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# Alpha-fetoprotein gene polymorphisms and risk of HCC and cirrhosis

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ABSTRACT

*Background:* Elevated level of alpha fetoprotein (AFP) is found in approximately 60% of hepatocellular carcinoma (HCC) cases. Other liver diseases including cirrhosis and chronic hepatitis are related with an increased level of AFP. The regulation of *AFP* gene expression has been relatively less studied although the gene has been suggested to play a role in HCC development. This study aimed at identifying genetic variations in *AFP* that might be associated with the presence of HCC and cirrhosis among ethnic Indonesians.
*Methods:* Direct DNA sequencing was carried out to sequence *AFP* promoter, exons, and 3′ untranslated region (UTR) in DNA samples isolated from 119 HCC, 119 cirrhosis and 105 control subjects. For each sample serum AFP level was determined and association studies with single nucleotide polymorphisms (SNPs) and haplotypes were performed.
*Results:* In this study we identified 47 SNPs in the *AFP* gene. Statistically significant associations with HCC and cirrhosis were detected for six individual SNPs in the *AFP* promoter, *AFP* intron 1 and intron 2 (rs6834059, rs3796678, rs3796677, rs3796676, rs28532518 and rs4646038). Furthermore, we identified two SNPs in *AFP* intron 7 and 3′UTR, rs2298839 and rs10020432, which are associated with increased risk of cirrhosis.
*Conclusion:* Genetic variants in the *AFP* gene may be associated with HCC and cirrhosis risk for ethnic Indonesians.


## 1. Introduction

Hepatocellular carcinoma (HCC) is one of the top 10 most frequent tumor types worldwide with short survival times and few treatment options. Although the disease may take 20–50 years to develop, early detection is not often achieved due to the lack of reliable markers [1]. HCC is a major health-care problem in Asia, where HBV infection is highly endemic. It has been estimated that worldwide 350 million individuals are suffering from chronic HBV infection and as many as 170 million persons are infected with HCV, and thus are at risk of developing cirrhosis and/or HCC [2–4]. Approximately 4.6% of the Indonesian population tested positive for HBV surface antigen (HBsAg), and the estimated HCV prevalence lies between 1 and 2.5% of the Indonesian population [5,6]. Wang et al. surveyed the demographic, clinical and virological characteristics of 414 HCC patients including 107 from China, 15 from India, 101 from Indonesia and 191 from Japan [5]. The most frequent cause for HCC is HBV infection in China, whereas HCV was more common in Japan. The patterns of Indonesia were in between those of China and Japan. The mean age ± SD for HCC patients is 53.7 ± 14.2 years, and male patients predominate with up to 75% of total HCC patients [5].

Alpha fetoprotein (AFP) is a well-recognized tumor marker for HCC; elevated serum AFP concentration is found in approximately 60% of HCC patients [7]. The cutoff concentration of AFP used for diagnosis determines the specificity and sensitivity of AFP as a diagnostic and/or prognostic marker [8]. Variations of serum concentration AFP are observed among HCC patients as well, thus contributing to the complexity in the diagnosis. Various reports have suggested the role of AFP in the cell as a superoxide dismutase [9] and as an apoptotic

*Abbreviations:* AFP, Alpha fetoprotein; HCC, Hepatocellular carcinoma; SNP, Single nucleotide polymorphism; HBV, Hepatitis B virus; HCV, Hepatitis C virus; UTR, Untranslated region; LD, Linkage disequilibrium; OR, Odds ratio; CI, Confidence interval; PCR, Polymerase chain reaction.

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factor [10–12]. AFP has also been reported to directly promote proliferation in cultured cells [10–16]. AFP may positively regulate cell proliferation by enhancing the apoptosis resistance via alteration of the p53/Bax/cytochrome c/caspase-3 signaling pathway in AFP-producing HCC cell line [17]. AFP forms complexes with caspase-8 in the cytoplasm of human hepatoma cell line Bel7402 and blocks onward transmission of signaling from caspase-8 [18]. Cytoplasmic AFP has also been shown to function in retinoic acid–retinoic acid receptor (RA-RAR) signaling to promote the growth of human hepatoma by inhibiting translocation of RAR-beta into the nucleus via competitive binding to RAR-beta with all-trans retinoic acid (ATRA) [19].

Most of the studies on the cellular function of AFP have been done *in vitro*, however the molecular background of HCC associated with increased AFP concentrations in HCC patients and the mechanisms underlying the association of *AFP* with the onset of HCC remain unclear. Using global mRNA expression analysis of 21 liver cancer cell lines that produce varying concentrations of AFP, Saito et al. identified 213 genes whose mRNA expression levels were significantly correlated with that of AFP ($P<0.0001$) [20]. In the study, a number of genes linked with HCC and other malignancies were found to be associated with *AFP* expression. Furthermore, *AFP* expression was reportedly correlated with the expression of several proteins in angiogenesis and in iron metabolism. Iron overload facilitates liver carcinogenesis by generating oxygen-reactive species and carcinogen oxidative damage [21].

