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Short communication

# Association study of microRNA polymorphisms with hepatocellular carcinoma in Korean population

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ABSTRACT

*Background:* Recent studies have suggested that common genetic polymorphisms alter the processing of microRNA (miRNA) and may be associated with the development and progression of cancer.
*Patients and methods:* The association of miRNA polymorphisms with HCC survival was analyzed in 159 HCC patients and 201 controls by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.
*Results:* The risk of HCC was significantly lower for the *miR-499*A>G, AG + GG in HCC patients (AOR = 0.603, 95% CI = 0.370–0.984) and hepatitis B virus (HBV)-related HCC patients (AOR = 0.561, 95% CI 0.331–0.950). In addition, the risk of HCC was significantly lower for the *miR-149*C>T, CT and CT + CC in HCC patients (CT; AOR = 0.542, 95% CI = 0.332–0.886, CT + CC; AOR = 0.536, 95% CI = 0.335–0.858) and HBV-related HCC patients (CT: AOR = 0.510, 95% CI 0.305–0.854, CT + CC: AOR = 0.496, 95% CI 0.302–0.813). The *miR-149*C>T polymorphism was also associated with survival rate of HCC patients in OKUDA II stage.
*Conclusions:* *miR-149*C>T and *miR-499*A>G were associated with HBV-related HCC. Further studies on larger populations will need to be conducted to confirm these results.



## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the common malignant tumors worldwide (Llovet et al., 2003), and has the third mortality rate in South Korea (Korea National Statistical Office, 2008; Park et al., 2007). The causes of HCC are known to be hepatitis B and C, aflatoxin B1, alcohol, and non-alcoholic steatohepatitis (Park et al., 2007). However, the causes of about 25% of HCC are unknown. Therefore, there is an increasing necessity for understanding other factors involved in tumor growth and progression.

MicroRNAs (miRNAs) are short, non-coding RNAs of approximately 23 nucleotides that regulate target genes (Bartel, 2009). miRNAs have been implicated in several biochemical pathways in the eukaryotic cells of various organisms (Lim et al., 2005; Wilfred et al., 2007). As a post-transcriptional regulator, miRNAs bind to complementary sequences on target messenger RNAs (mRNAs), resulting in mRNA degradation or translational repression (Rodriguez et al., 2004). Sequences encoding the miRNA precursor are usually transcribed by RNA polymerase II to generate primary miRNAs (pri-miRNAs) (Lee et al., 2004). Pri-miRNAs are processed to premature miRNAs (pre-miRNAs) by the DGCR8-DROSHA complex. Pre-miRNAs are transported to the cytosolic matrix in a process involving the shuttle protein, Exportin-5, and cleaved into a mature miRNA form by DICER, the RNase III (Gregory et al., 2006).

A strong association between miRNA and cancer has been recently reported (Bartels and Tsongalis, 2009; Chen et al., 2008; Czech, 2006; Esquela-Kerscher and Slack, 2006; Huang et al., 2010; Slack and Weidhaas, 2008; Waldman and Terzic, 2007). miRNAs have been proposed to contribute to oncogenesis as either tumor suppressors or oncogenes (Chen, 2005; Zhang et al., 2007). A single nucleotide polymorphism (SNP) is a DNA sequence variation of a single nucleotide, A (adenine), T (thymine), C (cytosine), or G (guanine), on genomic DNA (Mishra and Bertino, 2009; Wang et al., 1998). An SNP in an miRNA sequence may alter miRNA expression and/or maturation and be associated with the development and progression of cancer (Chen et al., 2009; Guo et al., 2009; Schetter et al., 2008; Wu et al., 2011).

*miR-146a*G>C, *miR-149*C>T, *miR-196a2*C>T, and *miR-499*A>G polymorphisms have been reported to be associated with lung cancer

*Abbreviations:* miRNA, microRNA; pri-miRNA, primary miRNA; pre-miRNA, premature miRNA; HCC, hepatocellular carcinoma; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.

