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INVITED REVIEW – OPEN ACCESS

A Transgenerational Genetic Marker of the Autism Spectrum Disorder

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ABSTRACT

Autism spectrum disorder (ASD) is an increasingly frequent neurodevelopmental disorder. A number of indications recently pointed to abnormal distributions of microRNAs (miRNAs) in autistic patients. The noncoding regulatory miRNAs are abundant in the developing brain and abnormal levels of expression of several of them were found in tissues of ASD patients. Here, we discuss the previously published results and compare them with our recent data identifying 6 miRNAs whose blood levels are downregulated in ASD patients. A similar although less pronounced decrease is hereditarily transmitted by the clinically unaffected parents of sick children and the sibling. Robustness of the finding was confirmed by similarly low levels of the six microRNAs in two established mouse models of the disease. Several hopeful avenues of research may be considered from these results including molecular mechanisms from the regulation of the miRNAs to the identification of their target genes and the non-Mendelian mode of inheritance of the autism-prone state. On the clinical side, they offer the possibility of a very early detection of the affected children.

Keywords: Autism, downregulation, heredity, microRNAs, mouse model, parent

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INTRODUCTION

The autism spectrum disease (ASD) is a group of apparently heterogeneous symptoms diagnosed in children by a lack of interest in activity, communication, and social interaction along with an oversensitivity to sound, contact with cloths and with repetitive behaviors (1). Several reports indicate a progressive increase in the rate of ASD occurrence, as high as 1 in 59 children in 2014 (Centers for Disease Control and Prevention). Studies on large groups of patients have evidenced that it is a heritable condition and a large number of genetic variants have been identified in ASD patients (2). None of them, however, accounts for the general occurrence of the disease (3), a defect in the neurodevelopmental processes that establish cognitive and social development (4). The risk factors are both genetic and environmental insults. Complex genetic variations were observed in autism patients including de novo multiple monogenic (5, 6) and tandem repeat variation (7), but common genetic risk variants have not been yet identified (8, 9). We extended the search for genetic alteration(s) common to genetically unrelated patients to a cohort of 45 patients and family members previously assembled at the Erciyes University of Kayseri with the proper controls. It allowed us to identify a group of six miRs (microRNAs) downregulated in the blood of ASD patients and their parenthood (10).

GENETIC DETERMINATION of ASD

In general, and often in the literature, the 90% incidence for ASD in identical twins, compared to 10% for dizygotic twins (11) is mentioned as a strong argument in favor of a genetic determinant of the disease. In view of recent developments (12), "genetic determinant" has to include either structural modifications of the genome or epigenetic determinants. In the human brain, the most complex organ created by evolution, the fact that 80% of genome is expressed illustrates the level of complexity expected for the genetic determination of neuropsychiatric disorders such as autism. The provisional conclusion was that pathological changes are generated by combinations of mutations in exons and/or in regulatory elements that impact the general development of the brain. In the case of ASD, one distinguishes syndromic (4, 13) occurrences (ASD symptoms are parts of a distinct syndrome) and non-syndromic forms (14). Syndromic ASD is an element of pathologies such as FMR, RETT, and a few others (13), while the majority of ASD occurrences are non-syndromic. A unique set of genetic modifications is one of the hypotheses for non-syndromic ASD, but they still have yet to be clearly determined. Epigenetic modifiers were also listed that could possibly transmit small effects to different genes. Polymorphisms of a nucleotide (single-nucleotide polymorphism) (15) has been proposed to contribute to the disease. However, these theoretical possibilities still lack experimental validation. We recently reported (10)

analytical evidence of modified microRNAs (miRNAs) profiles as a common genetic alteration, first in 45 non-syndromic ASD patients and their progenitors, then in two experimental mouse models of the disease.