The regulation of *AFP* gene expression is a complex process involving transcriptional activators and repressors that bind to the *AFP* promoter and enhancer; and the difference of serum AFP concentration in HCC patients and cell lines likely the result of the *AFP* enhancer and silencer activity [22,23]. The complex regulation of *AFP* expression is reflected in the high concentration of AFP in the fetus and the shutdown of AFP expression after birth. In HCC and certain cancers, serum AFP concentration is increased again in a mechanism that is not well understood.

Chen et al. [24] recruited 83 HCC patients and 28 controls of ethnic Chinese in Hong Kong. After re-sequencing 980 bp region of *AFP* promoter, they identified three novel SNPs associated with AFP concentration in serum and high risk of developing HCC. Those SNPs were located at −330, −401, and −692 positions; −401 and −692 SNPs have been implicated as putative binding sites of the known transcription factor. However, this interesting pathologically significant result was hindered by the small study population.

## 2. Materials and methods

### 2.1. Study participants

Subjects were recruited in the Division of Hepatology, Department of Internal Medicine, Cipto Mangunkusumo Hospital, Jakarta, "Klinik Hati" Jakarta, the Division of Gastroentero-Hepatology, Gatot Soebroto Hospital, Jakarta, and Division of Gastroentero-Hepatology, Department of Internal Medicine, Wahidin Sudirohusodo Hospital, Makassar from May 2006 until December 2008. Liver cirrhosis was diagnosed by liver function and ultrasonography. The diagnosis for HCC was on the basis of ultrasonography and an increased serum concentration of AFP (≥200 ng/ml). Fine needle aspiration biopsy procedure reconfirmed the diagnosis of HCC for samples in which the AFP concentration was low. Subjects with HIV co-infection or autoimmune hepatitis were excluded in the study. Control subjects were healthy individuals visiting the hospital for routine health checkup or treatment for non-cancer illness. Blood samples were collected from each subject at the time of the evaluation. The study was approved by the Institutional Ethic Committee and informed consent was obtained from each patient.

### 2.2. DNA isolation

DNA isolation from 200 µl whole blood of each sample was performed using PureLink™ Genomic DNA Mini Kit (Invitrogen Life Science, San Diego, CA) according to manufacturer's standard protocol. The genomic DNA concentration was measured using NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, USA) and adjusted to 10 ng/µl.

### 2.3. Primer design and polymerase chain reaction (PCR)

For identification of SNPs in the *AFP* gene, primers were used to amplify the 1500 bp region of the *AFP* promoter and 15 exons of the *AFP* gene including 500 bp regions upstream and downstream of each exon. The nucleotide positions for all primers were designed according to the published *AFP* sequence in the NCBI database (GenBank accession number: L34019, M16110). Primers, which were used to amplify the exons and exon flanking regions, the lengths of PCR product, and the SNPs identified within the amplified regions were listed in Table 1.

PCR was performed using AmpliTaq® Gold DNA Polymerase kit (Applied Biosystems, Foster City CA) or KOD Hot Start DNA polymerase (Novagen, EMD Bioscience, Darmstadt, Germany). Each reaction was carried out in 20 µl solution containing 1.5 mmol/l $Mg^{2+}$, 2 µmol/l dNTPs, 10 µmol of each primer, 10 ng genomic DNA as template and 0.625 U polymerase. All reactions had an initial denaturation step of 10 min at 95 °C, followed by 45 cycles at 95 °C for 30 s denaturation, annealing at the specific annealing Tm for 30 s, 60 s at 72 °C followed by a 10 min final extension step at 72 °C. Each PCR product was verified for correct amplification in 2% agarose DNA gel electrophoresis. Prior to sequencing, each PCR product was either purified using MultiScreen $PCR_{96}$ Filter Plate (Millipore, Billerica, MA), or subjected to incubation with 0.16 U of shrimp alkaline phosphatase (New England Biolabs, Ipswich, MA) and 0.16 U of exonuclease I (New England Biolabs, Ipswich, MA) at 37 °C for 45 min, followed by heat inactivation at 85 °C for 20 min.

### 2.4. DNA sequencing

PCR products were sequenced using an ABI Prism® BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's standard protocol. Each sequencing reaction contains 0.3 µl BigDye™ Terminator, 5 µl purified PCR product and 3.3 µmol/l sequencing primer. Sequencing reactions were performed using forward primer and repeated using reverse primer in most cases. All sequencing reactions were performed at least twice with DNA amplified from at least two independent polymerase chain (PCR) reactions. Sequencing cycling conditions consist of incubation at 94 °C for 30 s denaturation, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequencing products were purified using ethanol/EDTA precipitation method, the purified products were denatured at 95 °C for 5 min with 15 µl Hi-Di Formamide (Applied Biosystems, Foster City, CA) before subjected for sequencing on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) according to manufacturer's standard protocol.

