

Original Research Article

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Alterations of miR-320 Family Members as a Novel Diagnostic Biomarkers in Peripheral Blood of Schizophrenia Patients

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ABSTRACT

To date, the development of biomarkers of schizophrenia still remains a challenge. Recently, alterations in the expression of microRNAs (miRNAs) from peripheral blood, serum and post-mortem brain tissue have been linked to schizophrenia and other neurodevelopmental disorders. It is well known that miRNAs are expressed by various cell types and can modulate broad physiological functions. On this basis it is assumed that miRNAs could be useful predictive biomarkers for the diagnosis or prognosis of pathological conditions, like schizophrenia. In order to observe an association between miRNAs and schizophrenia, this study was designed to investigate expression profiling of miRNAs in peripheral blood. The aim of this study was to test whether whole blood miR-320 family members display differential expression profile in schizophrenia patients. The relative expression levels of miR-320 members were analyzed by stem-loop qRT-PCR assay in a cohort of 30 participants diagnosed with schizophrenia and 25 age- and gender-matched general population controls. The results indicated that miR-320b, miR-320c and miR-320d were expressed substantially higher in schizophrenia patients than in control subjects, and miR-320a showed the greatest change. Furthermore, a statistically significant difference of miR-320a level was revealed in schizophrenia subjects compared to healthy controls ($p = 0.0076$). Our results suggest that differentially expressed miR-320 family members might be involved in schizophrenia molecular pathways and blood-based miR-320 signature might be able to serve as potential prognostic biomarkers for schizophrenia. The results confirm that the blood-based miRNA profiling is a feasible way to identify biomarkers for schizophrenia.

Keywords

Schizophrenia,
MiRNAs,
Stem-loop qRT-PCR,
Whole blood

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Introduction

Schizophrenia is a severe chronic mental disorder, affecting approximately 1% of the

population worldwide. MicroRNA molecules (miRNAs) are large family of

small non-coding +RNAs that negatively regulate the expression of more than 60% of protein-coding genes (Friedman *et al.*, 2009). The nervous system has the most extensive profile of miRNA expression of all human tissues. Approximately 70% of all known miRNAs are expressed in the brain, where many of them are neuron's specific miRNAs (Krichevsky *et al.*, 2003). MiRNAs have received increased attention in genetic studies of schizophrenia, as some support the hypothesis that miRNAs play an important role in brain disorders (Ma *et al.*, 2013; Müller *et al.*, 2014; Kumar *et al.*, 2013; Shi *et al.*, 2012).

Several recent studies on postmortem cortices have identified numerous miRNAs that are differentially expressed in schizophrenia patients (Beveridge *et al.*, 2010; Perkins *et al.*, 2007). In a study of Chinese population, Xu *et al.*, described a potentially functional variant ss178077483 located in the pre-mir-30e that affected pre-miR-30e and was associated with schizophrenia (Xu *et al.*, 2010). Even though the samples were obtained from the peripheral blood, the findings of Xu *et al.* were comparable to the observations of Perkins *et al.*, who detected an increase in the expression level of miR-30e in the prefrontal cortex of patients with schizophrenia.

It is not surprising that investigation of miRNA expression in blood and postmortem brain tissue has revealed differences between schizophrenia patients and control group (Mellios and Sur., 2012; Beveridge *et al.*, 2012). Several miRNAs, such as miR-124, miR-132, miR-134, and miR-137 have been implicated in schizophrenia (Mellios and Sur., 2012).

In a recent work Gardiner *et al.*, 2011 and Lai *et al.*, 2011 speculated that

schizophrenia-associated miRNA expression signatures may also be detected in non-neuronal tissue such as whole blood. Gardiner *et al.* investigated the expression profile of miRNA in peripheral blood mononuclear cells (PBMCs) of 112 patients with schizophrenia and 76 non-psychiatric controls (Gardiner *et al.*, 2011). They identified 83 miRNAs that were significantly downregulated in the schizophrenia group, including a large subgroup of miRNAs transcribed from a single imprinted locus at the maternally expressed DLK1-DIO3 region on chromosome 14q32. In addition, similarly to Gardiner *et al.* Lai and co-workers identified a specific profile of seven miRNAs in an initial cohort of 30 patients with schizophrenia and 30 controls. The specific signature detected, included upregulated miR-564, miR-34a, miR-449a, miR-548d, miR-652 and miR-572, and down regulated miR-432 (Lai *et al.*, 2011). Results from that particular study were subsequently validated in an extended cohort of 60 schizophrenia patients and 30 controls. Authors carried out a miRNA expression profiling of PBMCs and found a group of dysregulated miRNAs in schizophrenia patients. Lai *et al.* had also shown that the miRNA expression signatures observed in PBMCs in schizophrenia patients may have the potential to serve as predictive biomarkers of schizophrenia.

