

RESEARCH COMMUNICATION

Genetic Variation in the MicroRNA-499 Gene and Hepatocellular Carcinoma Risk in a Turkish Population: Lack of Any Association in a Case-Control Study

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Abstract

MicroRNAs (miRNAs) are an abundant class of small non-protein-coding RNAs with posttranscriptional regulatory functions as tumor suppressors and oncogenes. It has been suggested that the presence of single nucleotide polymorphisms (SNPs) in miRNAs can alter miRNA processing, expression, and/or binding to target mRNA and represent another type of genetic variability that can contribute to susceptibility to cancer development in humans. An adenine to guanine polymorphism (rs3746444), located in the sequence of miR-499, results in a change from A:U to G:U in its stem region. To determine the association of this polymorphism with the risk of hepatocellular carcinoma (HCC) in a Turkish population, a hospital-based case-control study was designed consisting of 222 subjects with HCC and 222 cancer-free control subjects matched for age, gender, smoking and alcohol status. The genotype frequency of the miR-499 rs3746444 polymorphism was determined using a polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) assay. No statistically significant differences were found in the allele or genotype distributions of the miR-499 rs3746444 polymorphism among HCC and cancer-free control subjects ($P>0.05$). Our results demonstrate for the first time that the miR-499 rs3746444 polymorphism does not been any major role in genetic susceptibility to hepatocellular carcinogenesis, at least in the population studied here. Independent studies are need to validate our findings in a larger series, as well as in patients of different ethnic origins.

Keywords: HCC - microRNA-499 - rs3746444 A/G polymorphism - susceptibility - Turkey

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer death. Because of its high fatality rates, the incidence and mortality rates are approximately equal (Jemal et al., 2011). Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are the major cause of HCC. Nevertheless, only a fraction of infected patients develop HCC during their lifetime suggesting that genetic factors might modulate HCC development. So the search for genetic factors that could help to select high-risk populations and thus to modulate the indications of screening procedures are necessary (Lovet et al., 2004). Moreover, identification of predictive factors could lead to a better diagnosis and planning of new prevention strategies at-risk individuals (Lodato et al., 2006).

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, single-stranded, non-coding RNA molecules of ~20 nucleotides in length that function as regulators of gene expression at the posttranscriptional level (Bartel,

2004; Esquela-Kerscher and Slack, 2006). Bioinformatic data indicated that a single miRNA could bind to as many as 200 different target transcripts, it has been conjectured that miRNAs regulate the expression of approximately one third of the protein-coding mRNAs (Bartel, 2004; Vasudevan et al., 2007). miRNAs have been implicated in a wide range of physiologic and pathologic processes, including development, cell differentiation, proliferation, apoptosis and carcinogenesis (Bartel, 2004). In the pathogenesis of HCC, miRNAs have essential roles in progression and directly contribute to cell proliferation, avoidance of apoptotic cell death, and metastasis by targeting a large number of critical protein-coding genes (Huang and He, 2011).

A Homo sapiens miR-499 gene was mapped to 20q11.22. Analysis of the genomic location of the miR-499 gene showed it to be contained within the 20th intron of the beta-myosin heavy chain 7B (Myh7b) gene. The single nucleotide polymorphisms (SNPs), the most common type of genetic variation in human genome which can affect the functions of miRNA and in turn influence

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the individual susceptibility to cancer (Loktionov, 2004). Recently, functional SNP has been identified in the miR-499 gene (adenine to guanine [A/G]) (Hu et al., 2008). A/G polymorphism (rs3746444) is located in the stem region opposite to the mature miR-499 sequence (placing it in the passenger strand, 3p) and results in a change from A:U pair to G:U mismatch in the stem structure of miR-499 (Hu et al., 2008). The optimal free energy was decreased from -62.30 kcal/mol for A to -61.90 kcal/mol for G alleles, suggesting a less stable secondary structure for the G allele compared with the A allele (Hu et al., 2008). It has been observed that genetic variants in mature miRNA regions could change the conformation of the secondary structure and thereby directly affect both the binding to target mRNAs and the miRNA maturation process (Zeng and Cullen, 2003; Duan et al., 2007).