### 2.5. SNP identification

Sequencing results were aligned and analyzed for SNPs using BioEdit 7.0.0 program (Applied Biosystems, Foster City, CA). All SNPs detected in this study were located within the high quality region of the chromatogram. Samples from patient and control groups were tested in the same experimental batches to minimize batch-to-batch variations in genotyping.

**Table 1**
List of primers used in this study.

| Region | Primer name | Sequence (5′ to 3′) | Size (bp) | SNP | Position relative to transcriptional start site | AA change |
|---|---|---|---|---|---|---|
| Promoter | AFP_prom_3F | cgtgagcaaggcctgttttgt | 889 | rs4446279 | −1270 | |
| | AFP_prom_4R | gcagtgggtcaggtgcatcatt | | rs12651581 | −1000 | |
| Promoter | O12-AFP_Prom2F | cgaatgatgcacctgacccact | 700 | rs4018 | −567 | |
| | AFP + NcoI-R | caccatggttgctagttattttgtt | | rs6815213 | −542 | |
| | | | | rs1178736 | −499 | |
| | | | | rs4024 | −496 | |
| | | | | rs6834059 | −205 | |
| Exon 1 | O38-Exon1aF | ggcattgcctgaaaagagta | 957 | rs3796678 | 191 | |
| | O38-Exon1aR | tgttttcaactgcaaccaaga | | rs3796677 | 271 | |
| | | | | rs3796676 | 335 | |
| | | | | rs28532518 | 661 | |
| Exon 2 | O16-Exon2-F | gctgggatatgaatggcaaacc | 730 | rs41265655 | 920 | |
| | O17-Exon2-R | gcaccctgttggtactgatgaga | | rs16849384 | 990 | |
| | | | | rs4640638 | 1320 | |
| Exon 3 | O18-Exon3-F | gctccttgcacatccaaacct | 762 | rs16849388 | 1790 | |
| | O19-Exon3-R | gctgccctctttagcaattcaga | | rs7655393 | 1995 | |
| | | | | rs6446932 | 2238 | |
| Exon 4 | O40-Exon4aF | cccagcgtgcattacctatt | 1367 | rs6857080 | 4791 | |
| | O41-Exon4aR | gtgtgcccttagccagttgt | | | | |
| Exon 5 | O1-Exon5a-F | cagtgtccagttccaagtcag | 496 | rs35765619 | 6209 | K187Q |
| | O2-Exon5b-R | cccatttatagcaccctctcttt | | rs35924362 | 6244 | A198 |
| Exon 6 | O22-Exon6-F | gctctcagtgtcaagccgtgat | 831 | rs1981436 | 7397 | |
| | O23-Exon6-R | gctgacactcagtgaaggctgact | | rs3822100 | 7592 | |
| Exon 7 | O24-Exon7a-F | gcctcctttcctggattctc | 843 | rs41265657 | 8888 | L258V |
| | O25-Exon7a-R | cctcccgtcctccaagtaac | | rs2298839 | 8964 | |
| Intron 7 | O42-Exon7bF | gtggcattggcttatttttgg | 880 | rs6446933 | 9778 | |
| | O43-Exon7bR | ttgcccctaaacaacacttg | | rs16849431 | 9917 | |
| | | | | rs4694166 | 9950 | |
| | | | | rs4694167 | 10056 | |
| | | | | rs11936954 | 10234 | |
| | | | | rs11941100 | 10460 | |
| | | | | rs28693791 | 10488 | |
| Exon 8 | O26-Exon8-F | gcttctttccttcctccttccc | 725 | rs7667494 | 11289 | |
| | O27-Exon8-R | gcaatggtggccttgatatgag | | rs28482344 | 11313 | S286 |
| | | | | rs17182362 | 11676 | |
| Exon 9 | O28-Exon9-F | gcctctccaccttgggtatc | 784 | rs10031441 | 12769 | |
| | O29-Exon9-R | gcaagccactagctgctgactg | | rs10518114 | 12979 | |
| Exon 10 | O30-Exon10-F | gcagtcagcagctagtggcttg | 755 | rs34255749 | 13147 | |
| | O31-Exon10-R | gcaacctcggagcttcattga | | rs35252463 | 13361 | |
| | | | | rs41265659 | 13807 | |
| Exon 11 | O3-Exon11a-F | gctcctggagtttgctttttcat | 478 | rs1894264 | 14497 | S445 |
| | O4-Exon11b-R | gcctaaggccatctctacattgg | | | | |
| Exon 12 | O5-Exon12a-F2 | cccaaacaaatgggtaaatcc | 456 | rs35920062 | 16297 | G496 |
| | O6-Exon12b-R2 | ggggttgtcattcttttcca | | rs4235117 | 16450 | T547 |
| Exon 13 | O7-Exon13a-F | cacaacctgcacaactccag | 485 | rs7790 | 17658 | A570 |
| | O8-Exon13b-R | ccctcaatctgcttctcaatg | | | | |
| Exon 14 | O32-Exon14-F | gcagtgctttatctgcaaacct | 740 | rs16849445 | 19004 | |
| | O33-Exon14-R | gcacaccgaatgaagactcgt | | | | |
| Exon 15, 3′UTR | O34-Exon15-F | gcaaaaactcgtgctgctttgg | 1000 | rs3198039 | 19589 | |
| | O35-Exon15-R | gcaaaatgccctggtagcatc | | rs57618101 | 19677 | |
| | | | | rs6826233 | 19690 | |
| | | | | rs10020432 | 19719 | |