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(Hu et al., 2008), breast cancer (Hu et al., 2009), thyroid cancer (Jazdzewski et al., 2008, 2009), colon cancer (Landi et al., 2008; Lee et al., 2010), and gastric cancer (Peng et al., 2010). In particular, the *miR-196a2*C>T polymorphism is associated with the susceptibility to hepatitis B virus (HBV)-related HCC in male Chinese patients (Li et al., 2010; Qi et al., 2010) and in the Turkish population (Akkız et al., 2011a). The *miR-146a*G>C polymorphism has not been linked to the genetic susceptibility to hepatocellular carcinogenesis in the Turkish population (Akkız et al., 2011b). Also, association studies between *miR-499*A>G and HCC have been reported (Akkiz et al., 2011c; Xiang et al., 2012). However, little is known about whether SNPs in miRNAs are associated with HCC.

The aim of this study was to investigate whether polymorphisms of *miR-146a*G>C, *miR-149*C>T, *miR-196a2*C>T, and *miR-499*A>G are associated with the susceptibility to and survival of HCC.

## 2. Materials and methods

### 2.1. Study population

A total of 159 cases with HCC diagnosed at CHA Bundang Medical Center from June 1996 to August 2008 were enrolled. The control group consisted of 201 individuals randomly selected from health screening program participants to exclude those with a history of cancer and other medical diseases. The clinical stage of HCC was evaluated on the basis of the TNM classification and OKUDA stage system. The patients were classified by the Child–Turcotte–Pugh score as A, B, or C. The present study was approved by the Institutional Review Board of CHA Bundang Medical Center and written informed consent was obtained from all patients and control subjects of the study.

### 2.2. DNA extraction and genotyping

Genomic DNA was extracted from whole blood, purified using high-salt buffer methods, and diluted to 100 ng/μl with 1× tris-ethylenediaminetetraacetic acid buffer. *miR-146a*G>C, *miR-149*C>T, *miR-196a2*C>T, and *miR-499*A>G genotypes were analyzed using the polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. The four polymorphic regions were amplified using the following PCR conditions: 95 °C for 5 min, 35 cycles at 95 °C for 30 s, 59 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 5 min.

PCR primers were designed using PrimerQuest™ (Integrated DNA Technologies, Coralville, IA). PCR of the *miR-146a*G>C polymorphism was performed using the following primers to generate a 147-bp product: forward 5′-CAT GGG TTG TGT CAG TGT CAG AGC T-3′ and reverse 5′-TGC CTT CTG TCT CCA GTC TTC CAA-3′. For the *miR-149*C>T polymorphism, the following primers were used to generate a 263-bp product: forward 5′-CTG GCT CCG TGT CTT CAC TC-3′ and reverse 5′-TGA GGC CCG AAA CAC CCG TA-3′. For the *miR-196a2*C>T polymorphism, the following primers were used to generate a 149-bp product: forward 5′-CCC CTT CCC TTC TCC TCC AGA TA-3′ and reverse 5′-CGA AAA CCG ACT GAT GTA ACT CCG-3′. For the *miR-499*A>G polymorphism, the following primers were used to amplify a 146-bp fragment: forward 5′-CAA AGT CTT CAC TTC CCT GCC A-3′ and reverse 5′-GAT GTT TAA CTC CTC TCC ACG TGA TC-3′. Underlined bases in the primers above are mismatches with the complementary sequence.

The *miR-146a*G>C, *miR-149*C>T, *miR-196a2*C>T, and *miR-499*A>G polymorphisms were detected by digesting the PCR products with restriction endonucleases *Sac*I, *Alu*I, *Msp*I, and *Bcl*I, respectively. The endonucleases were purchased from New England Biolabs (Beverly, MA). The reaction products (12 μl) were run on a 3.0% agarose gel that was stained with ethidium bromide and directly visualized under ultraviolet illumination. In the case of *miR*-146G>C, an undigested 147-bp band was indicative of the G allele, while two bands of 122- and 25-bp represented the C allele. In the case of *miR*-149C>T, an undigested 263-bp band represented the T allele, while three bands of 163-, 69-, and 31-bp represented the C allele. In the case of *miR*-196a2C>T, an undigested 149-bp band represented the T allele, while two bands of 125- and 24-bp represented the C allele. In the case of *miR*-499A>G, an undigested 146-bp band represented the G allele, while two bands of 120- and 26-bp represented the A allele. For each of the *miR* polymorphisms, 30% of the PCR assays were randomly chosen for a second PCR assay followed by DNA sequencing to validate the RFLP findings. Sequencing was performed using an ABI3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). The concordance of the quality control samples was 100%.

