

## Impact of Genetic Variants in Mir-122 Gene and its Flanking Regions on Hepatitis B Risk

Sadegh Fattahi<sup>1</sup>, Mohammad Karimi Alivije<sup>2</sup>, Farhang Babamahmoodi<sup>3</sup>, Masomeh Bayani<sup>4</sup>, Mahmoud Sadeghi-Haddad-Zavareh<sup>4</sup>, Mohsen Asouri<sup>1</sup>, Maryam Lotfi<sup>1</sup>, Galia Amirbozorgi<sup>1</sup>, Morteza Gholami<sup>1,5,6</sup>, Haleh Akhavan-Niaki<sup>1,7\*</sup>

1. Cellular and Molecular Department, North Research Center, Pasteur Institute of Iran, Amol, Iran.
2. Department of Infectious Diseases, Imam Reza Hospital, Amol, Iran.
3. Department of Antimicrobial Resistance Research Center, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran.
4. Infectious Diseases and Tropical Medicine Research Center, Babol University of Medical Sciences, Babol, Iran.
5. Obesity and Eating Habits Research Center, Endocrinology and Metabolism Molecular–Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran.
6. Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran.
7. Department of Genetics, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran.

Submitted 27 Nov 2016; Accepted 29 Dec 2016; Published 14 Jan 2017

MicroRNAs are small non coding RNAs that are involved in gene expression regulation. Mir-122 was reported to inhibit hepatitis B virus (HBV), but little is known about the role of mir-122 polymorphisms on HBV infection development. This present study aimed to investigate the association between single nucleotide polymorphisms (SNPs) in mir-122 gene region with HBV infection. Study cases were HBV positive and negative individuals. 67 SNPs in mir-122 gene and its flanking regions were analyzed by sequencing method. mirVas software was used to assay the impact of polymorphisms on the secondary structure of mir-122 gene. 66 out of 67 studied SNPs were monomorphic and rs 17669 was the only polymorphic SNP in the studied population, with the T allele being four times more frequent than the C allele. However, there was no significant difference in alleles distribution between patient and control groups. Rs 17669 variant located near the mir-122 gene showed the highest impact for centroid, maximal expected accuracy, and minimal free energy structures in the arm, flank, and flank regions of mir-122, respectively. Therefore, rs17669 variant was predicted to exert an effect on mir-122 stability. The study of larger samples from different ethnicities may help to find a possible association between rs17669 genotype and HBV infection.

**Keywords:** Mir-122, HBV, single nucleotide polymorphism, rs17669, mirVas

MicroRNAs (miRNAs) are a large family of short (~22 nucleotides) non-coding RNAs that play important role in gene expression (1).

MiRNAs regulate gene expression at post-transcriptional level via binding to 3'-UTR complementary sequences on target mRNA and either

\*Correspondence: Department of Genetics, Faculty of Medicine, Babol University of Medical Sciences, Babol, Iran.  
E-mail: halehakhavan@yahoo.com

catalyze the target degradation or inhibit mRNA translation (2, 3). Furthermore, miRNAs have been associated with diverse biological processes, including cell proliferation, differentiation, apoptosis, development, immune response, tumorigenesis, DNA methylation and chromatin modification (4-9). To date, there are more than 8,800 miRNA encoding genes in humans which regulate the activity of more than half of all protein-coding genes (10). Recently, many studies have been performed in order to identify the role of cellular miRNAs in viral life cycles (11-15). Among the miRNAs identified, mir-122, is a 22 nucleotide miRNA that is expressed at high levels in hepatocytes, where it constitutes close to 70% of total liver miRNAs content (4). It has been reported that mir-122 is involved in a wide range of hepatic functions, including hepatocyte differentiation (16), lipid metabolism (17, 18), and hepatocellular carcinoma (19). Recent studies have also shown that mir-122 may enhance hepatitis C virus (HCV) replication by many different methods such as interaction with a 5' untranslated region of the HCV genome, and enhancing ribosomes detachment from the viral RNA (3, 20, 21). In contrast to HCV, mir-122 inhibits hepatitis B virus (HBV) by binding to the viral target sequence, up-regulating cyclin G1 followed by cyclin G1-p53 complex formation, and down-regulating heme oxygenase-1 (22-25). HBV is a hepatotropic double-stranded DNA virus with a ~3.2 kb genome that establishes persistent infections in liver tissue. More than 350 million people are estimated to be chronically infected with HBV worldwide, which may further result in hepatocellular carcinoma (HCC) (26). Although mir-122 expression and its role in hepatocarcinogenesis and the regulation of HCV and HBV infection has been widely studied, little is known regarding mir-122 gene polymorphism and its role in HBV infection. It has been shown that genetic variants within precursor miRNAs and miRNA target sites can affect miRNA regulation and are associated with various diseases