### 2.6. Serum AFP determination

The quantitative measurement of serum AFP concentration was performed using enzyme immunoassay method (Diagnostic System Laboratories, Webster, TX).

### 2.7. Statistical analysis

Results were expressed as percentage for categorical variables and as mean ± SD for the continuous variables. A $P < 0.05$ was considered to be statistically significant. Logistic regression was used to estimate the odds ratios (ORs) and 95% confidence intervals (CIs) with adjustment for age and gender. Each polymorphism was tested for Hardy Weinberg equilibrium in the case and control population. Statistical analysis was carried out using SPSS 12.0 (SPSS Inc., Chicago, IL). Tests for linkage disequilibrium (LD) and haplotype analysis were performed using Haploview [25].

## 3. Results

### 3.1. Demographic statistics of subjects

A total of 343 subjects comprising of 119 cirrhosis, 119 HCC patients and 105 controls were included in the study. Table 2 lists the demographic data of the subjects. The mean age for HCC, cirrhosis and control group was 54.4 ± 13.1, 54.2 ± 12.2 and 43.3 ± 11.5 years respectively. There was a slight male predominance in this study with a ratio of 3.25:1 for HCC and 1.3:1 for cirrhosis. HBsAg positivity was observed in the majority of HCC and cirrhosis subjects (61.34% and 45.38%), whereas HCV Antibody was present in 25.21% of HCC and 38.66% cirrhosis patients respectively. Among the study population, HBsAg and HCV Antibody could not be detected in 16 HCC and 15 cirrhosis subjects. Increased concentration of serum AFP concentration (≥200 ng/ml) was observed in 70 HCC subjects (58.82%), serum AFP concentration 200 ng/ml or below was detected in 49 HCC subjects

**Table 2**
Demographic statistics of study subjects

| Variable | HCC *n* (%) | Cirrhosis *n* (%) | Control *n* (%) | *P*-value | | | *P*-value (all) |
|---|---|---|---|---|---|---|---|
| | | | | HCC vs. control | Cirrhosis vs. control | HCC vs. cirrhosis | |
| Gender | | | | 4.28E$^{-08}$[a,*] | 0.0231[a,*] | 4.28E$^{-07}$[a,*] | 0.0021[a,*] |
| Male | 91 (76.47) | 67 (56.30) | 62 (59.05) | | | | |
| Female | 28 (23.53) | 52 (43.70) | 43 (40.95) | | | | |
| Age (y) | | | | 1.11E$^{-15}$[b,*] | 2.34E$^{-15}$[b,*] | 0.8995[b] | 2.65E$^{-18}$[b,*] |
| Mean ± SD | 54.4 ± 13.1 | 54.2 ± 12.2 | 43.3 ± 11.5 | | | | |
| Virological assay | | | | | | | |
| HBV DNA positive | 73 (61.34) | 54 (45.38) | – | | | 0.092[a] | |
| HCV DNA positive | 30 (25.21) | 46 (38.66) | – | | | 0.066[a] | |
| HBV+ HCV DNA positive | 0 (0) | 4 (3.36) | – | | | | |
| HBV+ HCV tests negative | 16 (13.45) | 15 (12.60) | – | | | | |
| AFP | | | | | | 1.11E$^{-09}$[a,*] | |
| AFP>200 ng | 70 | 2 | – | | | | |
| AFP<200 ng | 49 | 117 | – | | | | |

[a] $X^2$ analysis.
[b] Anova.
* Significant, $P<0.05$.

(41.18%); this concentration was considered nonspecific for HCC diagnosis [26]. Gender and age at diagnosis were associated with the presence of liver disease when all groups were considered in the evaluation as indicated by the statistically significant *P*-value of 0.0021 and 2.65E$^{-18}$ respectively. Among cirrhosis subjects, gender and serum AFP concentration were variables that showed a significant association with the presence of HCC (*P*-value = 4.28E$^{-07}$ for gender, *P*-value = 1.11E$^{-09}$ for serum AFP concentration).