A few molecular epidemiological studies have investigated the association between the miR-499 rs3746444 polymorphism and the cancer (Hu et al., 2008; 2009; Catucci et al., 2009; Tian et al., 2009; Okubu et al., 2010; Liu et al., 2010; Srivastava et al., 2010; George et al., 2011; Mittal et al., 2011; Zhou et al., 2011a; 2011b). According to our recent knowledge, to date, there was only one report on the relationship between miR-499 rs3746444 polymorphism and the risk of HCC (Zhou et al., 2011b). No similar study has been conducted yet in a Turkish population. To test the hypothesis that the rs3746444 miR-499 polymorphism is associated with risk of developing HCC, we performed genotyping analysis using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay in a hospital-based case-control study of 222 HCC patients and 222 age, gender, smoking and alcohol consumption matched cancer-free controls in Turkish population.

Materials and Methods

Study Population

The study population and subject characteristics were previously described elsewhere (Akkız et al., 2010; Akkız et al. 2011). This is an ongoing molecular epidemiologic study of HCC conducted in Adana, Turkey and the subject recruitment was approved by the Committee for Ethics of Medical Experiment on Human Subjects, Faculty of Medicine, Çukurova University. Briefly, all subjects were genetically unrelated Turkish and were from Çukurova and the surrounding regions of southern Turkey. Submission of the individuals to the study was conditioned by an obtained written informed consent form regarding the use of their blood samples for research studies. The study proceeded in agreement with the Helsinki declaration approved on the World Medical Association meeting in Edinburgh. Blood samples were collected from 222 consecutive patients with HCC seen in the department of gastroenterology between September 2005 and April 2011. During the same time, 222 unrelated community residents with no evidence of hepatocellular or other cancer who entered the hospital for health check-ups were enrolled as the control group. The 222 cancer-free control subjects did not have a history of liver disease and had no serological evidence of hepatitis B or C virus infection. Each control was pair-matched by

sex, age (± 3 years), smoking and alcohol consumption to a patient with HCC. The HCC diagnostic criteria was based on the guideline proposed by European Association for the Study of the Liver (EASL) (Bruix et al., 2001). We gave a diagnosis of HCC when a patient had one or more risk factors (i.e., HBV or HCV infection, or cirrhosis) and one of the following: >400 ng/mL α -fetoprotein (AFP) and at least one positive finding following examination using spiral computed tomography (CT), contrast-enhanced dynamic MRI, or hepatic angiography; or <400 ng/mL α -fetoprotein and at least two findings following CT, MR, or hepatic angiography. A positive HCC finding using dynamic CT or MRI is indicative of arterial enhancement followed by venous washout in the delayed portal/venous phase. In addition; we performed histopathological diagnosis for cases that did not fulfill all of the clinical non-invasive diagnostic criteria of HCC. Cirrhosis was diagnosed with liver biopsy, abdominal sonography, and biochemical evidence of parenchymal damage plus endoscopic esophageal or gastric varices (Tsai et al., 1994). Patients with cirrhosis were classified into three Child-Pugh grades based on their clinical status (Pugh et al., 1973). Serum HBsAg and Anti-HCV were assessed using an immunoassay (Abbott Laboratories, Abbott Park, IL, USA). Serum AFP concentration was measured by microparticle enzyme immunoassay (Abbott Laboratories, AXSYM, USA). Heavy alcohol intake was defined as a daily minimum consumption of 160 g alcohol for at least eight years.

DNA Extraction

A 5 mL sample of venous blood was collected from each subject into a test tube containing EDTA as anticoagulant. Genomic DNA was extracted from peripheral whole blood using High Pure PCR Template Preparation Kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis

PCR-RFLP analysis was performed to determine the genotype of the A/G polymorphism of miR-499 gene, as described previously (Hu et al., 2008). The 146 base pair (bp) fragment encompassing the A to G polymorphic site in miR-499 region was amplified using specific primers 5'-CAA AGT CTT CAC TTC CCT GCC A -3' and 5'-GAT GTT TAA CTC CTC TCC ACG TGA TC -3'. Amplification was performed in GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Singapore).

