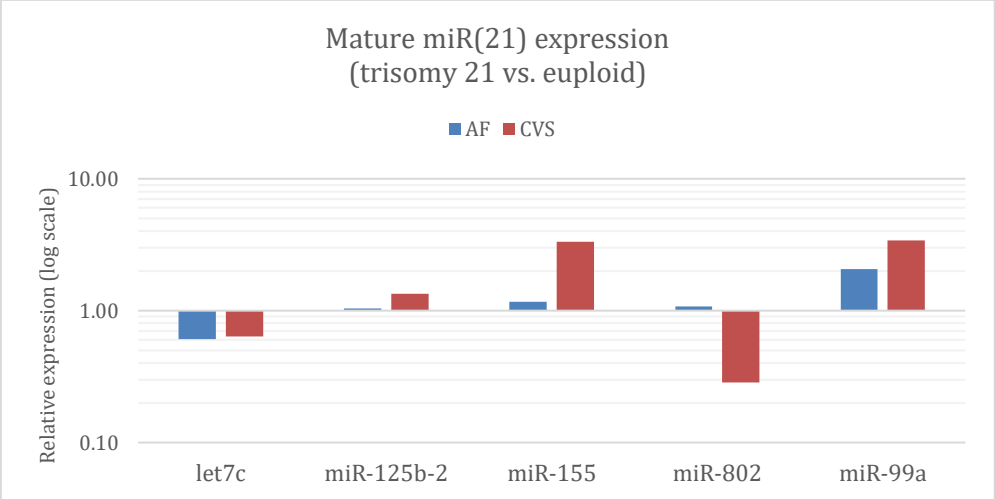

Fig 1. Change of mature mir(21) expression in CVS and AF biological samples.

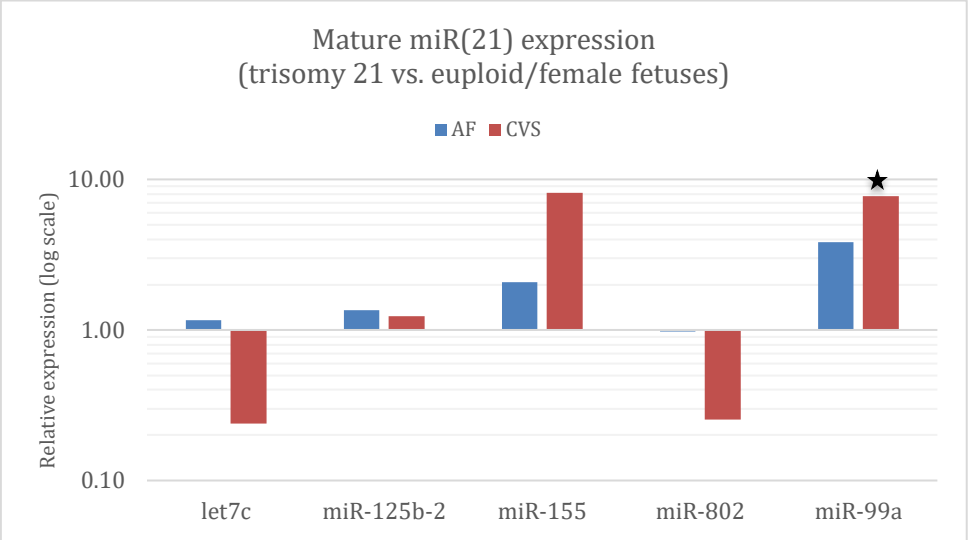

Fig 2. Change of mature mir(21) expression in CVS and AF, female fetuse biological samples; ★ P<0,001

Study 2. Quantification of mature miR-371-1/3 cluster expression in biological samples of amniotic fluid and chorionic villi

The evaluation of mature miR-371-1/3 expression in biological samples from DS and euploid pregnancies was performed by qRT-PCR, using the TaqMan MicroRNA Cells-to-Ct kit (Ambion) and specific TaqMan primers. This placenta specific, evolutionary conserved cluster served a biological control for viability of CVS/AF/samples.

miR-371 was excluded from our analysis because, in over 70% of the samples was amplified below the established detection limit (Ct = 40), thus being considered undetermined. All other microRNAs in the miR-371-1/3 cluster (miR-372, miR-373, miR-373*) were overexpressed in amniotic fluid samples from pregnancies with DS samples compared to euploid samples (Fig. 3). In samples of chorionic villi, with the exception of miR-373* (downregulated), all other microRNAs within the cluster are overexpressed. However, the change was statistically significant only for miR-373 (FC = 10.10; p = 0.02) (Fig 3).