### 3.2. SNPs identified in AFP gene

Table 1 contains the identified SNPs in the *AFP* genomic region analyzed in the study. In total 47 SNPs were detected in the partial *AFP* locus that covers the promoter, the exons, the exon–intron junctions and the 3′-UTR. The position of the SNPs relative to transcription start site is indicated in Table 1. Six from eight SNPs in the coding region of *AFP* were found to be homozygous, thus these polymorphisms did not result in an amino acid change. Additionally two SNPs in *AFP* Exon 7, rs 41265657, and in Exon 13, rs7790, were detected in HCC and control subjects with MAF of 0.01 and 0.02 respectively. In the *AFP* genomic region analyzed 31 SNPs were found to be homozygous (MAF<0.01). The genotype distribution and allele frequencies in our study population did not differ significantly compared to data from other Asian populations in reference/public databases. Genotype distribution and allele frequencies of polymorphisms in AFP with MAF ≥ 0.25 are listed in Tables 3 and 4. The allele frequencies were in Hardy Weinberg equilibrium, except the polymorphisms in intron 1, which deviated slightly from the Hardy Weinberg Equilibrium for the control population ($P \leq 0.05$).

### 3.3. Analysis of association between AFP genotypes and HCC or cirrhosis

In the AFP promoter region, rs6834059, showed significantly different genotype frequencies in HCC and cirrhosis subjects compared to the control group for GG genotype with crude *P*-values of 0.0047 and 0.0105 respectively. The gender and age stratification for rs6834059 resulted in adjusted *P*-values of 0.0025 and 0.0026 (Tables 3 and 4). Furthermore the GG genotype in rs6834059 was associated with reduced HCC and cirrhosis risk at adjusted OR = 0.167 (95%CI: 0.052–0.534) and 0.1811 (95%CI: 0.0595–0.552) respectively. The serum AFP concentration was lower in HCC subjects with the GG genotype (mean serum AFP concentration 2519 ng/ml) than subjects with CC or CG genotype (mean serum AFP concentration 31,911 or 19,256 ng/ml respectively) with a *P*-value of 0.028 and 0.082 (Fig. 1A). Similar observation was found in cirrhosis subjects, GG genotype was associated with lower serum AFP concentration compared to CC or CG genotype with a significant *P*-value of 0.011 or 0.017 respectively (Fig. 1B). CC genotype in rs6834059 was associated with increased risk for cirrhosis at adjusted *P* = 0.0072 and OR = 2.41 (95%CI: 1.07–3.21). The mean serum AFP concentration in subjects with CC genotype was higher than GG or CG genotypes (*P*-value 0.017 and 0.829, Dunnetts T3 test).

Subjects carrying the AA genotype in rs3796678 and the TT genotype in rs3796677, rs3796676 and rs28532518 showed a significant association with increased risk for HCC. The AA genotype in rs3796678 was associated with the presence of HCC at adjusted *P* = 0.0005 and OR = 6.240 (95%CI: 2.23–17.4). The TT genotype in rs3796677, rs3796676 and rs28532518 was associated with increased risk for HCC at adjusted *P* = 0.0001, 0.0005 and 0.0002 respectively and OR (95%CI) = 8.18 (2.80–23.9), 5.32 (2.07–13.6) and 6.26 (2.38–16.5) respectively (Table 3). Similarly among cirrhosis subjects, the AA genotype in rs3796678 and the TT genotype in rs3796677, rs3796676 and rs28532518 were correlated with a significantly increased risk of cirrhosis. The AA genotype in rs3796678 was associated with the presence of cirrhosis at adjusted *P* = 0.0015 and OR = 5.57 (95%CI: 1.93–16.0). The TT genotype in rs3796677, rs3796676 and rs28532518 was associated with increased risk for cirrhosis at adjusted *P* = 0.0027, 0.0023 and 0.0016 respectively and OR (95%CI) = 5.36 (1.79–16.0), 4.62 (1.72–12.4) and 5.37 (1.89–15.2) respectively (Table 4). HaploView analysis with pair-wise $r^2$ illustrating the Linkage Disequilibrium (LD) between SNPs with MAF ≥ 0.05 indicated that these 4 neighboring SNPs in intron 1 were correlated with pair-wise LD $r^2$-values of 0.874 and $D'$ of 0.937 (Supplementary data). This suggested that these SNPs are inherited together and one of the SNPs can be used as tag SNP in further analysis.

Polymorphism in intron 2, rs4640638, resulted in GG, GA and AA genotypes. GA genotype in this SNP was significantly associated with the presence of HCC (adjusted *P* = 0.0122, OR = 2.26 (95%CI: 1.19–4.27) albeit not with the presence of cirrhosis (Table 4). However, when HCC and cirrhosis subjects were compared for this particular polymorphism, G/A polymorphism at rs4640638 in individuals with liver cirrhosis was associated with increased risk for HCC (adjusted *P* = 0.0107, OR (95%CI) = 2.02 (1.18–3.48), Table 5). In addition, the GG genotype in rs4640638 showed a statistically significant association with the presence of cirrhosis alone (adjusted *P* = 0.0388, OR (95%CI) = 1.93 (1.03–3.60), Table 4).