In the human genome there are eight annotated miR-320 genes encoding five mature miRNA variants including miR-320a (Chr 8), miR-320b (Chr 1), miR-320c (Chr 18), miR-320e (Chr 19), miR-320d-1 (Chr 13) and miR-320d-2 (Chr X), last two with the same mature miRNA sequences. The current study aim to elucidate the expression profile of particular miR-320 family members through qRT-PCR assay.

Materials and Methods

Participants and Clinical Assessment

This study design and the Inform Consent Form (ICF) were approved by the Ethics Committee of Medical University of Plovdiv. The Institutional Review Board approved the use of the samples for this study. Thirty schizophrenia patients were recruited from State Psychiatric Hospital - Pazardzhik, Bulgaria after obtaining written informed consent.

Routine psychiatric examination and wide medical history were done by a certified psychiatrist to evaluate the diagnosis of paranoid schizophrenia, using Diagnostic and Statistical Manual of Mental Disorders fourth edition Text Revision (DSM IV TR) criteria. In order to exclude any other mental disorder of the study and control groups the Mini-International Neuropsychiatric Interview version 5.0 was also done. The sample population included 32 participants diagnosed with schizophrenia and 24 age- and gender-matched general population controls. Main inclusion criteria was that the participants did not receive any medication before blood sampling for at least 2 weeks. Persons with other medical illness or psychoactive substances (with exception of nicotine) users were excluded from the cohorts of interest.

Blood Collection and RNA Extraction

The peripheral blood samples (2.5 ml) for each participant (schizophrenia and healthy controls) were collected into PAX gene blood RNA tubes (PreAnalytiX). Total RNA was isolated using PAXgene blood miRNA kit (PreAnalytiX), according to the manufacturer's protocol. Assessment of A260/A280 ratios revealed that all analyzed RNA samples are with sufficient quality for

qRT-PCR analysis (1.93 - 2.10). RNA quality and purity were analyzed by Epoch Micro-Volume Spectrophotometer System (BioTek). RNA integrity of all samples (schizophrenia and healthy controls) was also checked by agarose gel electrophoresis.

Confirmation of Differentially Expressed miR-320 Family Members in Whole Blood by Stem-Loop qRT-PCR Analysis

Quantitative RT-PCR was used to measure the expression of individual miR-320 family members. MicroRNA specific cDNA was synthesized from equal mass inputs of 1 µg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) using miRNA-specific stem-loop primers. Five microliters of each miRNA specific cDNA were subjected to pre-amplification prior to the real-time PCR step with Long Range PCR Kit (Qiagen) to potentially enhance sensitivity of the assay. Copy DNA for the protein coding gene was synthesized from total RNA with oligo (dT)₁₈ primer using Revert Aid First Strand cDNA Synthesis Kit according to the assay protocol (Thermo Fisher Scientific, Waltham, MA USA) using 1 µg of total RNA samples. qRT-PCR was performed using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) with ABI PRISM 7500 (Applied Biosystems).

The qRT-PCR primers used are listed in Table.1. The expression levels of miRNAs for qRT-PCR were normalized to the level of human B Actin, and relative quantification (RQ) was calculated utilizing the $2^{-\Delta\Delta C_t}$ method. PCR amplification of the specific products corresponding to B Actin and each specific miR-320 amplicons was confirmed by monitoring the dissociation curve (Melting curve analysis) and by agarose gel electrophoresis.