The 20 μ L PCR mixture contained approximately 250 ng DNA, with 0.25 μ M of both primer, 0.1 mM of each dNTP, 1X PCR buffer, 1.5 mM MgCl₂ and 1U Taq polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: 95 °C for 5 min, followed by 30 cycles of 94 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min.

As a negative control, PCR mix without DNA sample was used to ensure contamination free PCR product. After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was digested overnight with 5 units BclI (from an Escherichia

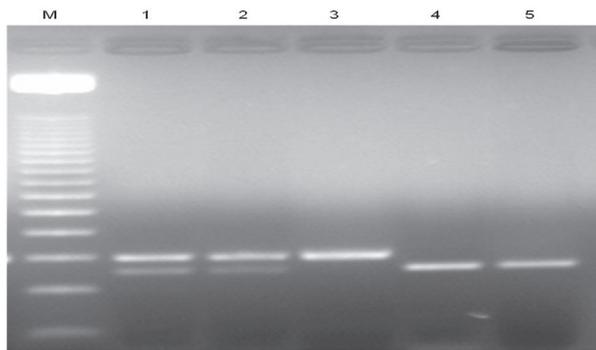


Figure 1. Analysis of MiR-499 rs3746444 Polymorphism.

A representative agarose gel picture showing PCR-RFLP analysis of miR-499 rs3746444 A/G genotypes in genomic DNAs of study subjects with restriction endonuclease enzyme BclI. M: 50-bp DNA ladder, Lane 1 and 2: AG heterozygous (146 bp, 120 bp and 26), Lane 3: G/G homozygous (146 bp), Lane 4 and 5: AA homozygous (120 bp and 26 bp)

coli strain that BclI gene from *Bacillus caldolyticus*, recognizing the sequence 5'-TGATCA-3') enzyme at 50°C (New England Biolabs Inc., Beverly, MA) and electrophoresed on 3% agarose gel containing 0.5 µg/ml ethidium bromide and visualized under UV illumination. PCR products with A at the polymorphic site were digested into two fragments, 120 bp and 26 bp, while those with G were not because of the absence of a BclI restriction site. Samples yielding 120 bp and 26 bp fragments were scored as AA, those with single 146 bp fragments as GG, and 146 bp, 120 bp and 26 bp as AG. This assay was illustrated in Figure 1. To ensure quality control, genotyping was performed without knowledge of the subjects' case/control status and a 15% random sample of cases and controls was genotyped twice by different persons; reproducibility was 100%.

Statistical Analysis

The sample size was calculated using the QUANTO 1.1 program (hydra.usc.edu/gxe) considering the minor allele frequency (MAF) of the miR-499 rs3746444 polymorphism in Caucasian population. The sample size of 222 cases and 222 controls was adequate to give us a power of 80% (probability of not making Type II error). Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS; SPSS, Inc., Chicago, IL, USA) for Windows (version 10.0). Differences in the distributions of demographic characteristics between the cases and controls were evaluated using the Student's t-test (for continuous variables) and χ^2 test (for categorical variables). The observed genotype frequencies were compared with expected values calculated from Hardy-Weinberg equilibrium theory ($p^2 + 2pq + q^2 = 1$; where p is the frequency of the wild-type allele and q is the frequency of the variant allele) by using a χ^2 test with degree of freedom equal to 1 among cases and controls, respectively. Pearson's χ^2 test was used to determine whether there was any significant difference in allele and genotype frequencies between patients and controls. The associations between miR-499 rs3746444 genotypes and the risk of HCC were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs)

Table 1. Distribution of Selected Characteristics in Patients with Hepatocellular Carcinoma and Controls