### 2.3. Statistical analyses

To analyze baseline characteristics, we used Chi-square tests for the categorical data and Mann–Whitney tests for continuous data when comparing patient and control baseline data. Associations between miRNA polymorphisms and HCC risk were estimated using adjusted odds ratios (AORs) and 95% confidence intervals (95% CIs) from multivariate logistic regression, which was used to adjust the effect factor (i.e., age, gender, hypertension, diabetes mellitus, body mass index, smoking, and drinking).

Survival time was calculated from the date of HCC diagnosis to the date of death or last follow-up. Survival analysis was estimated using the Kaplan–Meier method, log-rank test, and Cox-proportional hazards regression model. MST was presented as median survival time. The significance level of all tests was set at $P<0.05$. The analyses were performed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA) and Medcalc version 11.1.1.0 (Medcalc Software, Mariakerke, Belgium).

## 3. Results

The characteristics of the HCC patients and control subjects are shown in Table 1. The HCC patients and control subjects were matched for age and sex ($P=0.069$ and 0.570, respectively). There were significant differences between the patients and control subjects for hypertension and smoking ($P=0.002$ and 0.001, respectively), but there were no significant differences for diabetes mellitus, body mass index, and drinking. Hepatitis B and C virus infections were identified in 127 (79.9%) and 16 (10.0%) patients, respectively. The genotype distribution in all of the groups did not deviate from Hardy–Weinberg equilibrium.

The *miR-146a*G>C, *miR-149*C>T, *miR-196a2*C>T, and *miR-499*A>G polymorphisms were detected, and their genotype distributions in HCC patients and control subjects are shown in Table 2. We found a significant decrease in cancer risk with the variant homozygote CT and CT + CC of the *miR-149*C>T polymorphism compared with wild-type homozygote HCC patients (CT type; AOR = 0.542, 95% CI = 0.332–0.886, $P=0.015$, CT + CC type; AOR = 0.536, 95% CI = 0.335–0.858, $P=0.009$) and HBV-related HCC patients (CT type: AOR = 0.510, 95% CI = 0.305–0.854, $P=0.010$, CT + CC type: AOR = 0.496, 95% CI = 0.302–0.813, $P=0.005$). In addition, the risk of HCC was significantly lower for the *miR-499*A>G, AG + GG genotype compared to the AA genotype in HCC patients (AOR = 0.603, 95% CI = 0.370–0.984, $P=0.043$) and HBV-related HCC patients (AOR = 0.561, 95% CI = 0.331–0.950, $P=0.032$). However, the *miR-146a*G>C and *196a2*C>T polymorphisms were not significantly different between the HCC patients and the control subjects.

Survival analysis for HCC patients at the OKUDA II stage showed statistically significant differences in the MST between the *miR-149*CC genotype (55 month), CT genotype (18 month), and TT genotype (14 month; log-rank $P=0.032$). Multivariate Cox proportional hazard regression analysis also showed a significant survival benefit for the *miR-149*CC genotype compared to the TT genotype (adjusted HR = 0.183, 95% CI = 0.043–0.778, $P=0.022$). In addition, the *miR-149*CT + CC genotype, compared to the TT genotype (log-rank $P=0.017$, adjusted HR = 0.497, 95% CI = 0.260–0.947, $P=0.034$), and the *miR-149*CC genotype compared to the TT + CT genotype (log-rank $P=0.041$) were statistically significant ($P<0.05$; Table 3). Stepwise Cox proportional hazard analysis