The G/A polymorphism in rs2298839 at intron 7 gave rise to GG, GA and AA genotypes. The GG genotype in this SNP was associated with the presence of cirrhosis (adjusted *P* = 0.0184, OR (95%CI) = 2.23 (1.14–4.33)), however this particular genotype was not significantly associated

**Table 3**
Genotype frequencies of SNPs in partial AFP locus in HCC and control subjects.

| SNP | Genotype | Control | | HCC | | Allele | MAF | | HCC vs. controls | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | | Control | HCC | Adjusted *P*-value[a] | Adjusted OR[a] | 95%CI |
| rs6834059 | CC | 40 | 38.10 | 52 | 43.70 | C | 0.6143 | 0.6975 | NS | 1.570 | 0.835–2.95 |
| | CG | 49 | 46.67 | 62 | 52.10 | G | 0.3857 | 0.3025 | NS | 1.19 | 0.648–2.18 |
| | GG | 16 | 15.24 | 5 | 4.20 | | | | 0.0025 | 0.167 | 0.052–0.534 |
| rs3796678 | AA | 8 | 7.62 | 26 | 21.85 | A | 0.5147 | 0.5336 | 0.0005 | 6.24 | 2.23–17.4 |
| | AT | 89 | 84.76 | 75 | 63.03 | T | 0.4853 | 0.4664 | 1.40E-05 | 0.144 | 0.0603–0.346 |
| | TT | 5 | 4.76 | 18 | 15.13 | | | | 0.0281 | 3.81 | 1.15–12.6 |
| rs3796677 | TT | 7 | 6.67 | 28 | 23.53 | T | 0.5049 | 0.5252 | 0.0001 | 8.18 | 2.80–23.9 |
| | TA | 89 | 84.76 | 69 | 57.98 | A | 0.4951 | 0.4748 | 1.24E-06 | 0.110 | 0.0451–0.268 |
| | AA | 6 | 5.71 | 22 | 18.49 | | | | 0.0111 | 4.24 | 1.39–12.9 |
| rs3796676 | TT | 10 | 9.52 | 29 | 24.37 | T | 0.5294 | 0.5420 | 0.0005 | 5.32 | 2.07–13.6 |
| | TA | 88 | 83.81 | 71 | 59.66 | A | 0.4706 | 0.4580 | 2.92E-06 | 0.128 | 0.0540–0.303 |
| | AA | 4 | 3.81 | 19 | 15.97 | | | | 0.0075 | 5.75 | 1.60–20.720 |
| rs28532518 | TT | 9 | 8.57 | 31 | 26.05 | T | 0.4951 | 0.5462 | 0.0002 | 6.26 | 2.38–16.5 |
| | TC | 83 | 79.05 | 68 | 57.14 | C | 0.5049 | 0.4538 | 8.05E-05 | 0.218 | 0.102–0.465 |
| | CC | 10 | 9.52 | 20 | 16.81 | | | | NS | 1.75 | 0.681–4.500 |
| rs 4640638 | GG | 51 | 48.57 | 52 | 43.70 | G | 0.6524 | 0.6765 | NS | 0.830 | 0.470–1.38 |
| | GA | 35 | 33.33 | 57 | 47.90 | A | 0.3476 | 0.3235 | 0.0122 | 2.26 | 1.19–4.26 |
| | AA | 19 | 18.10 | 10 | 8.40 | | | | 0.0160 | 0.259 | 0.0988–0.679 |
| rs2298839 | GG | 28 | 26.67 | 46 | 38.66 | G | 0.4905 | 0.5798 | NS | 1.82 | 0.942–3.53 |
| | GA | 47 | 44.76 | 46 | 38.66 | A | 0.5095 | 0.4202 | NS | 0.865 | 0.466–1.60 |
| | AA | 30 | 28.57 | 27 | 22.69 | | | | NS | 0.613 | 0.306–1.22 |
| rs10020432 | GG | 34 | 32.38 | 49 | 41.18 | G | 0.5865 | 0.6303 | NS | 1.54 | 0.810–2.95 |
| | GA | 54 | 51.43 | 52 | 43.70 | A | 0.4135 | 0.3697 | NS | 0.746 | 0.405–1.37 |
| | AA | 16 | 15.24 | 18 | 15.13 | | | | NS | 0.825 | 0.355–1.92 |

[a] Adjusted for age and gender.

with the presence of HCC (adjusted $P=0.0745$, OR (95%CI) = 1.82 (0.942–3.53)). Upon further analysis we detected that the risk for developing cirrhosis indicated by the GG genotype in rs2298839 was more significant in male compared to female as indicated by a *P*-value of 0.0166 (adjusted) and an OR (95%CI) of 2.44 (1.081–5.49) (Table 6).