Characteristic	Patients (%)	Controls (%)	p value ^a
Age (years) mean (±SD) (range)	60.53±11.13 (20-87)	60.14±10.94 (20-90)	NS
Male sex	178 (80.2%)	178 (80.2%)	NS
Smoking Status			NS
Ever	107 (48.2%)	107 (48.2%)	
Never	115 (51.8%)	115 (51.8%)	
Alcohol status			NS
Drinker	63 (28.4%)	63 (28.4%)	
Non-drinker	159 (71.6%)	159 (71.6%)	
Viral Infection			
HBsAg positive	132 (59.4%)	-	
Anti-HCVAb positive	53 (23.9%)	-	
Both positive	3 (1.4%)	-	
Both negative	34 (15.3%)	-	
Liver cirrhosis			
Present	180 (81.1%)	-	
Absent	42 (18.9%)	-	
Child-Pugh classification			
A	39 (21.7%)	-	
B	62 (34.4%)	-	
C	79 (43.9%)	-	
α -Fetoprotein (ng/mL)			
<100	101 (45.5%)	-	
100-400	33 (14.9%)	-	
>400	88 (39.6%)	-	

NS, not significant; n, total number of case patients or control subjects; ^ap-values were derived from Pearson χ^2 test except age; student's t-test was used for age; all p-values are two-sided

from binary logistic regression analysis. The homozygous genotype for the AA allele of miR-499 rs3746444 was used as the reference in calculating ORs and 95% CIs. Statistical modeling was performed on the relative risk of the GG genotype or the AG genotype against the AA genotype independently. Furthermore, to estimate the recessive or dominant effect of miR-499 rs3746444 genotype on risk, statistical modeling was performed on the relative risk of the GG genotype against the AG+AA genotype (recessive model) or the GG+AG genotype against the AA genotype (dominant model). Probability levels less than 0.05 were used as a criterion of significance.

Results

General Characteristic of the Subjects

A total of 444 Turkish subjects were enrolled in our study. General characteristic of the subjects are summarized in Table 1. As expected, no significant difference was found between case patients and control subjects with regard to age and sex ($P=0.70$ and $P=1.00$, respectively) which implied that age and sex matched adequately. Similarly, there were no significant differences in smoking status and alcohol consumption between case and control group. In addition to these, Table 1 shows the distribution of a series of demographic variables such as AFP, marker of hepatitis, cirrhosis and Child-Pugh grade of cases.

Table 2. Alleles and Genotypes Frequency Distribution of MiR-499 rs3746444 Polymorphism Among Cases and Controls as Well as the Association with Hepatocellular Cancer Risk

	Cases (%), n = 222	Control (%), n = 222	P value ^a	OR (95% CI)
Allele frequency				
A	177 (39.9%)	187 (42.1%)		1.00 (Reference)
G	267 (60.1%)	257 (57.9%)	0.50	1.10 (0.84-1.43)
General genotype				
AA	45 (20.3 %)	47 (21.2%)		1.00 (Reference)
AG	87 (39.2%)	93 (41.9%)	0.93	0.97 (0.59-1.61)
GG	90 (40.5%)	82 (36.9%)	0.60	1.15 (0.69-1.90)
Dominant genotype				
AA	45 (20.3%)	47 (21.2%)		1.00 (Reference)
AG + GG	177 (79.7%)	175 (78.8%)	0.82	1.06 (0.67-1.67)
Recessive genotype				
AA + AG	132 (59.5%)	140 (63.1%)		1.00 (Reference)
GG	90 (40.5%)	82 (36.9%)	0.44	1.17 (0.80-1.71)

^aData were calculated by logistic regression analysis

Genotype Frequency Distribution miR-499 rs3746444 Polymorphism

The frequency distributions of the different genotypes for miR-499 rs3746444 polymorphism are shown in Table 2. The genotypic frequencies of the control (n = 222; $\chi^2 = 0.004$ df = 1, P=0.95) were in Hardy–Weinberg equilibrium, suggesting that there was no population stratification and no sampling bias. The patients' frequencies were also in Hardy–Weinberg equilibrium (n = 222; $\chi^2 = 0.000$ df = 1, P= 1.00). The allelic frequencies of case subjects (A, 0.40; G, 0.60) were not significantly different from those of the control subjects (A, 0.42; G, 0.58) (P=0.50). Thus, genotypic frequencies in the cases were similar to that of the controls ($\chi^2 = 0.62$, df = 2, P = 0.74).