Table 1. MiR-320 specific qRT- PCR primer sets

MiRNAs	Primer sequence 5'- 3'
miR-320b SL	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTTGCCCTC
miR-320b For	ACACTCCAGCTGGGAAAAGCTGGGTTGAGA
miR-320a SL	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTCGCCCTC
miR-320a For	ACACTCCAGCTGGGAAAAGCTGGGTTGAGA
miR-320c SL	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGACCCTCTC
miR-320c For	ACACTCCAGCTGGGAAAAGCAGGGTTGA
miR-320d SL	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTCCTCTCA
miR-320d For	ACACTCCAGCTGGGAAAAGCTGGGTTG
Universal Rev	GTCGGCAATTCAGTTGAG
B ActinFor	AGTGTGACGTGGACATCCGCA
B ActinRev	GCCAGGGCAGTGATCTCCTTCT

Statistical Analysis

All statistical calculations were performed using the Statistical Package for the Social Sciences software, version 20.0 (SPSS Inc., Chicago, IL, USA). Analysis of variance (ANOVA) *t*-test of Ct was used to examine differences in expression levels of miR-320 members across healthy controls and schizophrenia subjects, considering Kolmogorov-Smirnov criteria for normal distribution and Fisher–Snedecor distribution. A Spearman correlation analysis was also done for searching relation between expression levels and demographic characteristics of the tested individuals. The expression level of each miRNA was quantified by its normalized threshold cycle number ΔCt , in which $\Delta Ct = [Ct(miRNA)] - [Ct(B Actin)]$, and the relative expression level was calculated as $2^{-(\Delta Ct)}$, which is commonly used in miRNAs profiling studies (Schmittgen *et al.*, 2008). Logistic Regression analysis was done to determine predicted probabilities of miRNA 320 expression levels. Subsequent ROC (receiver operating characteristic) curve analyses was performed so specificity and sensitivity can be obtained.

Results and Discussion

MiRNA expression was analyzed in peripheral blood of 32 patients with schizophrenia and 24 non-psychiatric controls using miR-320 family specific stem-loop qRT-PCR assay. The PCR amplification of the specific products corresponding to B actin and miR-320 amplicons was confirmed by monitoring the dissociation curve (melting curve analysis). Our analytical approach allows the quantification of specific miR-320 family members as alterations in expression of individual miRNAs in schizophrenia. While qRT-PCR validation showed that all of the analysed miRNAs were up-regulated (miR-320b, miR-320c, miR-320d), only miR-320a showed statistically significant difference ($p = 0,0114$) (Figure 2). Two-dimensional hierarchical clustering of differentially expressed miR-320 genes between schizophrenia and control group subjects as observed by stem-loop qRT-PCR are shown in Figure 1. Alterations in miRNA levels are presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the healthy

Figure.2 Schizophrenia associated miR-320 up regulation in peripheral blood. Quantitative real-time RT-PCR reaction analysis of miRNA genes in schizophrenia versus control group. Expression levels (left diagram) of miR-320a, miR-320c (\log_2 (RQ) = 0.35), miR-320d (\log_2 (RQ) = 0.35) and miR-320b (\log_2 (RQ) = 0.47), respectively. Bars represent fold change in expression. Box plot of miR-320a expression (right diagram), indicating a 0,79 fold increase for miR-320a in cases relative to controls

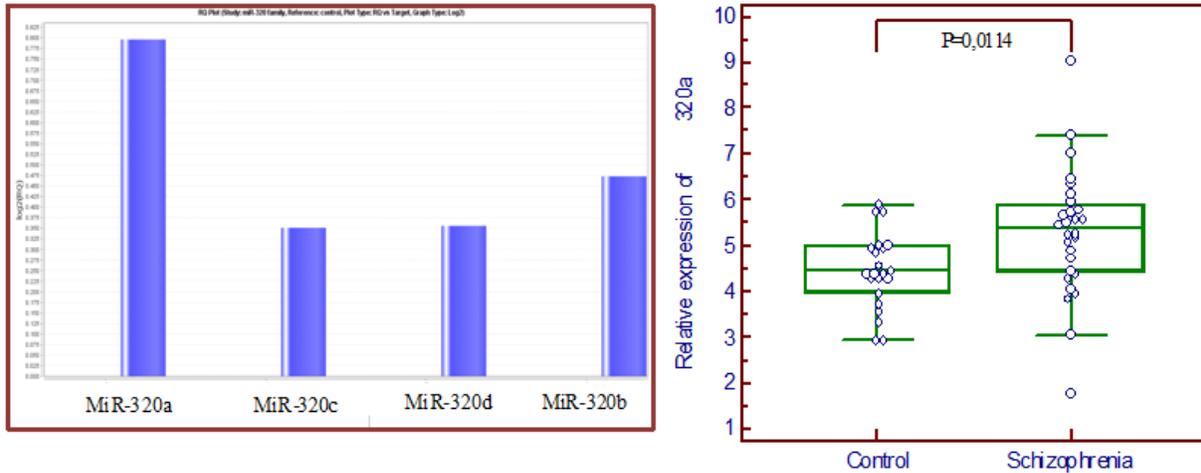


Figure.3 Performance of the miR-320 prediction model. The ROC for miR-320 gene expression was performed to evaluate the prediction accuracy. The dotted diagonal line represents random classification accuracy (AUC 0.5). ROC curves were drawn for miR-320a, miR-320b, miR-320c and miR-320d which yielded 0,722, 0,574, 0,529, 0,524 as AUC values, respectively.