microRNAs

Several hundred microRNAs, non-coding RNA molecules 18–25 nt long, are found in all multicellular species. Many are expressed in multiple cells and some are unique to a specific lineage. In humans, more than 2000 of them determine functions at the translational level miRNA-directed target degradation pathway of their target messengers (16, 17). In addition, new finding brought explanation for instability of some miRNAs, because the length of pairing with target could shorten the microRNA's life span (18, 19). miRNAs are degradated by cellular nucleases with pathway of target-directed miRNA degradation (20, 21). miRNAs were also reported as circulating vectors in RNA vehicles (exosomes) that could be secreted and reach distant tissues (22, 23). Individual miRNAs are critical determinants of the levels of the thousands of transcripts in a developing organism (21). Variations of individual miRNAs levels have been described in a number of diseases, resulting from either genetic changes or environmental variations (24). As summarized below, we reported one instance of a stepwise reduction in the levels of a group of six miRNAs from parents to children, correlated with the behavioral alterations characteristic of ASD.

This line of research may hopefully have several useful outcomes. One immediate application would be to provide a diagnostic tool based on a strict quantitative molecular assay, relatively inexpensive and applicable at the earliest ages, even to the future parents. It then generates a new set of questions for future research, identification of new targets of the modified miRNAs on one side and the factors and genes that regulate the accumulation of the regulatory miRNAs on the other.

miRNA VARIATIONS IN AUTISM: CURRENT REPORTS

A comparative review of microRNA expression patterns in autism spectrum disorder (ASD) has been collated by Hicks and Middleton in 2016 (3). It summarized expression in the postmortem cerebellum and central nervous system (CNS), in periphery and in cell culture in addition with parallel studies in the serum and saliva of children. However, on several points, results from different groups are inconsistent and in addition, they differ from one study to another. As an attempt to make the approaches more comparable, here we will not discuss instances with obvious causes of variability such as the postmortem analysis and cell cultures which may be subject to variations in gene expression. Even then inconsistent results are still observed in assays performed on blood and saliva. We propose for future studies a homogeneous strategy for the analysis of microRNAs as a routine platform. It is clear in general that blood, serum, and saliva samples are to be preferred. As shown by the current results of seven blood studies; however, multiple causes of discrepancy will still remain, including variations generated by various techniques, limited numbers of microRNAs tested, not to mention the lack of parallel studies on parents and siblings and possibly on animal models of the disease.

The report by Da Silva Vaccaro et al. (25) analyzes 26 miRNAs in the blood of seven male patients with ASD with an average age of 7.5 years (sd 2.5) and four male controls. Blood expression profiles were established by real-time quantitative polymerase chain reaction (RT-qPCR) using a stem-loop RT-PCR technique. Among the 26 microRNAs analyzed four were found upregulated (miR34c-5p, miR-145-5p, miR92a-2-5p, and miR199a-5p) and three downregulated (miR19b-1-5p, miR27a-3p, and miR193a-5p).

Huang, 2015 (26), reports a study of 5 autistic patients (1 girl and 4 boys; average age 4.5 years) and five controls by peripheral blood tests using a RiboArray miDETECT Human Array. A total of 77 miRNAs were found expressed differentially. In addition to the microarray analysis, 15 other patients (two girls and 13 boys; average age: 4.3–1.623) and 15 controls were analyzed by qRT-PCR, indicating that miR-557 and miR-486-3p expression levels were significantly increased. They also validated 24 upregulated miRNAs and 20 miRNAs downregulated. It is of interest to note that one of them miR-19b-3p was repeatedly found to be abnormally expressed in the peripheral blood of autism patients.

Nakata et al. 2019 (27) reported miRNA expression profile in peripheral blood of adults with high-functioning ASD, and healthy age and genus-matched controls which were performed by miRNA microarrays. They reported 18 miRNAs (two upregulated and 16 downregulated) that showed significant changes between ASD and control groups. In particular, miR-6126, miR-3156-5p, miR-1227-5p, miR-6780a-5p, and miR- 328-3p were most significantly dysregulated in ASD, especially miR-6126 as being highly downregulated in ASD and correlated with the severity of social deficits.