(27-32). Recently, Cammaerts et al. showed that genetic variants present not only within miRNA sequences or their target sites, but also those present in flanking regions of microRNA genes may have an impact on miRNAs expression and function (33). The aim of the present study was to investigate for the first time the association between genetic variants within mir-122 gene and its flanking region with HBV infection.

## Materials and methods

### Subjects

18 people (8 healthy individuals and 10 patients infected with HBV) were included in the study. All subjects were consenting volunteers among HBV suspect cases referred to the North Research Center, Pasteur Institute of Iran, Amol, for molecular diagnosis of HBV. All experimental protocols complied with the guidelines of Medical Ethics Committee, Ministry of Health, Iran. The diagnosis of acute or chronic HBV infection was based on serology for the hepatitis B surface antigen (HBsAg) and the presence of HBV DNA by real-time PCR kit.

### DNA extraction

Genomic and viral DNA were extracted from 200 µL blood sample using Qiagen's QIAamp DNA Blood Mini Kit (Cat# 51104), and Qiagen viral DNA mini kit according to manufacturers' instructions, respectively.

### Single nucleotide polymorphisms analysis

67 variants located either in mir-122 gene or its flanking upstream and downstream regions were studied. Primer sets for the amplification and sequencing analysis of mir-122 were designed based on Gen-Bank sequences (Ref. Genome seq.; NC\_000018). PCR was performed using forward (5'-TGGTGGGCAGGAGTTCACATA -3') and reverse (5'-ACAACAGCATGTGAGAGGCA -3') primers. Amplification was achieved by an initial denaturation at 95 °C for 5 min followed by 35 cycles each consisting of denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, extension at 72 °C

for 45 s, and a final elongation step at 72 °C for 7 min. The 1042 bp PCR product was used for further sequencing by ABI 3730XL instrument by Bioneer Co, Korea

### Impact of variants on mir-122

miRvAs software was used to predict the impact of variants on mir-122 (34). Also, this software had a RNA fold tool for representing different secondary structures, and VARNA tool to generate predicated images (35, 36). The centroid (CEN), maximal expected accuracy (MEA) and minimal free energy (MFE) structures with 1000 bp 3' and 5' flanking bases were used to assess the structural impact.

### Statistical analysis

Genotype and allelic frequencies in the patient and control groups were determined by using  $\chi^2$  and Fisher's tests. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were calculated. P value of  $\leq 0.05$  was considered as statistically significant. Data analysis was performed using SPSS 22 software.

## Results

The investigation of 67 variants present in mir-122 and its flanking regions in normal and HBV infected individuals by direct sequencing, revealed that only one single nucleotide polymorphism (SNP) (rs17669) was polymorphic in the studied population (Table 1). The T and C allele frequencies

were found to be 0.8125 and 0.1875, respectively among the normal subjects, and 0.7 and 0.3, respectively among hepatitis subjects. The distribution of TT and CT genotypes in normal individuals were 75% and 12.5%, respectively compared to 60% and 20%, respectively in hepatitis patients. There was no significant difference in genotype frequencies between controls and affected patients ( $P > 0.05$ ). Figure 1 shows the rs17669 chromatogram pattern after direct sequencing.