Gender oriented analysis of miR-371-1/3 expression did not identify significant changes in the trisomic samples of amniotic fluid or chorionic villi when compared with euploid samples.

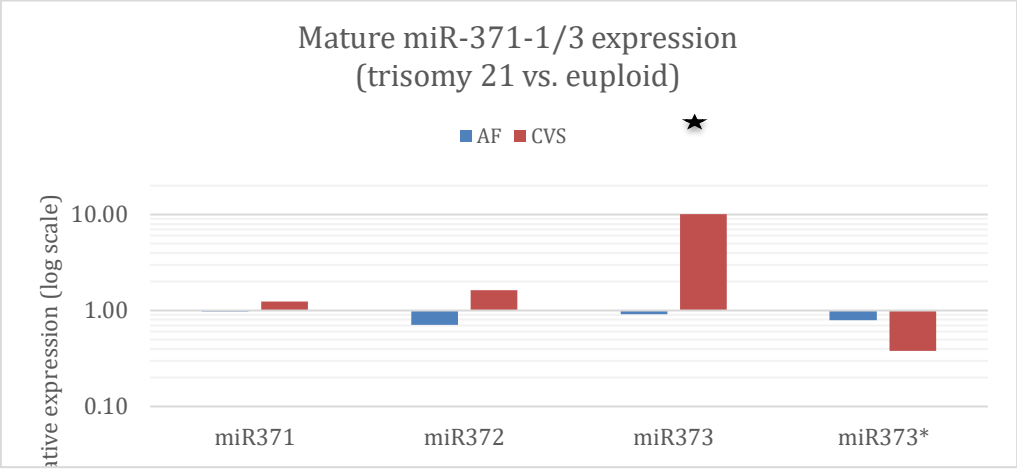

Fig 3. Change of mature mir-371-1/3 expression in CVS and AF biological samples; ★ p<0,005

Study 3. Quantification of pri-microRNA expression in biological samples of amniotic fluid and chorionic villi

We decided to quantify 4 pri-microRNAs (pri-let-7c, pri-miR-99a, pri-miR-155, pri-miR-373), corresponding to mature microRNAs previously shown to have statistically significant altered expression levels; to this end, we used the TaqMan Gene Expression Cells-to-Ct kit (Ambion) and specific primers.

Excepting pri-miR-99a, slightly elevated in amniotic fluid samples (FC = 1.11), all other quantified pri-miRs are down-regulated in trisomic samples, both in amniotic fluid, as well as in chorionic villi. However, none of the pri-microRNA analyzed showed statistically significant expression changes (Fig. 4).

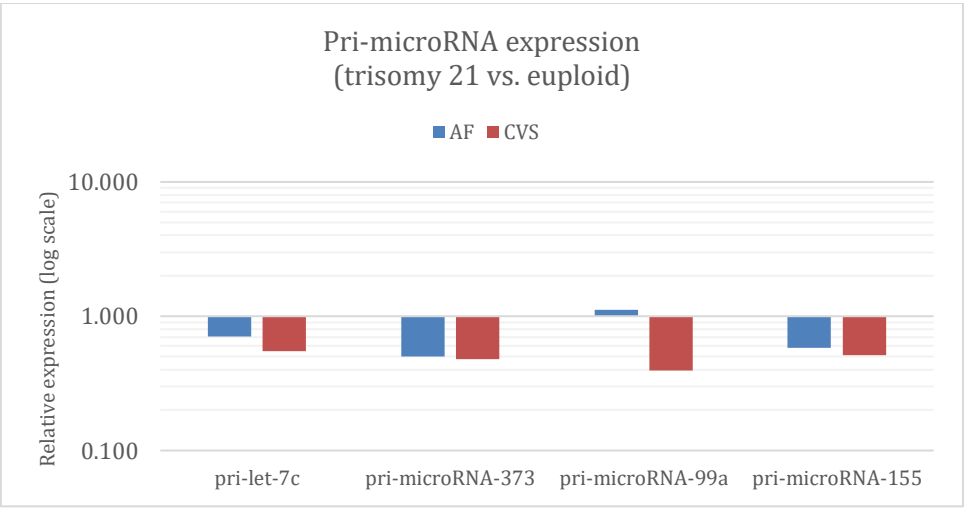

Fig 4. Change of pri-mir(21) expression in CVS and AF biological samples.