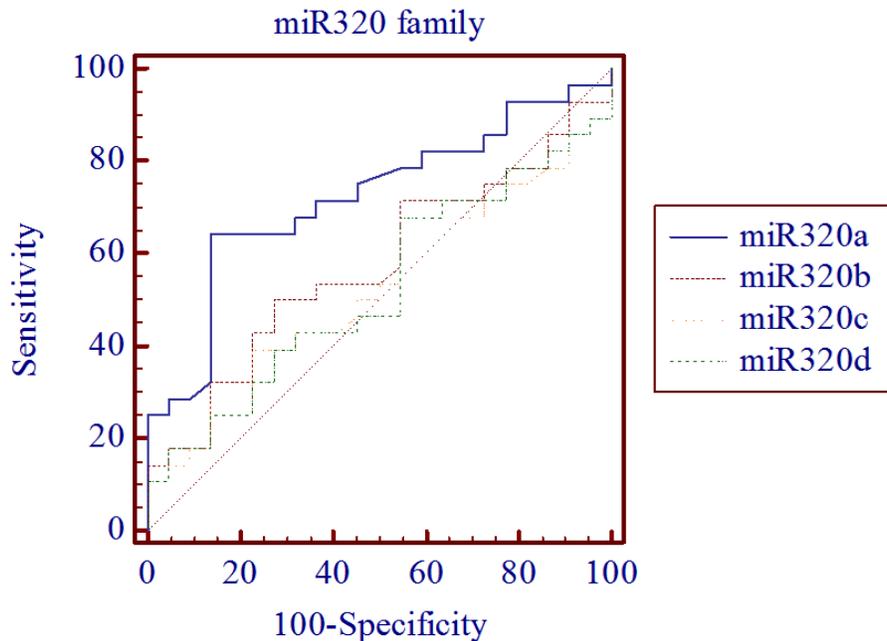


Figure.4 Combined ROC curve of the differentially expressed miR-320 family members (miR-320a, miR-320b, miR-320c and miR-320d). The diagonal line represents random classification accuracy (AUC 0.5). Diagnostic sensitivity of combined classifiers were 85,7% with the corresponding specificity of 95,5%

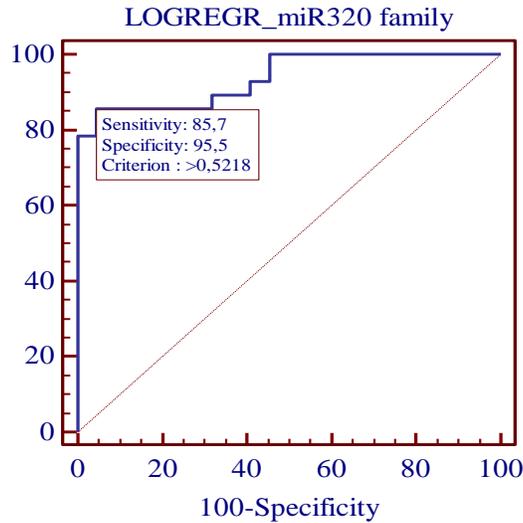


Figure.5 Sex-dependent variations in the miR-320a expression pattern between analyzed groups, according to Kruskal-Wallis test