Vasu et al. 2014 (28) analyzed by SYBR Green quantitative PCR the profile of microRNAs in the serum of 55 individuals with ASD and age-and-sex-matched controls. Altered expression of 14 miRNAs in ASD samples compared to controls, eight downregulated (MiR-151a-3p, miR-181b-5p, miR-320a, miR-328, miR-433, miR-489, miR-572, and miR-663a) and six upregulated miR-101-3p, miR-106b-5p, miR-19b-3p, miR-195-5p, miR-130a-3p, and miR-27p.

Hicks and Middelton, 2016 (29), reported sequence analysis of the salivary miRNAs present in at least half the sample from 24 ASD subjects. Of these, 14 miRNAs showed significant changes in expression in the ASD group compared to controls. Ten of the miRNAs were up-regulated in ASD subjects and four were down-regulated. One miRNA with the largest average difference in abundance between ASD and control subjects was miR-628-5p. Individually, miR-335-3p had the largest AUC and miR-30e-5p had the highest accuracy in predicting ASD diagnosis at 76%.

On the other hand, a genetic model of Fragile X Syndrome, which is the most common inherited mental retardation disorder including ASD, the loss of FMRP or FXR1P leads to downregulation of brain specific miRNAs (30). We note that miR-19b-3p was again found as one of the possible targets of FXR1 (31).

This comparative overview of the published miRNA analysis in ASD patients shows an apparently extensive degree of heterogeneity. Studies differ by the technique used for quantitative estimates, making it difficult to draw a detailed comparison. Still, one impor-

tant point appears obvious at this first degree of analysis: While some of the microRNAs tested were statistically more represented in patients, none of these alterations is common to all of them.

Our own study (10) was launched on a cohort of 37 families, with one or two autistic children each altogether 45 patients, with associated healthy age-and-sex controls. Expression profiles were established for 384 miRNAs using the specific Taq-man probe real-time RT-q-PCR technique. None of them was found overregulated in the patients or controls. Six of them (miR-19a-3p, miR-361-5p, miR-3613-3p, miR-150-5p, miR-126-3p, and miR-499a-5p) were downregulated at low to very low levels (90%) in all children with autism compared to healthy controls. Interestingly, they were also downregulated by 50% in either one or both of their apparently healthy parents and by 70% in other siblings but never in genetically independent controls.

Altogether reported articles identified 63 up-regulated and 52 downregulated miRNAs, only three of them evidenced in two separate studies (miR92a, miR-19b-3p, and miR-34a-5p) (3). Confirmed in addition by our results (above), the case of the miR-19 RNAs appears of specific interest. Either miR-19 a or b was found in patient blood in four studies by real-time RT-q-PCR either two-fold downregulated [miR19-b-1-5p (25)] including our results for miR-19a-3p or in contrast miR-19b-3p was found to be two-fold upregulated (28, 32). miR-19a and b are polycistronic transcribed from the same loci. The alteration observed in multiple studies and circumstances underline the levels of miR-19a/b as being highly important for behavioral development. Interestingly, in two independent mouse models (see below), we observed a decrease in miR-19a and b and their 5p and 3p sequences in all tissues, including blood and semen. Here, we propose that the optimal levels of miR-19a/ or b, which are crucial for balanced behavior, appear to be determined at the transcriptional level affecting the production both of 5p/3p. The detection of miR-19 in all four (25, 28, 32) studies was performed by real-time RT-q-PCR and appears to be reproducible in independent trials. In addition, the miR-19b is one of the miRNAs that are varying by food and are upregulated in the testes and sperm of mouse models kept under a high-fat diet (33).

Apart from miR-19, comparing our results with the conclusions of previous studies is not exploitable, because the other five miRs identified by our studies (miR-361-5p, miR-3613-3p, miR-150-5p, miR-126-3p, and miR-499a-5p) either were not included in these studies or were analyzed by methods that, as of today, are essentially disregarded for their degree of variability.

