RESEARCH ARTICLE

Single Nucleotide Polymorphisms in miR-149 (rs2292832) and miR-101-1 (rs7536540) Are Not Associated with Hepatocellular Carcinoma in Thai Patients with Hepatitis B Virus Infection

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Abstract

MicroRNAs directly and indirectly influence many biological processes such as apoptosis, cell maintenance, and immune responses, impacting on tumor genesis and metastasis. They modulate gene expression at the post-transcriptional level and are associated with progression of liver disease. Hepatocellular carcinoma (HCC) is a cancer which mostly occurs in males. There are many factors affect HCC development, for example, hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV), co-infection, environmental factors including alcohol, aflatoxin consumption and host-related factors such as age, gender immune response, microRNA and single nucleotide polymorphisms (SNPs). Chronic infection with the hepatitis B virus is the major factor leading to HCC progression since it causes the liver injury. At present, there are many reports regarding the association of SNPs on miRNAs and the HCC progression. In this research, we investigated the role of miR-149 (rs2292832) and miR-101-1 (rs7536540) with HCC progression in Thai population. The study included 289 Thai subjects including 104 HCC patients, 90 patients with chronic hepatitis B virus infection (CHB) and 95 healthy control subjects. The allele and genotype of rs2292832 and rs7536540 polymorphisms were determined by TaqMan real-time PCR assay. Our results revealed no significant association between miR-149 (rs2292832) and miR-101-1 (rs7536540) and the risk of HCC in our Thai population. However, this research is the first study of miR-149 (rs2292832) and miR-101-1 (rs7536540) in HCC in Thai populations and the results need to be confirmed with a larger population.

Keywords: miR-149 - rs2292832 - miR-101-1 - rs7536540 - hepatocellular carcinoma - HBV - Thailand

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Introduction

Hepatitis B virus infection is the most common risk factor for cirrhosis and liver cancer. Liver cancer ranked the fifth most common cancer in the world (Jemal et al., 2011). Considerably, chronic hepatitis B virus can cause at least 60% of circumstance of liver cancer. Hepatocellular carcinoma (HCC) is the major type of primary liver cancer. The highest prevalence of liver cancer can be found in East, the middle zone of Eastern Asia and in sub-Saharan Africa (El-Serag et al., 2007). HCC is the most frequent subtype of the liver cancer which accounts for 70-85% (Perz et al., 2006).

Single nucleotide polymorphism (SNP) is one of many mechanisms accounted for genetic variation in human, described by a single-nucleotide substitution of one base into another in DNA sequences. The abnormal forms of SNPs were recorded that SNPs on coding region can result in the malformation of protein structures and affect its functions. Moreover, SNPs on regulatory regions of genome like promoter, operator and enhancer can affect gene expression while SNPs on non-protein-coding region, which encode for RNAs that regulate translation such as microRNA (miRNA) mechanism (Ramirez-Bello et al., 2013), can create alternative polyadenylation signals that lead to loss of microRNA regulation (Thomas et al., 2012).

MicroRNAs (miRNAs) are small non-coding RNA with approximately 17-22 nucleotides in length which have been shown the important role in post-transcriptional regulation of gene expression process. The mechanism of miRNAs processing are counted on the complementary interaction between miRNAs and mRNA of target genes on 3'UTR region that effect a translational repression or mRNA degradation of target genes. Normally, miRNAs are transcribed by RNA polymerase II in order to generate

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primary miRNAs (pri-miRNAs) before endonuclease enzyme named "Drosha" cleaves pri-miRNAs to generate a hair-pin structure which are approximately 60-80 nucleotides in size called precursor miRNAs (pre-miRNAs). After that, pre-miRNAs are transported to cytoplasm followed by it will be cleaved into mature miRNA duplexes (20-22 nucleotides) by enzyme called Dicer. The miRNAs are translocated to RNA-induced silencing complex (RISC) and then separated to single strand(s). The RISC complex moves to 3´ UTR of the target gene resulted in mRNA degradation or translational inhibition. The previous study suggested that miRNAs can be either oncogenes or tumor suppressor genes (Chen et al., 2005). Therefore, the polymorphisms on miRNA sequences might be associated with a progression and development of liver cancer.

At present, there are many previous studies have been reported about the association between liver cancers and SNPs on miRNAs such as miR-371-373 (rs3859501) (Kwak et al., 2012) and miR-149c (rs2292832) (Kim et al., 2012) involved in the decrease of cancer risk while miR-101-1 (rs7536540), miR101-2 (rs12375841), miR34b/c (rs4938723) (Bae et al., 2012), and miR-106b-25 (rs999885) (Liu et al., 2012) were found to be related to the increase in the risk of liver cancer. On the other hands, miR-499 (rs3746444) (Akkiz et al., 2011; Kim et al., 2012; Xiang et al., 2012) was found an ambiguous relationship with liver cancer among different nationality.