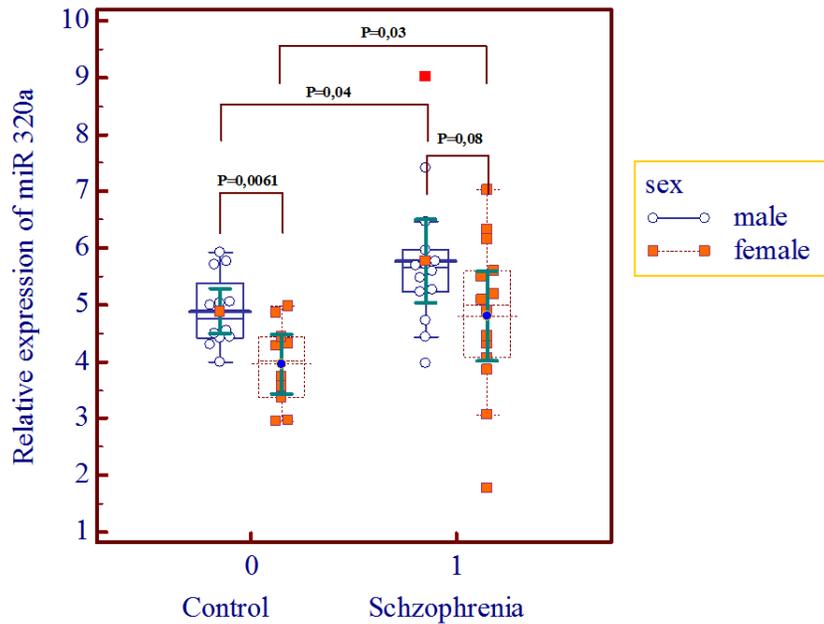
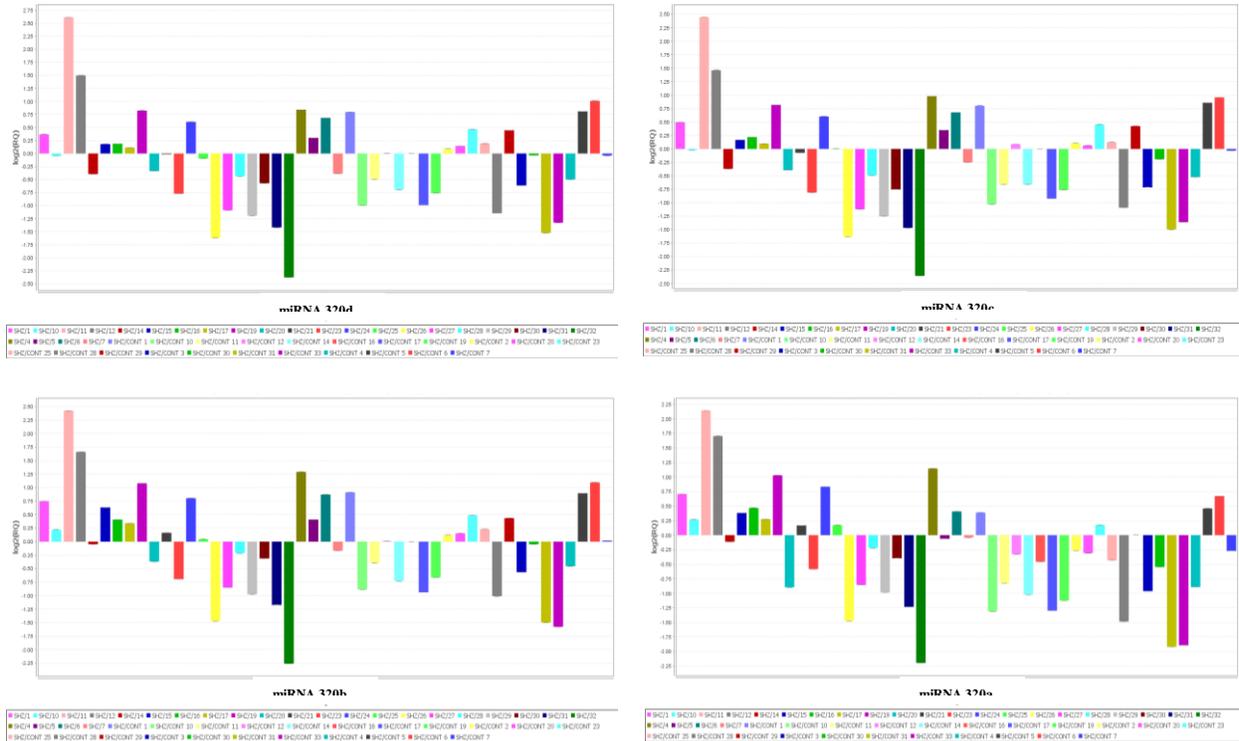


Figure.6 Relative miRNAs levels are shown for miR-320b, miR-320d, miR-320c, and miR-320a that demonstrate differential expression levels when comparing 32 individuals with schizophrenia and 24 healthy controls. miRNA levels were normalized to the endogenous reference gene relative to the healthy control patients. Whole blood samples were analyzed for the expression of selected miRNAs by stem-loop RT-qPCR. Data represent the expression fold changes (RQ) relative to healthy controls