MOUSE MODELS

Mouse models of autism with genetic mutation and or induced by the environment are essential to validate molecular mechanisms of the diseases. Mice are a robust model with homogeneous genetics, which provide tools to develop diagnostic and ultimately effective treatments. Mouse behavioral tasks have been developed (34, 35) and now molecular tools with analogies in humans (3, 4, 7, 36–42) could provide the approach to multiple diagnostic symptoms to detect diseases. Because of important genetic variation in human for analysis of complex disease, animal model cannot be ignored.

In parallel, our finding in human have been validated with two well-established mouse models with autism-characteristic behavioral alterations and six miRNAs downregulation (10). One of them is established by injection of valproic acid (VPA) to young males (2 weeks old), known to induce autism in humans (43–58) and rodents (45). The second mouse model was inbred heterozygotes of a mutation (Lac-z insertion) in the Cc2d1a locus. Family cases of Cc2d1a heterozygosity have been associated with the constellation of autism in humans (49–51), and mice (52). Cc2d1a protein is a transcriptional repressor of one of the serotonin receptors [Serotonin1A (5-HT1A)] involved in brain development. We found five (miR-19a-3p, miR-361-5p, miR-150-5p, miR-126-3p, and miR-499a-5p) out of six the same "miRNAs" that are altered in the blood, hippocampus, and semen in these two mouse models as cited above in the human patient serum. Sixth (miR-3613-3p) does not exist in Mus musculus. In both cases, the characteristic profile of 5-miRNAs diminution was inherited with symptoms of the disease in the offspring of crosses with healthy animals. Even apparently disease-free Cc2d1a+/+ offspring generated in crosses between diseased heterozygous was found to maintain the modified miRNA profile. It, therefore, appears, both in humans and mice, as a heritable trait associated with the disease. Once properly extended to other genes involved in ASD and developed, these findings can provide valuable tools for the very early identification of affected children and potential ancestors.

In 2016, Hara et al. (47) have reported that among three miRNAs (miR-9, miR-124, and miR-132) tested after exposure VPA to prenatal (E12.5) in mice, an immediate response was observed only for miRNA levels specific and brain-enriched. These effects are observed only in the mouse's embryonic brain, it is not specified which region of the brain nor if it can be found later in adult mice. In addition, RNA sequence analysis revealed that prenatal VPA exposure caused changes in other miRNAs than miR-132 in the entire embryonic brain. However, none of them are present in our shortlist of "Six-miRNAs" and at the moment no rational explanation can be proposed apart from the differences in the techniques used. Still these results confirm alterations in miRNA levels dependent on neural activity produced by prenatal VPA exposure as an environmental insult.

DISCUSSION

miRNAs in human patients

miRNAs and RNA targets in ASD

"Our" six miRNAs (10) are all expressed in the brain, where they are expected to target several hundreds of RNAs. Most of them target several mRNAs already known to be involved in the developing brain and ASD. Target mRNA research can only validate the discovery of ASD genes that are targeted by these miRNAs but this does not allow for a major breakthrough or rapid understanding because the same miRNAs also regulate large numbers of other mRNAs. However, identification of miRNAs altered in autism patients along with their mRNAs targets, which are among gene candidates in autism, could facilitate the search for molecular mechanisms.

What does heredity of miRNA downregulation mean?