This study emphasized on the association between SNPs on miR-149 (rs2292832) and miR-101-1 (rs7536540) and liver cancer in Thai population. Allele frequencies and genotypes of miR-149 (rs2292 832) and miR-101-1 (rs7536540) were determined by the *Taq*Man real-time PCR.

Materials and Methods

Study population

Totally, 289 Thai subjects including 104 Hepatocellular carcinoma (HCC) patients, 90 cases with chronic hepatitis B virus (CHB) infection and 95 healthy controls. Both HCC and CHB patients were received from Chulalongkorn hospital, Bangkok, Thailand whereas healthy controls were collected from National Blood Centre Thai Red Cross Society, Bangkok. HCC patients were diagnosed with CHB positive, diagnostic ultrasound or/and biopsy and serology test. CHB patients were positive for HBsAg and anti-HBc test while the healthy control subjects were negative against HBV and HCV infection and had no record for liver diseases and cancers. All data of individual subjects were obtained from case records including sex, age, HBsAg, anti-HBc, aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TB) and Albumin levels. All subjects have perceived and provided their written information and consent form for this study. The protocol was approved by ethical committee at faculty of Medicine, Chulalongkorn University (IRB No. 361/56).

Detection of SNPs on miR-149 (rs2292832) and miR-101-1 (rs7536540)

One hundred microliters of individual peripheral blood mononuclear cells (PBMCs) were used for DNA extraction by phenol-chloroform: isoamyl alcohol as described previously (Sopipong et al., 2013). Concentration and purity of DNA were determined by using NanoDrop 2000c spectrophotometer (Thermo scientific, USA). The SNPs on miR-149 (rs2292832) and miR-101-1 (rs7536540) were identified using a real-time PCR protocol based on the pre-validated *Taq*Man MGB probe for allelic discrimination assay (Applied Biosystems). The reactions carried out on a StepOne Plus Real-Time PCR System (Applied Biosystems). The PCR reaction consisted of 5 μl of 2X ABI Master mix (Applied Biosystems), 0.25 μl of 40X primers and probe mix (Applied Biosystems), 1 µl of genomic DNA (50-500 ng/µl) and nuclease-free water in a total volume of 10 μl. The thermocycling conditions were conducted according to the manufacturer's instructions. Briefly, initial denaturation at 95°C for 10 min, then followed by 40 cycles of amplification including denaturation at 92°C for 10 sec, and annealing/extension at 60°C for 1 min. Fluorescent signals (FAM and VIC) were acquired at the end of each cycle. Positive controls for each allele and negative controls were included in each experiment in order to ensure correct interpretation. Allelic discrimination was analyzed using StepOneTM software (version 2.2, Applied Biosystems).

Statistical analysis

The equation was used to calculate the sample size (Kadam et al., 2010). According to the calculation, the sample size should be at least 53 samples in each group. The association of miR-149 (rs2292832) and miR-101-1 (rs7536540) and the progression of HCC using the odd ratio (OR) with 95% confidence intervals (CIs) was calculated by MedCalc statistical software (http://www.medcalc.org/calc/odds_ratio.php). P value < 0.05 was statistical significance.

Table 1. Characteristics of the Subjects in this Study

Characteristic	HCC cases N=104 (%)	CHB subjects N=90 (%)	P value	Healthy subjects N=95	P value
Age (years) mean	52.87 ± 7.07	52.08 ± 8.74	0.49	51.43± 3.45	0.08
Male	93 (89.42)	61 (61.78)	0.002	59 (62.11)	0.001
AST (IU/L)	87.89 ± 77.97	31.00 ± 19.43	<0.0001		
ALT (IU/L)	60.17 ± 55.21	35.38 ± 29.72	0.0002		
TB (mg%)	1.04 ± 0.58	0.62 ± 0.26	0.0033		
Alb (mg%)	3.74 ± 0.70	4.42 ± 0.29	0.0005		

Results

Genotype distributions and allele frequencies of miR-149 (rs2292832)

The genotypic distributions of rs2292832 among groups were comparable (Table 2). HCC group (N=104) contained 63.46% (N=66) of homozygous major genotype (TT), 10.58% (N=11) of homozygous minor genotype (CC) and 25.96% (N=27) of heterozygous genotype (CT). Similarly, in the CHB group (N=90) revealed 66.67% (N=60), 13.33% (N=12) and 20.00% (N=18) for homozygous major genotype (TT), homozygous