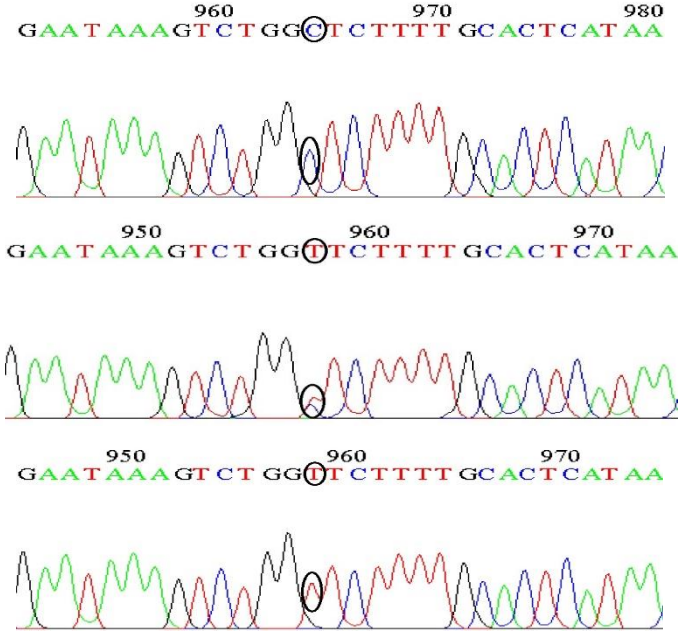
### Variant rs17669 and different secondary structures in mir-122

Based on sequencing data, all variants in or near mir-122 gene were monomorphic in the studied population, except rs17669 variant. Hence this variant was selected to assess the impact of the variant on nearby mir-122 secondary structure. As shown in figures 2-4, based on the secondary structure prediction location, rs17669 variant was 104 nt outside the mir-122 hairpin structure and the highest impact for CEN, MEA and MFE was observed in the arm, flank, and flank regions, respectively. Based on data obtained from miRvAs software, CEN\_ΔG, MEA\_ΔG and MFE\_ΔG for wild type allele were -66.05, -109.80 and -136.80 kcal/mol, respectively and -96.70, -120 and -138.90 kcal/mol, respectively for the variant form. Consequently, ΔΔG was not equal to zero and rs17669 variant was predicted to exert an effect on the miRNA stability.

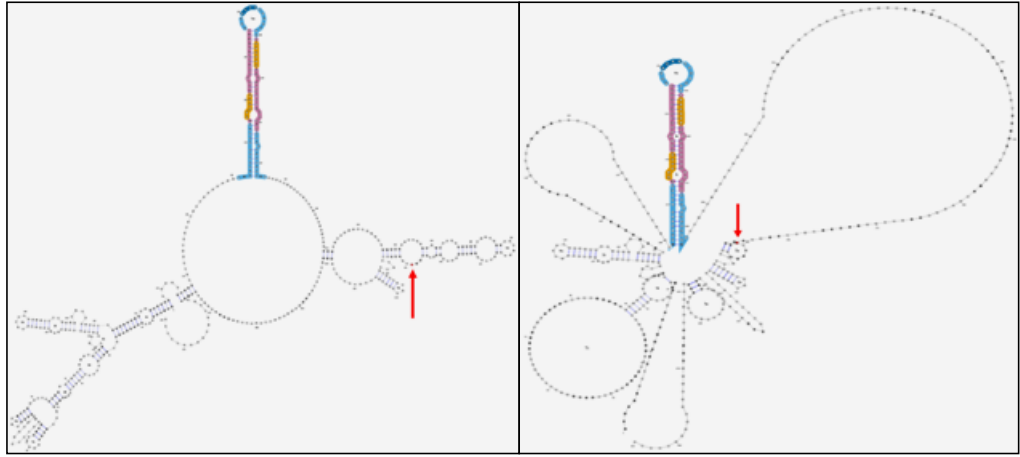
**Table 1.** Single nucleotide polymorphisms examined in mir-122 locus