miR-499 rs3746444 Polymorphism and Risk of Hepatocellular Carcinoma

To evaluate the risk of HCC according to the miR-499 rs3746444 genotype, logistic regression analysis was conducted (Table 2). Using the AA genotype as the reference genotype, GG genotype increased risk of HCC (OR = 1.15; 95% CI = 0.69-1.90, P= 0.60) but AG genotype exhibited with 0.97-fold (95% CI = 0.59-1.61, P=0.93) decreased HCC risk although neither association reached statistical significance. Study subjects combined with the miR-499 rs3746444 AG and GG genotypes had a non-significant increase HCC risk (OR = 1.06; 95% CI = 0.67-1.67, P=0.82), compared this with the miR-499 rs3746444 AA genotype. When we used AA and AG genotypes as a reference, we found that the OR of the GG genotype was 1.17 (95% CI = 0.80-1.71, P= 0.44).

Stratified Analyses

To observe whether the effect of genetic variation was modified by epidemiologic factors, HCC patients and controls were stratified on the basis of various host characteristics including sex, HCC etiology (viral infection status) and age. Subgroup analysis revealed that the effect of gender and viral infection status were not significantly different among miR-499 rs3746444 genotypes (data not given). Age at diagnosis with HCC (mean±standard deviation) was not significantly different

among miR-499 rs3746444 genotypes (AA: 59.02±11.54, AG: 60.85±11.18 and GG: 60.98±10.87, P= 0.60). In addition to this, we did not find any significant association between different genotypes of miR-499 rs3746444 polymorphism and demographical variables like liver cirrhosis, Child Pugh grade of cases and AFP levels (data not given).

Discussion

Identifying genetic biomarkers of HCC susceptibility and their application in conjunction with traditional diagnosis, staging and prognosis, it might be possible to reduce HCC mortality through early diagnosis, patients care and personalized therapy (Ludwig and Weinstein, 2005). miR-499 deserves growing attention as an ideal biomarker for carcinogenesis due to its participate in many biological processes such as cellular senescence, apoptosis, inflammation and immune response which are all crucial in the development and progression of cancer (Lafferty-Whyte et al., 2009; Hu et al., 2010; Wang et al., 2011). Multiple mechanisms of cellular senescence induction exist including telomere shortening, oxidative stress, oncogene expression and DNA damage signaling (Lafferty-Whyte et al., 2009). Lafferty-Whyte et al.(2009) have reported that miR-499 had the potential to regulate all 4 of the cellular senescence induction types. Furthermore, Wang et al. (2011) found that modulation of miR-499 levels affects apoptosis and also p53 transcriptionally downregulates miR-499 expression. Recent work demonstrates that level of miR-499 from the serum may serve as a noninvasive predictor for the overall survival of non–small-cell lung cancer (Hu et al., 2010). Recently, Lui et al. (2011) identified that miR-499 expression was frequently increased in colorectal cancer cell lines and lymph node-positive colorectal cancer specimens. Additionally, enhancing the expression of miR-499 promoted colorectal cancer cell migration and invasion in vitro and lung and liver metastasis in vivo, while silencing its expression resulted in reduced migration and invasion. In addition to this, to identify mRNA targets for miR-499, we searched miRBase database using the microrna.org, TargetScan and Micro-Cosm programs and found

more target genes that some participate in immune and inflammatory responses (the microRNA Database).