In the 3′ untranslated region (UTR) of *AFP*, the G/A polymorphism in rs10020432 was associated with the presence of cirrhosis albeit not with HCC (adjusted $P=0.0312$, OR (95%CI) = 2.02 (1.7–3.84) for cirrhosis and adjusted $P=0.187$, OR (95%CI) = 1.54 (0.810–2.85) for HCC, Tables 3 and 4). Interestingly the susceptibility to cirrhosis for GG genotype in rs10020432 was more significant in female as opposed to male as shown by a *P*-value of 0.0103 (adjusted) and OR (95%CI) = 5.32 (1.48–19.1) (Table 6).

### 3.4. Haplotype analysis of SNPs in AFP gene

We further evaluated rs6834059, rs3796678, rs4640638, rs2298839 and rs10020432 for haplotype distribution in our study population. At

**Table 4**
Genotype frequencies of SNPs in partial AFP locus in cirrhosis and control subjects.

| SNP | Genotype | Control | | Cirrhosis | | Allele | MAF | | Cirrhosis vs. controls | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | | Control | Cirrhosis | Adjusted *P*-value[a] | Adjusted OR[a] | 95%CI |
| rs6834059 | CC | 40 | 38.10 | 61 | 51.26 | C | 0.6143 | 0.7311 | 0.0072 | 2.41 | 1.07–3.21 |
| | CG | 49 | 46.67 | 52 | 43.70 | G | 0.3857 | 0.2689 | NS | 0.797 | 0.432–1.47 |
| | GG | 16 | 15.24 | 6 | 5.04 | | | | 0.0026 | 0.1811 | 0.0595–0.552 |
| rs3796678 | AA | 8 | 7.62 | 27 | 22.69 | A | 0.5147 | 0.5588 | 0.0015 | 5.57 | 1.93–16.0 |
| | AT | 89 | 84.76 | 79 | 66.39 | T | 0.4853 | 0.4412 | 0.0003 | 0.196 | 0.0817–0.472 |
| | TT | 5 | 4.76 | 13 | 10.92 | | | | NS | 2.74 | 0.757–9.91 |
| rs3796677 | TT | 7 | 6.67 | 25 | 21.01 | T | 0.5049 | 0.5378 | 0.0027 | 5.36 | 1.79–16.0 |
| | TA | 89 | 84.76 | 78 | 65.55 | A | 0.4951 | 0.4622 | 0.0002 | 0.187 | 0.0783–0.449 |
| | AA | 6 | 5.71 | 16 | 13.45 | | | | 0.0467 | 3.315 | 1.017–10.8 |
| rs3796676 | TT | 10 | 9.52 | 27 | 22.69 | T | 0.5294 | 0.5672 | 0.0023 | 4.62 | 1.72–12.4 |
| | TA | 88 | 83.81 | 81 | 68.07 | A | 0.4706 | 0.4328 | 0.0003 | 0.197 | 0.0817–0.476 |
| | AA | 4 | 3.81 | 11 | 9.24 | | | | 0.0903 | 3.49 | 0.822–14.9 |
| rs28532518 | TT | 9 | 8.57 | 25 | 21.01 | T | 0.4951 | 0.5504 | 0.0016 | 5.37 | 1.89–15.2 |
| | TC | 83 | 79.05 | 81 | 68.07 | C | 0.5049 | 0.4496 | 0.0070 | 0.340 | 0.155–0.745 |
| | CC | 10 | 9.52 | 13 | 10.92 | | | | NS | 1.08 | 0.388–3.03 |
| rs 4640638 | GG | 51 | 48.57 | 69 | 57.98 | G | 0.6524 | 0.7395 | 0.0388 | 1.93 | 1.03–3.60 |
| | GA | 35 | 33.33 | 38 | 31.93 | A | 0.3476 | 0.2605 | NS | 0.961 | 0.507–1.82 |
| | AA | 19 | 18.10 | 12 | 10.08 | | | | 0.0052 | 0.260 | 0.101–0.669 |
| rs2298839 | GG | 28 | 26.67 | 51 | 42.86 | G | 0.4905 | 0.5756 | 0.0184 | 2.23 | 1.14–4.33 |
| | GA | 47 | 44.76 | 35 | 29.41 | A | 0.5095 | 0.4244 | 0.1568 | 0.633 | 0.3368–1.1917 |
| | AA | 30 | 28.57 | 33 | 27.73 | | | | 0.3295 | 0.714 | 0.3635–1.4042 |
| rs10020432 | GG | 34 | 32.38 | 57 | 47.90 | G | 0.5865 | 0.6681 | 0.0312 | 2.02 | 1.07–3.84 |
| | GA | 54 | 51.43 | 45 | 37.82 | A | 0.4135 | 0.3319 | NS | 0.633 | 0.341–1.17 |
| | AA | 16 | 15.24 | 17 | 14.29 | | | | NS | 0.678 | 0.295–1.56 |

[a] Adjusted for age and gender.