rs781380452	rs111237676	rs534136761	rs144352578	rs770081919	rs773714009	rs73959642
rs770549297	rs774051406	rs561961609	rs76821324	rs556624161	rs759229547	rs767154096
rs775028435	rs574971146	rs527940975	rs760460015	rs147372032	rs570185349	rs188170153
rs113204803	rs74623305	rs527441144	rs547693654	rs545694397	rs564008548	rs531525440
rs191437296	rs139491862	rs142518440	rs150923640	rs112087268	rs369868032	rs757244990
rs559216977	rs564329755	rs111436359	rs751678746	rs748643064	rs772608604	rs111316406
rs747356956	rs770586288	rs184005834	rs146738801	rs775046700	rs374041450	rs764055409
<u>rs751314950</u>	<u>rs761608653</u>	<u>rs41292412</u>	<u>rs554204767</u>	<u>rs755220609</u>	<u>rs779183355</u>	<u>rs748336334</u>
rs752772071	rs756437097	rs371363087	rs778068633	rs747459659	rs771353069	rs373818185
<b>rs745573208</b>	rs557777663	<b>rs17669</b>	rs771602049			

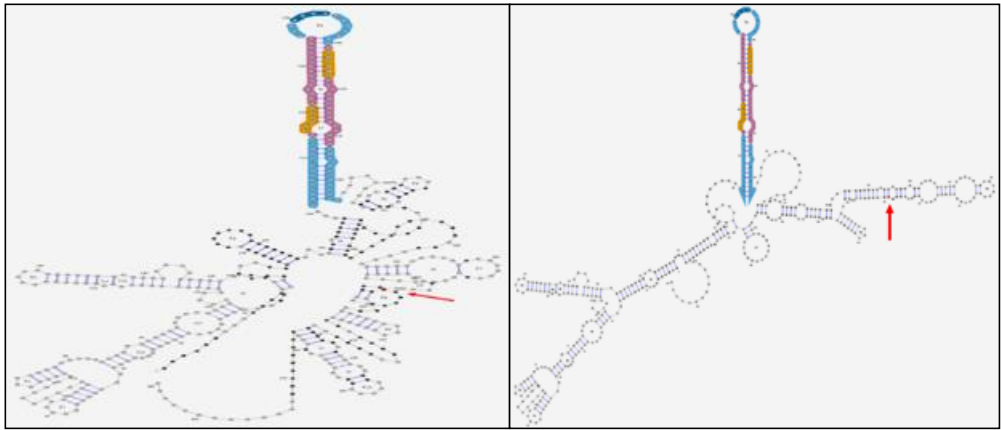
underline: SNPs within mir-122 gene.



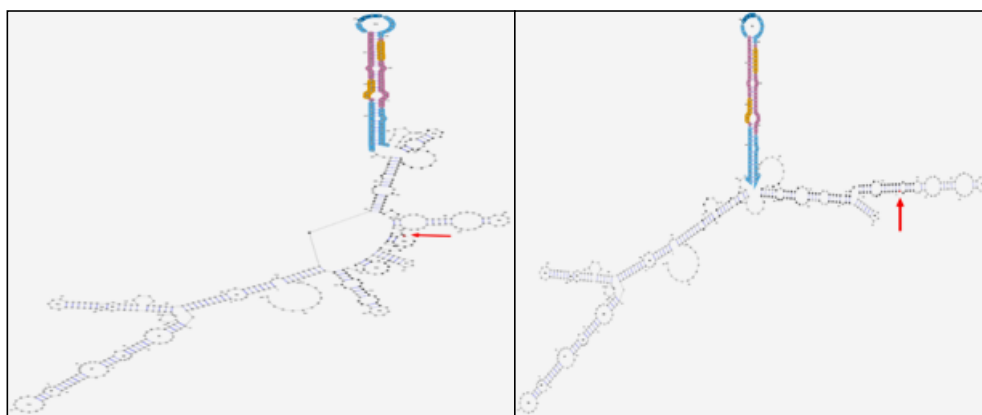
**Figure 1.** Chromatogram of sequences containing rs17669 polymorphism. Visualization with Chromas program. Upper panel: CC genotype; middle panel: CT genotype; lower panel: TT genotype.