Table 2. Genotype Distributions and Allele Frequencies of miR-149 (rs2292832)

SNP rs2292832	HCC N=104	CHB N=90	Healthy N=95	CHB + Healthy N=185	HCC vs. CHB	HCC vs. Healthy	HCC vs. CHB + Healthy
					OR (95% CI) P values	OR (95% CI) P values	OR (95% CI) P values
Genotype							
TT	66 (63.46%)	60 (66.67%)	62 (65.26%)	122 (65.95%)	1	1	1
CC	11 (10.58%)	12 (13.33%)	9 (9.47%)	21 (11.35%)	1.64 (0.59-4.50) 0.340	0.92 (0.33-2.60) 0.876	1.23 (0.51-2.94) 0.647
CT	27 (25.96%)	18 (20.00%)	24 (25.26%)	42 (22.70%)	1.36 (0.68-2.72) 0.379	1.06 (0.55-2.02) 0.868	1.19 (0.67-2.10) 0.552
Allele							
Major T	159 (76.44%)	138 (76.67%)	148 (77.89%)	286 (77.30%)	1	1	1
Minor C	49 (23.56%)	42 (23.33%)	42 (22.11%)	84 (22.70%)	1.01 (0.63-1.62) 0.959	1.09 (0.68-1.74) 0.730	1.05 (0.70-1.57) 0.815
Dominant							
TT	66 (63.46%)	60 (66.67%)	62 (65.26%)	122 (65.95%)	1	1	1
CC+CT	38 (36.54%)	30 (33.33%)	33 (34.74%)	63 (34.05%)	1.15 (0.64-2.08) 0.641	1.08 (0.61-1.93) 0.791	1.12 (0.68-1.84) 0.671
Recessive							
TT+CT	93 (89.42%)	78 (86.67%)	86 (90.53%)	164 (88.65%)	1	1	1
CC	11 (10.58%)	12 (13.33%)	9 (9.47%)	21 (11.35%)	0.77 (0.45-2.86) 0.555	1.13 (0.45-2.86) 0.796	0.92 (0.43-2.00) 0.840

Table 3. Genotype Distributions and Allele Frequencies of miR-101-1 (rs7536540)

SNP rs7536540	HCC N=104	CHB N=90	Healthy N=95	CHB + Healthy (N=185)	HCC vs. CHB	HCC vs. Healthy	HCC vs. CHB + Healthy
					OR (95% CI) P values	OR (95% CI) P values	OR (95% CI) P values
Genotype							
GG	37 (35.58%)	25 (27.78%)	39 (41.05%)	64 (34.59%)	1	1	1
CC	16 (15.38%)	20 (22.22%)	13 (13.68%)	33 (17.84%)	1.42 (0.66-3.06) 0.375	0.96 (0.42-2.23) 0.931	1.20 (0.60-2.38) 0.612
CG	51 (49.04%)	45 (50.00%)	43 (45.26%)	88 (47.57%)	0.77 (0.40-1.46) 0.419	1.25 (0.68-2.29) 0.470	1.00 (0.59-1.71) 0.993
Allele							
Major G	125 (60.10%)	95 (52.78%)	121 (63.68%)	216 (58.38%)	1	1	1
Minor C	83 (39.90%)	85 (47.22%)	69 (36.32%)	154 (41.62%)	0.74 (0.50-1.11) 0.147	1.16 (0.78-1.75) 0.462	0.93 (0.66-1.32) 0.687
Dominant							
GG	37 (35.58%)	25 (27.78%)	39 (41.05%)	64 (34.59%)	1	1	1
CC+CG	67 (64.42%)	65 (72.22%)	56 (58.95%)	121 (65.41%)	0.70 (0.38-1.28) 0.246	1.26 (0.71-2.24) 0.427	0.96 (0.58-1.58) 0.867
Recessive							
GG+CG	88 (84.62%)	70 (77.78%)	82 (86.32%)	152 (82.16%)	1	1	1
CC	16 (15.38%)	20 (22.22%)	13 (13.68%)	33 (17.84%)	0.64 (0.31-1.32) 0.224	1.15 (0.52-2.53) 0.734	0.84 (0.44-1.61) 0.594

minor genotype (CC) and heterozygous genotype (CT), respectively. The healthy control group contained 65.26% (N=62) for homozygous major genotype (TT), 9.47% (N=9) for homozygous minor genotype (CC) and 25.26% (N=24) of heterozygous genotype (CT). The frequencies of the major (T) and minor (C) alleles were comparable among each group. In the HCC group, 76.44% and 23.56% had major (T) and minor (C) alleles, respectively. In the CHB group, 76.67% and 23.33% had major (T) and minor (C) alleles, respectively. The healthy group contained 77.89% of major (T) allele and 22.11% of minor (C) allele (Table 2).