miRNA expression is a fluid process, varying during development and in life, throughout various regions of the brain (21–23, 27). Why a decrease in miRNAs levels in the parents of autistic children are inherited even at lower level in ASD patients? This is especially shown in mice (see below) where the decrease of miRNAs in the blood is also observed in sperm cells. Reversion as a back and forth event of gene expression is proposed to occur during adaptation to environment, here we observe gradual diminution from parents to sick children. There are numerous perspectives for miRNAs molecules in a tuning model, comparable to what is proposed for both genetic and epigenetic variation in a "rheostat model." A mechanism for reversible forms, with a non-Mendelian or transgenerational effects has not yet been elucidated. It is tempting to compare miRNAs alteration to these "Rheostat effects" in imprinting events (53) which are finely regulated during development and epigenetic heredity. miRNAs could be the perfect mediators in those processes. Transcription variation in germ-line is frequent and occurs normally and could probably be an advantage during severe selection conditions. miRNAs variation provides the potential for variants to contribute to variability of gene expression, without varying the primary sequence of the genome. Here, we are faced with phenomena of progressive miRNAs decreasing events. Under evolutionary theory, the mutations are deleterious, except in rare cases giving an advantage in reproduction but slight variations in miRNA levels are intriguing.

From our results, it appears that the downregulation of the six miRNAs is not reset in the germ cells at least in one generation. In fact, miRNAs levels are at down level in patients compared to the controls but not at the completely off position. The "Six-miRNAs" levels decrease is already started from parents of autistic children. This seems to indicate that once the "miRNAs/level" is disturbed it cannot always completely return at least in one generation to an optimal level during transmission to the next generation.

The molecular mechanisms underlying miRNA dysregulation in ASD are one of the fascinating research programs for the future. Non-syndromic ASD scans are observed among children who have been subjected to an early insult (embryonic development) with the environmental modifiers as drug (VPA), prenatal hypoxia, and inflammation such as strong immune responses. One explanation is that some miRNAs sensitive to these pressures are altered and could not recover easily after insult to their normal profiles? These hypotheses can now be tested in animal models at least for the drug VPA [see above (10)], hypoxia, or inflammation. In hypoxia, several studies have reported miRNAs alterations, two of these miRNAs downregulated are: miR-19 and miR-126. Both were found in our studies. With regard to miRNA candidates in inflammation, the data are so broad and variable at the experimental level that it could not enter the present review. Departing from these comparisons, all points must now be precisely tested and validated more precisely with animal models to validate the phenotype and miRNAs as molecular candidates for diagnosis.

miRNAs "Valuable Markers" for ASD

ASD is described as a complex disease due to a series of multi-factorial effects already listed from the environment, gene, organs, and finally the expression of phenotypes in patients. Non-coding RNAs such as miRNAs are perfect candidates for inducing or filling all these constellation elements. Children with ASD are increasingly diagnosed. Alterations especially diminution in miRNA profiles of peripheral blood sites can be explained by circulating miRNA that must pass through all tissues. Peripheral sources such as blood miRNA, most of the tissues contribute and they are kept stable and abundant to be transported by exosomes. Thus, while miRNAs are decreased in a given tissue, such as serum and/or saliva the other tissues will also be affected. Serum and saliva are an easy and optimal source to track CNS pathophysiology and are the ideal tool for the diagnosis of ASD.

The potential of miRNAs as biomarkers in ASD could be explored not only in a given patient but also in the serum of parents and siblings of children with autism. In our own recent study, involving 45 children with ASD, a set of six serum miRNA showed a consistent 50% decrease in parents to patients over 95% to control subjects. This highlights how the familial study of miRNA with a new generation of RT-q-PCR precise and sensitive technique presents a robust method and extends beyond the simple diagnosis of ASD to predict offspring/or phenotype and asses in offspring phenotype and severity.

Transgenerational Studies

Our results then validate the assumption that the laboratory animal truly provides a model of the mechanisms operating in the patients. Moreover, animal models provide unlimited access to physiological mechanisms much more difficult to reach in humans (brain functions) and they also provide an experimental approach of an obviously fundamental question, that of the inheritance of the pathological defect.

It is important to follow with a systematic analysis of the behavioral test on mutant mice targeted heterozygously for a large number of candidate genes of the autism spectrum. In relatively known genetic variation these models now accelerate the validation of biochemical or neuroanatomical markers for autism.