**Figure 2.** CEN secondary structure predictions of mir-122 for rs17669 variant. Colored region correspond to mir-122, and red arrow shows the position of rs17669 variant.



**Figure 3.** MAE secondary structure predictions of mir-122 for rs17669 variant. Colored region correspond to mir-122, and red arrow shows the position of rs17669 variant.



**Figure 4.** MFE secondary structure predictions of mir-122 rs17669 variant. Colored region correspond to mir-122, and red arrow shows the position of rs17669 variant.

## Discussion

The present study investigated the association between SNPs present in mir-122 gene region with HBV infection. It has been reported that mir-122 is involved in a wide range of hepatic functions and constitute close to 70% of total liver miRNAs content (37). We tested 67 SNPs in mir-122 gene and its flanking regions. Our data showed that 66 SNPs out of 67 were monomorphic and only one of them (rs17669) was polymorphic. At rs17669, the allele frequencies were similar in patient and control groups, and T allele was four times more abundant than the C allele. There was no significant difference between the distribution of different genotypes in control and patient groups. In addition, we assessed the impact of rs17669 variant on secondary structures of mir-122 by mirVas software. According to mirVas data, it was predicated that rs17669 variant can cause changes in the secondary structure of mir-122 locus. Therefore, a structural change in this region could impact on the processing and the expression of the miRNA. Regarding the role of miRNAs in hepatitis development, other studies demonstrated the importance of these regulatory small non-coding RNAs. Relatively, mir-196A2 polymorphism was shown to be associated with susceptibility to HBV-related hepatocellular carcinoma (HCC) in Chinese population (38). Some mir-122 polymorphisms were also reported to play roles in clinical phenotype modulation in inflammatory bowel disease (39). Mir-146a gene

polymorphism and mir-122-binding site polymorphism in the interleukin-1 $\alpha$  3' untranslated region have been associated with the risk for HCC (40, 41). Mir-122 is a completely conserved liver-specific miRNA and plays an important role in the maintenance of liver homeostasis and liver's differentiated state preservation (42). Mir-122 expression is reduced in HBV positive HCC (43), and it inhibits HBV replication in HCC cells *in vitro* (44). It was also suggested that rs4309483, in the upstream regulatory region of mir-122 gene, may regulate the expression of mir-122 and increase the risk for HCC development in HBV carriers, while protecting from chronic HBV infection (45).

Although in the present study no significant association was found between SNPs in mir-122 gene region and HBV infection, our data suggest that polymorphism in the flanking region of mir-122 gene induces structural changes in those regions and therefore may have an impact on mir-122 maturation process. Further analysis of mir-122 expression in patients presenting different rs17669 genotypes may help to understand the role of this variant in mir-122 processing.

## Conflict of interest

The authors declared no conflict of interest.