The pri-microRNA analysis in male fetus biological samples of chorionic villi and amniotic fluid associated did not reveal statistically significant expression changes.

In the chorionic villi samples collected from pregnancies with female fetuses, all four pri-microRNAs showed statistically significant expression variations: pri-let-7c (FC = 0.094; p = 0.038), pri-miR-99a (FC = 0.198; p = 0.019) and pri-miR-373 (FC = 0.092; p = 0.033) (Fig. 5). In the amniotic fluid samples, all pri-miR show an increased, none of which are statistically significant (FC = 1.01-1.05) (Fig. 5).

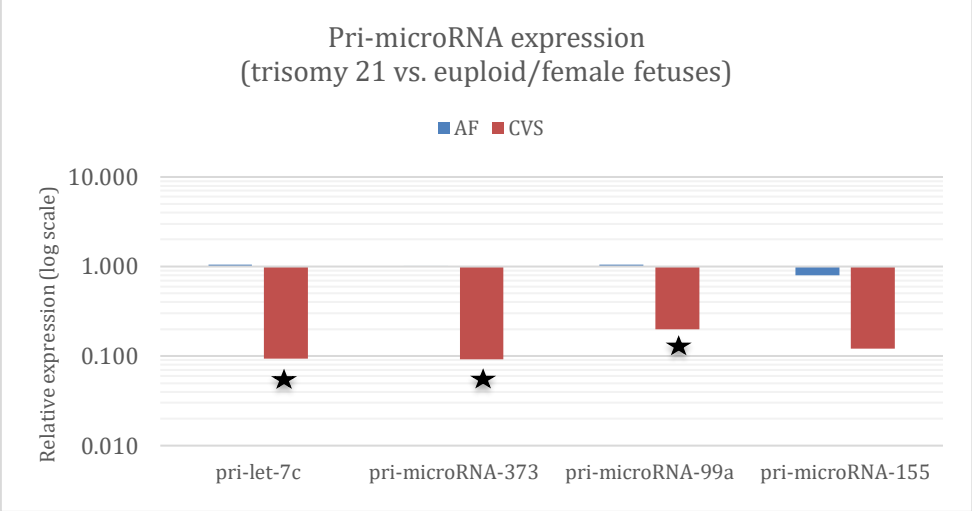

Fig 5. Change of pri-miR expression in CVS and AF, female fetuse biological samples; P<0,05

Study 4. Quantification of let-7c expression in placenta samples from Dp (16)1Yey/+ transgenic mice

For microRNA and pri-microRNA quantification in mouse placenta samples, we opted for a small RNA isolation and purification method (mirVana miRNA Isolation, Ambion kit), followed by reverse transcription (TaqMan MicroRNA Reverse Transcription kit, Applied Biosystems) and quantification by qPCR (Universal Master Mix no UNG II kit, Applied Biosystems) using specific primers.

Similar to expression in human CVS samples, let-7c is also under-expressed in mouse placenta samples (FC = 0.7), but the changes are not statistically significant (p value = 0.17) in (16)1Yey/+ transgenic placenta compared to normal mice. Gender oriented analyses did not find any statistically significant changes in transgenic placentas vs normal, control ones.

Study 5. Bioinformati analysis of statistically significant changes of mature microRNAs.