Genotype distributions and allele frequencies of miR-101-1 (rs7536540)

The genotypic distributions of rs7536540 among groups were also comparable (Table 3). The (GG) homozygous major genotype was found in HCC, CHB and healthy control for 35.58%, 27.78% and 41.05%, respectively. Moreover, the (CC) homozygous minor genotype was detected in HCC (15.38%), CHB (22.22%) and healthy control (13.68%) whereas the (CG) heterozygous genotype was identified in HCC (49.04%), CHB (50.00%) and healthy control (45.26%). The allele frequencies were not significant different among each group. The major (G) alleles were found in HCC (60.10%), CHB (52.78%) and healthy control (63.68%) whereas the minor (C) allele was identified in HCC, CHB and healthy control for 36.90%, 47.22% and 36.32%, respectively (Table 3).

SNPs (rs2292832 and rs7536540) and their association with HBV-related HCC

The genotypes of miR-149 (rs2292832) and miR-101-1 (rs7536540) were not significantly associated (P value > 0.05) with the development of HCC when comparing with CHB group, healthy group and CHB + healthy group in Thai population. After adjusting for variables such as age and sex in the logistic regression analysis, minor allele of rs2292832 and rs7536540 were not statistically associated (P value > 0.05) with the development of HCC in Thai population. The overall odd ratio (95% CI) and P value were summarized in Table 2 and 3.

Discussion

Until now, the knowledge about SNPs in miRNAs has been studied worldwide in order to be necessary in medical treatment. There are many studies indicated associations between miRNA regulation to diseases progression including cancer (Li et al., 2010; Kim et al., 2012). The insight of this biological regulation will provide benefits of the prognosis and diagnostic in cancer development. In this research, the associations of SNPs on miR-149 and miR-101-1 genes with the risk to HCC were analyzed in HCC with CHB patients and CHB patients in Thai population. The statistical analysis showed that the number of the sample size were sufficient to calculate the reliable results. The characteristic of the subjects in each group found were matched with age (P values=0.49, >0.005). Considerably, the data suggested that gender was uncorrelated among each group of study (p-value=0.002). This might be the limitation of this study because the gender is considered as the confounding factor of HCC incident (Tangkijvanich et al., 1999; Parkin et al., 2003). SNPs on miR-149 is a tumor suppressor in human gastric cancer (Wang et al., 2012) and as a tumor suppressor, this SNPs may be involved in the proliferation and invasion of GM cells via blockade of the AKT1 signaling (Pan et al., 2012). There are studies revealed that the SNPs on miR-149 (rs2292832) with HCC. In Korean population, 159 of HCC and the 201 of healthy controls, revealed CC+TC (AOR=0.536, 95% CI=0.335-0.858, p-value=0.009) and TC (AOR=0.542, 95% CI=0.332-0.886, p-value=0.015) genotype were significantly tended to reduce the risk of HCC when compare with TT genotype (Kim et al., 2012). Furthermore, in 2014, study has confirmed this association between this SNP (rs2292832) and HCC. The results showed that T allele of miR-149 (rs2292832) associated with increasing risk of Hepatitis B virus associated HCC (Wang et al., 2014).

Furthermore, miR-101-1 could repress the expression of FOS oncogene in HCC (Li et al., 2009). The study in Korean population including 404 spontaneously recovered controls and 1035 carrier cases can be divided in to three groups which are 315 CHB, 305 cirrhosis and HCC 417, found that miR-101-1 (rs7536540) may be associated with liver cirrhosis occurring in a dominant model (OR=0.63, 95%CI=0.42-0.93, P=0.02) and may be related to HCC occurrence in a dominant (OR=0.63, 95%CI=0.46-0.85, P=0.003) and co-dominant model (OR=0.79, 95%CI=0.65-0.97, P=0.02) (Kwak et al., 2012).

In conclusion, this study was the first reported considering to study the association of rs2292832 and rs7536540 polymorphisms and the impressibility to HCC in Thai population. Results showed that there were no significant associations between rs2292832 and rs7536540 polymorphisms with the risk of HCC in Thai population. Therefore, rs2292832 and rs7536540 polymorphisms may not be able to use as a genetic markers in Thai population. However, this study had the limitation in unmatched gender among each group. Therefore, further case-control study with gender matched among each group in order to control confounding factor and confirm the association of rs2292832 and rs7536540 polymorphisms with the susceptibility HCC in Thai population.

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