Despite some ethical limitations, sperm is relatively easy to collect in humans. We reported the case of the father of three children born to different mothers among them a girl with autism. Interestingly, the same "Six-miRNAs" downregulated in the blood of the father and his three children were also downregulated in his sperm RNA compared to three controls sperm. This unique case should now be repeated in other families. However, at these initial stages of investigations, mouse models are important because of the downregulation of the "six-miRNAs" found in sperm transcripts and their maintenance in two mouse models at transgenerational passages. The coordinated decrease in the six-miRNAs is indicative of a common genetic variation and strengthens the validity of the approach. RNA-mediated epigenetic heredity has been proven in mice first by genetic models (c-kit locus) and later by environmentally induced factors [stress (54), drugs (55–57), and food (58)] by several reports. It is important to note that in each of them non-coding RNAs, including miRNAs alterations are highlighted as the vector of transmission. Apart of VPA treated male cited above, in our recent report on the Balb/c line with a mutation introduced by genetic engineering in the Ccd2d1a locus, we observed an alteration in behavior and at the molecular level the alteration of miRNAs in heterozygous and wild genotype

from the same litters. Unlike the normal Balb/c mouse has never been crossed with Cc2d1a+/- mutants. Mice in the Cc2d1a+/- / colony maintained as heterozygous with two types of crosses, either over-crossing to wild type or inbreeding between Cc2d1a+/- heterozygous showed a phenotypic variation in behavior in offspring compared to controls. The Cc2d1a+/- mutants are viable and healthy, but appear different from wild-type animals in several behavioral parameters. Surprisingly, we found that not only did heterozygous animals have altered phenotypes but also wild-type animals born to heterozygous parents. Modified phenotypes vary if offspring are obtained from Cc2d1a+/- crossed to Cc2d1a+/+ versus Cc2d1a+/- crossed at Cc2d1a+/-. Analysis of blood, hippocampus, and sperm RNAs confirms that the same "Six-miRNAs" are regulated in the offspring obtained from these crosses such as heterozygotes (+/-) or homozygotes (+/+). Although it is not possible at this stage to explain, the relationship of the molecular effect with the disease, the definition of a high-risk heritable state before the development of symptoms is real. Intermediate levels of miRNAs expression were observed as long as the disease was not medically reported (parents and sibling of sick children), in contrast to the much lower level in human patients and clinically affected mice.

Based on current results, the physiological consequence of low expression of the "Six-miRNAs" would lead to increased expression of their target mRNA. Among the predicted genes by computational approach possibly regulated are indeed genes for which abnormal expression patterns have been reported in symptomatic ASD patients. Since the initial occurrence of the disease and its transgenerational maintenance appear to correlate with a change in the levels of regulatory miRNAs in somatic tissues and in semen, a general hypothesis may be made that a stable expression regimen of miRNAs and their target gene(s) is critical and is determined in the earliest developmental period. Because, reduced level is not recovered neither at the level of germ cells and is transmitted to the next generation, a crucial event would then be the level of expression of the miRNAs during the formation of zygotes depending to the partner miRNAs level.

Clinical Assessments of ASD

A uniform technical platform for miRNA analysis is required for very early detection of ASD (e.g., in newborns) in family with a high risk factor. A simple, inexpensive test for the identification of affected children would improve the ability to provide them with optimal conditions for their development. Four independent works using real time RT-q-PCR appear as a technique of choice for blood miRNAs examination in the autism assessment. Routine medical standard tests could be derived and established as quickly as possible.

CONCLUSION

It is clear that miRNA profiles are altered in several types of tissue in people with ASD. Six miRNAs showed consistent dysregulation through human and animal studies (miR-19a-3p, miR-361-5p, miR-3613-3p, miR-150-5p, miR-126-3p, and miR-499a-5p). Functional analysis should be designed in vivo to support the idea that these miRNAs are systematically modified in the brain related to neurodevelopment and involved in ASD. RNA networks targeted by these miRNAs and their RNA products are involved in ASD and have an important role in brain development. Animal models to test these miRNAs markers will be essential in defining the ASD knowledge base.

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