## References

1. Bartel D P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281-97.
2. Janas M M, Novina C D. Not lost in translation: stepwise

- regulation of microRNA targets. *EMBO J.* 2012;31:2446-7.
3. Li S, Xing X, Yang Q, et al. The effects of hepatitis C virus core protein on the expression of miR-122 in vitro. *Virology*. 2013;10:98.
  4. Girard M, Jacquemin E, Munnich A, et al. miR-122, a paradigm for the role of microRNAs in the liver. *J Hepatol.* 2008;48:648-56.
  5. O'Connell R M, Rao D S, Chaudhuri A A, et al. Physiological and pathological roles for microRNAs in the immune system. *Nat Rev Immunol.* 2010;10:111-22.
  6. Xu J, Zhu X, Wu L, et al. MicroRNA-122 suppresses cell proliferation and induces cell apoptosis in hepatocellular carcinoma by directly targeting Wnt/beta-catenin pathway. *Liver Int.* 2012;32:752-60.
  7. Ghildiyal M, Zamore P D. Small silencing RNAs: an expanding universe. *Nat Rev Genet.* 2009;10:94-108.
  8. Moazed D. Small RNAs in transcriptional gene silencing and genome defence. *Nature.* 2009;457:413-20.
  9. Chen D, Fu L Y, Zhang Z, et al. Dissecting the chromatin interactome of microRNA genes. *Nucleic Acids Res.* 2014;42:3028-43.
  10. Meneely P. Genetic Analysis: Genes, Genomes, and Networks in Eukaryotes: Oxford University Press, USA; 2014.
  11. Mosca N, Castiello F, Coppola N, et al. Functional interplay between hepatitis B virus X protein and human miR-125a in HBV infection. *Biochem Biophys Res Commun.* 2014;449:141-5.
  12. Dai X, Zhang W, Zhang H, et al. Modulation of HBV replication by microRNA-15b through targeting hepatocyte nuclear factor 1alpha. *Nucleic Acids Res.* 2014;42:6578-90.
  13. Jin J, Tang S, Xia L, et al. MicroRNA-501 promotes HBV replication by targeting HBXIP. *Biochem Biophys Res Commun.* 2013;430:1228-33.
  14. Hu W, Wang X, Ding X, et al. MicroRNA-141 represses HBV replication by targeting PPARA. *PLoS One.* 2012;7:e34165.
  15. van der Ree M H, de Bruijne J, Kootstra N A, et al. MicroRNAs: role and therapeutic targets in viral hepatitis. *Antivir Ther.* 2014;19:533-41.
  16. Xu H, He J H, Xiao Z D, et al. Liver-enriched transcription factors regulate microRNA-122 that targets CUTL1 during liver development. *Hepatology.* 2010;52:1431-42.
  17. Filipowicz W, Grosshans H. The liver-specific microRNA miR-122: biology and therapeutic potential. *Prog Drug Res.* 2011;67:221-38.
  18. Esau C, Davis S, Murray S F, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab.* 2006;3:87-98.
  19. Burchard J, Zhang C, Liu A M, et al. microRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. *Mol Syst Biol.* 2010;6:402.
  20. Jopling C L. Regulation of hepatitis C virus by microRNA - 122. *Biochem Soc Trans.* 2008;36:1220-3.
  21. Garcia-Sastre A, Evans M J. miR-122 is more than a shield for the hepatitis C virus genome. *Proc Natl Acad Sci U S A.* 2013;110:1571-2.
  22. Zhang X, Zhang E, Ma Z, et al. Modulation of hepatitis B virus replication and hepatocyte differentiation by MicroRNA-1. *Hepatology.* 2011;53:1476-85.
  23. Qiu L, Fan H, Jin W, et al. miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. *Biochem Biophys Res Commun.* 2010;398:771-7.
  24. Chen Y, Shen A, Rider P J, et al. A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. *FASEB J.* 2011;25:4511-21.
  25. Li C, Wang Y, Wang S, et al. Hepatitis B virus mRNA-mediated miR-122 inhibition upregulates PTTG1-binding protein, which promotes hepatocellular carcinoma tumor growth and cell invasion. *J Virol.* 2013;87:2193-205.
  26. Reyes-del Valle J, Hodge G, McChesney M B, et al. Protective anti-hepatitis B virus responses in rhesus monkeys primed with a vectored measles virus and boosted with a single dose of hepatitis B surface antigen. *J Virol.* 2009;83:9013-7.
  27. Cammaerts S, Strazisar M, De Rijk P, et al. Genetic variants in microRNA genes: impact on microRNA expression, function, and disease. *Front Genet.* 2015;6:186.
  28. Hu Z, Liang J, Wang Z, et al. Common genetic variants in pre-microRNAs were associated with increased risk of breast cancer in Chinese women. *Hum Mutat.* 2009;30:79-84.
  29. Wang G, van der Walt J M, Mayhew G, et al. Variation in the miRNA-433 binding site of FGF20 confers risk for Parkinson disease by overexpression of alpha-synuclein. *Am J Hum Genet.* 2008;82:283-9.
  30. Kapeller J, Houghton L A, Monnikes H, et al. First evidence for an association of a functional variant in the microRNA-510 target site of the serotonin receptor-type 3E gene with diarrhea predominant irritable bowel syndrome. *Hum Mol Genet.* 2008;