This molecular epidemiological study investigated whether the miR-499 rs3746444 polymorphism could have an impact on susceptibility to HCC. miR-499 rs3746444 polymorphism was selected as the candidate polymorphism because recent evidences indicated that miR-499 can play a role of mediator in a wide spectrum of biological processes, such as cellular senescence, apoptosis, immune response, tumorigenesis and metastasis (Lafferty-Whyte et al., 2009; Hu et al., 2010; Lui et al., 2011; Wang et al., 2011). Contrary to our expectation, in the present hospital-based case-control study in Turkish population, distribution of miR-499 rs3746444 genotype was not different between HCC cases and controls. No significant association emerged between risk of HCC and miR-499 rs3746444 polymorphism in overall statistical analyses. Our finding support previous data showing that there was an no association between miR-499 rs3746444 polymorphism and increased risk for hepatocellular carcinoma in the Chinese population (Zhou et al., 2011b).

However, the negative results were perhaps due to the small sample size, so replication of these findings in larger samples are needed. Also, our results in line with previous findings showing that there was an no association between miR-499 rs3746444 polymorphism and risk for various cancers including non-small cell lung cancer (Hu et al., 2008), breast cancer (Catucci et al., 2009), lung cancer (Tian et al., 2009), gastric cancer (Okubu et al., 2010), squamous cell carcinoma of head and neck (Liu et al., 2010), gallbladder cancer (Srivastava et al., 2010), bladder cancer (Mittal et al., 2011). Nonetheless, it should be noted that some molecular epidemiological studies have suggested that heterozygous AG genotype of miR-499 rs3746444 polymorphism is associated with increased risk of cervical squamous cell carcinoma (Zhou et al., 2011a) and prostatic cancer (George et al., 2011).

In contrast to Catucci et al. (2009), Hu et al. (2009) have reported that GG genotype of miR-499 rs3746444 polymorphism is associated with increased risk of breast cancer. It is possible that the significant difference in the results of studies may be due to differences in the studied population, as well as on several environmental and other factors that influence that population. Geographic or ethnic differences have been reported regarding the genotype frequency of several polymorphisms (NCBI dbSNP CEU HapMap Phase 3, 2011). For instance, A allele frequency of miR-499 rs3746444 polymorphism among the different ethnicities is as follows: 0.819 in Caucasians, 0.833 in Japanese, 0.860 in Chinese and 0.500 in Africans (NCBI dbSNP CEU HapMap Phase 3, 2011). Another rational explanation for this cancer-dependent difference in risk conferred by the miR-499 rs3746444 may be attributable to differences in the pathways of carcinogenesis among the various types of human cancers.

The limitations of our study are as follows. First limitation of the present study is that it was hospital-based case-control study, and patients were selected at a single institution (Çukurova University, Balcalı Hospital) and thus may have been unrepresentative of HCC patients in

the general population. In addition, it should be noted that the control subjects were recruited at the same hospital. However, in the control group, the agreement between the observed distribution of miR-499 rs3746444 genotype frequencies with the expected according to the Hardy-Weinberg equilibrium model suggested no selection bias. Second, this study is limited by the relatively small number of cases and controls. Therefore further studies with a larger number of subjects are needed to clarify this issue. Third, we also limited our study to Turkish population due to variation in allele frequency between different ethnic groups has been observed for miR-499 rs3746444. Fourth, this study only focused on single locus on single gene without taking into consideration gene-environment, gene-gene interactions and interactions between different locuses on the same gene, which may affect individual susceptibility to HCC. Because of advances in high-throughput genotyping techniques, it is likely that future association studies on hepatocellular carcinoma will need to investigate multiple polymorphisms within miRNA genes and will need to use recently developed haplotype-based methods to evaluate the haplotypic effects. Fifth, due to the lack of data on miR-499 expression according to rs3746444 genotypes in our HCC group, future work need to be done in order to explore the correlation between levels of miR-499 in normal liver and HCC tissues in the context of different genotypes of miR-499 rs3746444 polymorphism.

In conclusion, our results demonstrate for the first time that miR-499 rs3746444 polymorphism have not been any major role in genetic susceptibility to hepatocellular carcinogenesis in Turkish population. Further independent studies are required to validate our findings in a larger series, as well as in patients of different ethnic origins, it will contribute to the understanding molecular mechanisms of miR-499 rs3746444 polymorphism that underlie HCC tumorigenesis and susceptibility HCC.

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