To fully understand the biological significance of miR-99a overexpression in amniotic fluid samples, we used the miRWalk3.0 algorithm to determine the target gene list of miR-99a and obtained 2134 target genes (unique inputs). Then, we compared this list with the set of differentially expressed (Geo2R analysis, adjusted p <0.05) genes in SD amniocytes (GSE16176) and fetal heart tissue (GSE1397, GSE1789) and obtained two sets of 187 and, respectively, 208 differentially expressed, miR-99a target geness (175,176). Subsequent ontological analysis using the GATHER algorithm (adjusted p <0.05, Bayes factor ≥10) revealed two significant signaling pathways as potential targets of miR-99a in both amniocytes and in trisomic cardiac fetal cells: focal adhesion and cytokine-cytokine receptor interaction.

Pentru a evalua semnificația biologică a expresiei scăzute a let-7c în probele de vilozități coriale, am analizat impactul pe care acesta îl are asupra expresiei genice la nivel placentar. In urma analizei de predicție miRWalk3.0 am obținut 1828 gene care interacționează semnificativ cu let-7c și a căror expresie poate fi influențată de alterarea expresiei let-7c. Pentru a evalua care dintre aceste gene sunt diferențial exprimate în placente provenite din sarcini cu sindrom Down, am interogat setul de date GSE70102 (GEO DataSets), iar în urma analizei Geo2R am obținut 1786 gene diferențial exprimate în probele de placentă cu trisomie 21 (p<0,05, dupa corecție Benjamini & Hochberg) (217). Dintre acestea, 214 gene se regăsesc în lista de gene care interacționează cu let-7c conform miRWalk, fiind potențiale ținte ale expresiei alterate a let-7c. Analiza ontologica efectuată ulterior folosind algoritmul GATHER (p ajustat <0.05, factor Bayes ≥10) a evidențiat cinci căi de semnalizare semnificativ exprimate în placenta sarcinilor trisomice ca potențiale ținte ale let-7c: Jak-STAT, adeziuni focale, interacțiunea citokine-receptori pentru citokine, căile de semnalizare ale insulinei și apoptoza.

In order to understand the biological significance of the let-7c change of expression, we analyzed the impact that it might have on placental transcriptome. Following miRWalk3.0 predictive analysis, we obtained 1828 genes that significantly interact with and whose expression may be influenced by let-7c. To evaluate which of these genes are differentially expressed in placentas from pregnancies with DS, we queried the GSE70102 dataset, and, following the Geo2R analysis, we obtained 1786 differentially expressed genes in trisomy 21 placenta samples (p <0.05, after correction Benjamini & Hochberg) (217). Of these, 214 genes are found in the list of genes predicted to interact with let-7c, thus being potential targets for altered expression of let-7c. Further ontological analysis using the GATHER algorithm (adjusted p <0.05, Bayes factor ≥10) revealed five significant signaling pathways expressed in the placenta of trisomic pregnancies: Jak-STAT, focal adhesions, cytokine-cytokine-receptor interaction, insulin signaling pathways and apoptosis.

Conclusion and discussions

Our starting hypothesis postulates that the presence of three copies (total or partial) of chromosome 21 determines the overexpression of miR(21) in chorionic villi and amniotic fluid samples. Our data indicate that the expression of the majority of miR (21) is not statistically significantly altered in samples of amniotic fluid and chorionic villi derived from trisomy 21 pregnancies compared to normal pregnancies. However, analysis of miR(21) expression in amniotic fluid samples from female fetuses and DS compared to euploid pregnancies of the same sex showed significantly increased expression of miR-99a, but not of pri-miR-99a , which suggests alterations of the mechanism of formation and/or maintenance of hsa-miR-99a stability in female fetuses. The results are promising but it is clear that more studies, including a larger number of cases are needed to demonstrate the utility of miR-99a as a biomarker for prenatal (possibly non-invasive?) diagnosis of DS.

Another aim of this study is to evaluate the link between the differentially expressed miR(21) and the congenital heart defects in DS. Overexpression of miR-99a in amniotic fluid samples from trisomic pregnancies iwith female fetuses can significantly alter signaling pathways involved in cardiac morphogenesis. Cardiac development involves multiple, partially overlapping stages, controlled by an evolutionarily conserved network of signaling pathways and transcription regulators, of which microRNA in general (and in particular miR-99a, by altering Nodal1/Smad2 signaling) plays an important role (181, 187, 222). Multiple lines of evidence indicate the association of miR-99a expression with cardiac defects in female fetuses with SD (180). MiR-99a is involved early in cardiomyogenesis, and increased plasma levels of miR-99a have been associated with the presence of congenital fetal heart defects. Given the rapid bidirectional communication between the fetus and the amniotic fluid, it is absolutely plausible that the fetal heart is a possible source of plasma miR-99a (182,183).