- 17:2967-77.
31. Abelson J F, Kwan K Y, O'Roak B J, et al. Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science*. 2005;310:317-20.
32. Song F J, Chen K X. Single-nucleotide polymorphisms among microRNA: big effects on cancer. *Chin J Cancer*. 2011;30:381-91.
33. Cammaerts S, Strazisar M, Smets B, et al. Schizophrenia-Associated MIR204 Regulates Noncoding RNAs and Affects Neurotransmitter and Ion Channel Gene Sets. *PLoS One*. 2016;10:e0144428.
34. Cammaerts S, Strazisar M, Dierckx J, et al. miRVaS: a tool to predict the impact of genetic variants on miRNAs. *Nucleic Acids Res*. 2016;44:e23.
35. Lorenz R, Bernhart S H, Honer Zu Siederdisen C, et al. ViennaRNA Package 2.0. *Algorithms Mol Biol*. 2011;6:26.
36. Darty K, Denise A, Ponty Y. VARNAs: Interactive drawing and editing of the RNA secondary structure. *Bioinformatics*. 2009;25:1974-5.
37. Girard M, Jacquemin E, Munnich A, et al. miR-122, a paradigm for the role of microRNAs in the liver. *J Hepatol*. 2008;48:648-56.
38. Qi P, Dou T-h, Geng L, et al. Association of a variant in MIR 196A2 with susceptibility to hepatocellular carcinoma in male Chinese patients with chronic hepatitis B virus infection. *Hum Immunol*. 2010;71:621-6.
39. Ciccacci C, Politi C, Biancone L, et al. Polymorphisms in MIR122, MIR196A2, and MIR124A Genes are Associated with Clinical Phenotypes in Inflammatory Bowel Diseases. *Mol Diagn Ther*. 2016:1-8.
40. Gao Y, He Y, Ding J, et al. An insertion/deletion polymorphism at miRNA-122-binding site in the interleukin-1 $\alpha$  3' untranslated region confers risk for hepatocellular carcinoma. *Carcinogenesis*. 2009;30:2064-9.
41. Xu T, Zhu Y, Wei Q-K, et al. A functional polymorphism in the miR-146a gene is associated with the risk for hepatocellular carcinoma. *Carcinogenesis*. 2008;29:2126-31.
42. Krützfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs in vivo with 'antagomirs'. *Nature*. 2005;438:685-9.
43. Coulouarn C, Factor V M, Andersen J B, et al. Loss of miR-122 expression in liver cancer correlates with suppression of the hepatic phenotype and gain of metastatic properties. *Oncogene*. 2009;28:3526-36.
44. Hu J, Xu Y, Hao J, et al. MiR-122 in hepatic function and liver diseases. *Protein & cell*. 2012;3:364-71.
45. Thakral S, Ghoshal K. miR-122 is a unique molecule with great potential in diagnosis, prognosis of liver disease, and therapy both as miRNA mimic and antimir. *Curr Gene Ther*. 2015;15:142-50.