Diagnostic sensitivities of miR-320a, miR-320b, miR-320c and miR-320d, for schizophrenia were 64,3 %, 50,0 %, 39,3 % and 67,9 %, respectively. The corresponding specificities were 86,4%, 72,7%, 77,3% and 45,5%, and ROC curve areas (95% CI) were 0,722 (0,577 - 0,839%), 0,574 (0,426 - 0,713%), 0,529 (0,383 - 0,672%) and 0,524 (0,378 - 0,667%), respectively. Additionally, a combined ROC curve of the differentially expressed miR-320 family members was done. Diagnostic sensitivity and specificities of combined classifiers are shown in Figure 4. We also analysed sex-dependent variations in the miR-320a expression pattern between analyzed groups. As shown in Figure 5, no significant

difference of miR-320 a expression level was found between male and female schizophrenia patients ($P = 0.08$). However, there was significant difference between male and female healthy controls in miR-320a expression ($P=0.0061$). There was significant difference in miR-320 a expression level between female and male schizophrenia patients compared to healthy controls ($P=0.03$) and ($P=0.04$), respectively.

Multiple studies have shown that defective function and neuronal plasticity in neurodevelopmental disorders may have resulted from impaired miRNA mediated post transcriptional regulation. Numerous

expression studies have described dysregulation of members of the miR-320 family in patients with various neuropsychological disorders. Lopez *et al.*, conducted miRNA expression profiling in postmortem brain samples (Brodmann area 44) of individuals with major depressive disorder, committed suicide, using a TaqMan qRT-PCR absolute quantitation

The use of postmortem brain tissue has several limitations, including availability, potential for long delay between death and sample collection, during which rapid cellular and molecular changes can occur, the possible lack of medication history and the difficulty in separating the effect of disease from other confounding factors (Popova *et al.*, 2008). Since brain tissue is not readily accessible, a new focus in research on biomarkers is blood-based expression profiling of miRNAs. In order to determine the expression profile of miRNAs in peripheral blood leukocytes in people affected by Parkinson disease (PD), Soreq *et al.*, conducted SOLiD high throughput sequencing on small RNAs from peripheral blood leukocytes of PD patients followed by alignment to miRBase. (Soreq *et al.*, 2013). In this particular study miR-320a, miR-320b and miR-320c show statistically significant downregulation in the group of PD affected patients compared with healthy controls.

Tabares-Seisdedos *et al.*, reviewed the short arm of chromosome 8 as a potential site for CNV alterations, closely related to different neurodevelopment disorders such as depression, schizophrenia and autistic spectrum disorder (ASD). Among the 484 genes located in this region 7 are microRNA genes, including miR-320. Based on evidence obtained in molecular genetics and cerebral developmental studies, authors suggest that alterations in FGF17, miR-124-1 and miR-320 should be considered to endow

standard curve approach. In this study authors show that miR-320c displays statistically significant upregulation in suicide completers compared to controls. They also found significant negative correlation between miR-320c levels and the levels of the SAT1 gene, whose expression is referred to be lower in brain tissues of suicide completers (Lopez *et al.*, 2014) susceptibility to mental illness (Tabares-Seisdedos *et al.*, 2009). The authors, however do not specify the exact member of the miR-320 family in their review. However, assuming that miR-320a gene is the only one in the family located on the chromosome 8, we deduce that this is the specific micro RNA gene the authors have referred to.

In this study we have performed, miRNA specific stem loop qRT-PCR assay in order to identify microRNAs involved in schizophrenia. Despite the fact that we do not know whether the aberrant expression of the miRNAs were also present in the patients' CNS, the majority of them are detectable in the brain according to the literature (Hsu *et al.*, 2008; Wheeler *et al.*, 2006). The changes in the post-transcriptional regulatory environment of peripheral blood in patients with schizophrenia not only have implications for the pathophysiology of the disorder (Perkins *et al.*, 2005; Beveridge *et al.*, 2010) but also provide a suitable source of non-invasive biomarkers for schizophrenia. The study of the differential expression of miRNA molecules is very important since dysregulation in the expression levels of specific miR-320 members are expected to influence the transcription/translation of a number of protein-encoding genes. We found that miR-320b, miR-320c, miR-320d are up-regulated in schizophrenia and miR-320a is the most up-regulated. To our knowledge, this is the first study to show evidence from whole blood where the expression of miR-

320a is dysregulated in subjects with schizophrenia.

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