The KEGG signaling pathway analysis using the GATHER algorithm of potential miR-99a targets expressed in SD amniocytes and fetal cardiac tissue indicates two conserved signaling pathways: focal adhesion interactions and cytokine-cytokine receptor interactions.

Our data suggest that miR-99a overexpression in amniotic fluid is not a transcriptional event, but rather an alteration of the mechanism of degradation of the mature form of microRNA. MicroRNA destruction is less known than microRNA biogenesis, and many factors (from genomic organization, structural heterogeneity and post-transcriptional modifications, to the targets and biological context) are known as modulators of microRNA degradation (208).
To the best of our knowledge, this is the first report indicating altered stability / degradation of a microRNA in the trisomic environment, providing a new possible molecular mechanism for understanding gene dosage in Down syndrome.

Although miR-99a is the only statistically significant DS miR(21), it is important to note that, contrary to our expectations, let-7c has a low expression in both amniotic fluid and chorionic villi samples, concordant with the expression of the primary transcript, pri-let-7c. The results were confirmed in placenta

samples of Dp(16)1Yey/+ transgenic mice, but, similar to hsa-let-7c, the changes were not statistically significant. These data suggest that expression changes of mature forms of microRNA in DS may be due to different, transcriptional or post-transcriptional events.

The bioinformatic analysis of let-7c target genes differentially expressed in the trisomic placenta identified 5 signaling pathways: Jak-STAT, focal adhesions, cytokine - cytokine-receptor interaction, insulin signaling pathways and apoptosis (the effect of let-7c expression on the first three being, most likely, mediated by the interaction with lin28) (105-107).

Although we are aware that additional data on cardiac abnormalities are needed in the DS pregnancies, we dare to propose a novel association between the expression level of miR-99a in amniotic fluid and cardiac defects in female fetuses in in general, and DS in particular. Further studies, on a much larger batch of samples are needed to understand whether the increased significant level of miR-99a expression in amniotic fluid is predictive of fetal heart defects and whether the suggested alterations of post-transcriptional/degradation processing are also present in DS male fetuses (222).

References:

18. Asim A, Kumar A, Muthuswamy S, Jain S, Agarwal S. "Down syndrome: an insight of the disease". J Biomed Sci. 2015;22:41.
24. Lana-Elola E, Watson-Scales SD, Fisher EM, Tybulewicz VL. Down syndrome: searching for the genetic culprits. Dis Model Mech. 2011;4(5):586-95.
25. Randall J. Roper RHR. Understanding the basis for Down syndrome phenotypes. PLoS Genet. 2006;2(3):e50.
1. Lola Cartier LM-K. Counselling Considerations for Prenatal Genetic Screening. J Obstet Gynaecol Can. 2012;34(5):489-93.
4. O'Connor C. Trisomy 21 causes Down syndrome. Nature Education 2008;1(1):42.
62. Gratacos E, Nicolaides K. Clinical perspective of cell-free DNA testing for fetal aneuploidies. Fetal Diagn Ther. 2014;35(3):151-5.
67. Gil MM, Quezada MS, Revello R, Akolekar R, Nicolaides KH. Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: updated meta-analysis. Ultrasound Obstet Gynecol. 2015;45(3):249-66.
98. Friedman RC FK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92-105.
131. Tarang S, Weston MD. Macros in microRNA target identification: a comparative analysis of in silico, in vitro, and in vivo approaches to microRNA target identification. RNA Biol. 2014;11(4):324-33.
132. Patrick S. Mitchell RKP, Evan M. Kroh, Brian R. Fritz, Stacia K. Wyman, Era L. Pogosova-Agadjanyan, Amelia Peterson, Jennifer Noteboom, Kathy C. O'Briant, April Allen, Daniel W. Lin, Nicole Urban, Charles W. Drescher, Beatrice S. Knudsen, Derek L. Stirewalt, Robert Gentleman, Robert L.

