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Original Article

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

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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

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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

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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 X^2 and fisher's tests. Odds ratios (ORs) and their 95% confidence intervals (95% CI) were calculated. P value of ≤ 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). Figure1 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
rs751314950	rs761608653	rs41292412	rs554204767	rs755220609	rs779183355	rs748336334
rs752772071	rs756437097	rs371363087	rs778068633	rs747459659	rs771353069	rs373818185
rs745573208	rs557777663	**rs17669**	rs771602049			

underline: SNPs within mir-122 gene.

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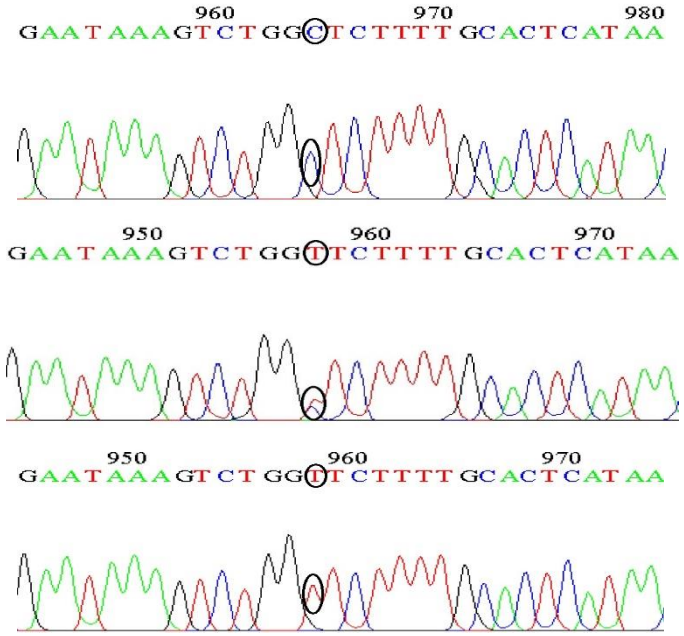


Figure 1. Chromatogram of sequences containing rs17669 polymorphism. Visualization with Chromas program. Upper panel: CC genotype; middle panel: CT genotype; lower panel: TT genotype.

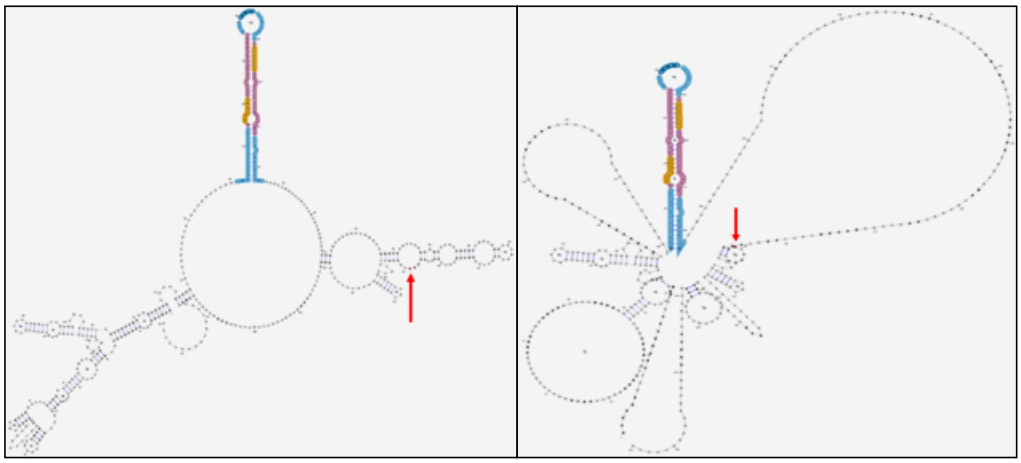


Figure 2. CEN secondary structure predictions of mir-122 for rs17669 variant. Colored region correspond to mir-122, and red arrow shows the position of rs17669 variant.

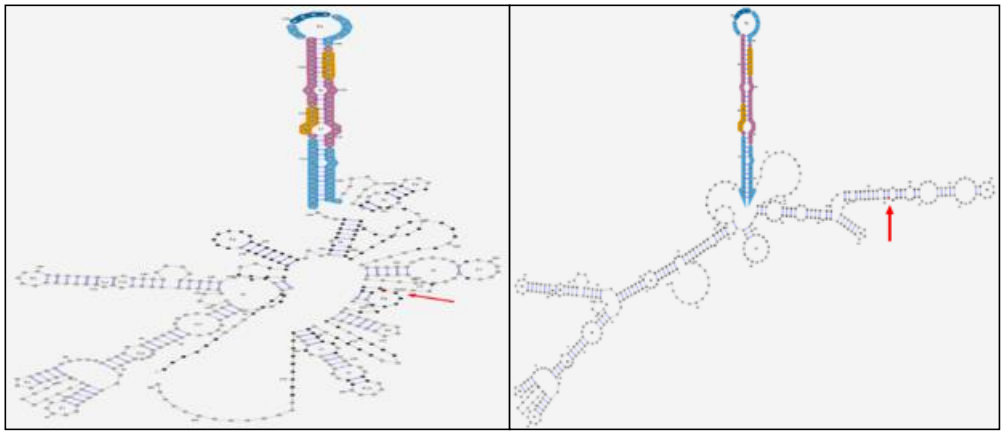


Figure 3. MAE secondary structure predictions of mir-122 for rs17669 variant. Colored region correspond to mir-122, and red arrow shows the position of rs17669 variant.

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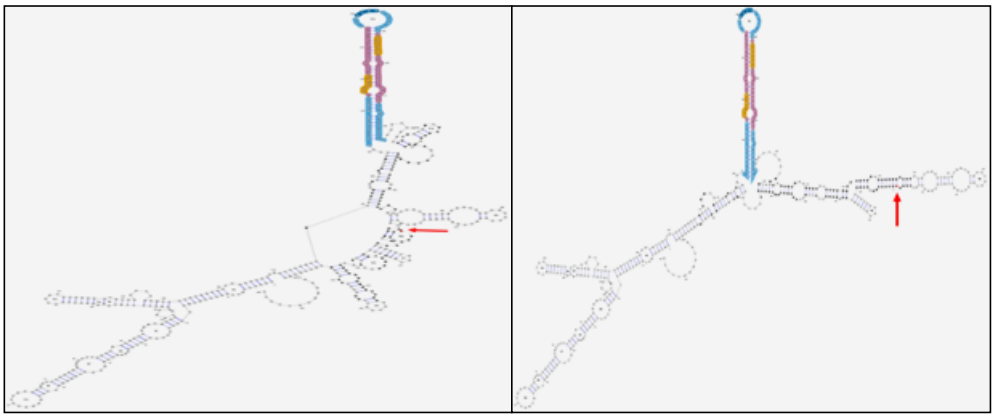


Figure 4. MFE secondary structure predictions of mir-122 rs17669 variant. Colored region correspond to mir-122, and red arrow shows the position of rs17669 variant.

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 sclose 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-1α 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.

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