Vessella, Peter S. Nelson, Daniel B. Martin, Muneesh Tewari. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513-8.
133. Gilad S ME, Yogev Y, Benjamin S, Lebanony D, et al. . Serum MicroRNAs Are Promising Novel Biomarkers. PLoS ONE 2008;3(9):e3148.
134. Arroyo JDea. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. Proc Natl Acad Sci USA 2011;108(12):5003-8.
135. Vickers KCea. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. Nat Cell Biol 2011;13(4):423-33.
136. Wang Kea. Export of microRNAs and microRNA-protective protein by mammalian cells. Nucleic Acids Res 2010;38(20):7248-59.
137. Hunter MP. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One 2008;3(11):e3694.
168. Katerina Kotlabova JD, Daniel Chudoba, Pavel Calda, Klara Dlouha, Ilona Hromadnikova Extracellular chromosome 21-derived microRNAs in euploid & aneuploid pregnancies. Indian J Med Res 2013;138:935-43.
175. Ionim DK, Koide K, Johnson KL, Tantravahi U et al. Functional genomic analysis of amniotic fluid cell-free mRNA suggests that oxidative stress is significant in Down syndrome fetuses. Proc Natl Acad Sci U S A. 2009;106(23):9425-9.
176. Mao R, Wang X, Spitznagel EL Jr, Frelin LP et al. Primary and secondary transcriptional effects in the developing human Down syndrome brain and heart. Genome Biol. 2005;6(13):R107.
217. Bianco K, Gormley M, Farrell J, Zhou Y et al. Placental transcriptomes in the common aneuploidies reveal critical regions on the trisomic chromosomes and genome-wide effects. Prenat Diagn. 2016; 36(9):812-22.
181. Coppola A, Romito A, Borel C, Gehrig C, Gagnebin M, Falconnet E et al. Cardiomyogenesis is controlled by the miR-99a/let-7c cluster and epigenetic modifications. Stem Cell Res. 2014; 12(2):323-37.
187. Katz MG, Fargnoli AS, Kendle AP, Hajjar RJ, Bridges CR. The role of microRNAs in cardiac development and regenerative capacity. Am J Physiol Heart Circ Physiol. 2016; 310(5):H528-41.
222. Vizitiu Anda Cornelia, Stambouli Danae, Pavel Anca-Gabriela, Mureșan Maria-Cezara, Anastasiu Diana Maria, Bejinar Cristina, Alexa Anda, Marian Cătălin, Sîrbu Ioan-Ovidiu, Sima Laurențiu. Mature miR-99a upregulation in the amniotic fluid samples from female fetus Down syndrome pregnancies: a pilot study. *Medicina* 2019, *55*, 728; doi:10.3390/medicina55110728.
180. Izzo A, Manco R, de Cristofaro T et al. Overexpression of Chromosome 21 miRNAs May Affect Mitochondrial Function in the Hearts of Down Syndrome Fetuses. Int J Genomics. 2017;2017:8737649.
182. Kehler L, Biro O, Lazar L, Rigo J, Nagy B. Elevated hsa-miR-99a levels in maternal plasma may indicate congenital heart defects. Biomedical Reports. 2017; 3(6):869-873.

183. Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. J Perinatol. 2005; 25(5):341-8.
208. Marzi MJ, Ghini F, Cerruti B, de Pretis S, Bonetti P et al. Degradation dynamics of microRNAs revealed by a novel pulse-chase approach. Genome Res 2016; 26(4):554-65.
105. Nachmani D, Zimmermann A, Oiknine Djian E, Weisblum Y, Livneh Y, Khanh Le VT, et al. MicroRNA editing facilitates immune elimination of HCMV infected cells. PLoS Pathog. 2014;10(2):e1003963.
106. Haruhiko Siomi MCS. Posttranscriptional Regulation of MicroRNA Biogenesis in Animals. Molecular Cell 2010;38(3):323-32.
107. Julia Starega-Roslan EK, Piotr Kozlowski, Wlodzimierz J. Krzyzosiak The role of the precursor structure in the biogenesis of microRNA. Cell Mol Life Sci. 2011;68:2859–71.