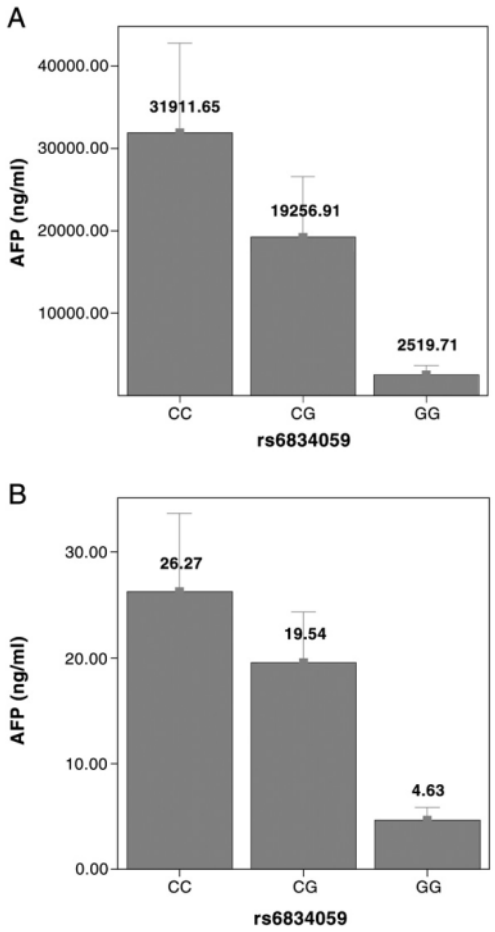


**Fig. 1.** A. Serum AFP concentrations in HCC subjects with different genotypes in rs6834059. *P*-value for genotype GG vs. CC or CG was 0.028 or 0.082 respectively (Dunnett's T3 test). Numbers on bars indicate mean AFP value. Standard error bars are shown. B. In cirrhosis subjects, *P*-value for genotype GG vs. CC or CG was 0.011 or 0.017 respectively (Dunnett's T3 test). Numbers on bars indicate mean AFP value. Standard error bars are shown.

least 16 haplotypes were derived from these 5 SNPs in HCC, cirrhosis and control groups. The two most common haplotypes were haplotype CAGGG and GTAAA. Estimated CAGGG haplotype frequencies in case and control were 40.1% for HCC, 38.2% for cirrhosis and 30.5% for control group, whereas haplotype GTAAA was detected [illegible]9%, 12% and 17% of HCC, cirrhosis and control subjects respectively. None of the identified haplotypes showed statistically significant association with HCC or cirrhosis development (Supplementary data).

**Table 5**
Analysis of association between rs4640638 in cirrhosis subjects and HCC.

| SNP | Genotype | HCC | | Cirrhosis | | HCC vs. cirrhosis | | |
|---|---|---|---|---|---|---|---|---|
| | | *n* | % | *n* | % | Adjusted *P*-value[a] | Adjusted OR[a] | 95% CI |
| rs4640638 | GG | 52 | 43.70 | 69 | 57.98 | 0.0255 | 0.547 | 0.323–0.929 |
| | GA | 57 | 47.90 | 38 | 31.93 | 0.0107 | 2.02 | 1.18–3.48 |
| | AA | 10 | 8.40 | 12 | 10.08 | NS | 0.792 | 0.311–2.01 |

[a] Adjusted for age and gender.

**Table 6**
Analysis of association between rs2298839 or rs10020432 and cirrhosis by gender.

| SNP | Gender | Genotype | Control | Cirrhosis | Cirrhosis vs. controls | | |
|---|---|---|---|---|---|---|---|
| | | | *n* | *n* | Adjusted *P*-value[a] | Adjusted OR[a] | 95% CI |
| rs2298839 | Male | GG | 17 | 32 | [illegible]18 | 2.435 | 1.08–5.49 |
| | | GA | 30 | 20 | NS | 0.633 | 0.286–1.402 |
| | | AA | 15 | 15 | NS | 0.574 | 0.232–1.423 |
| | Female | GG | 11 | 19 | NS | 1.944 | 0.600–6.33 |
| | | GA | 17 | 15 | NS | 0.531 | 0.181–1.56 |
| | | AA | 15 | 18 | NS | 1.068 | 0.365–3.12 |
| rs10020432 | Male | GG | 24 | 31 | NS | 1.324 | 0.611–2.870 |
| | | GA | 32 | 28 | NS | 0.776 | 0.360–1.67 |
| | | AA | 6 | 8 | NS | 0.945 | 0.294–3.04 |
| | Female | GG | 10 | 26 | 0.0103 | 5.316 | 1.48–19.0 |
| | | GA | 22 | 17 | NS | 0.413 | 0.142–1.20 |
| | | AA | 10 | 9 | NS | 0.489 | 0.137–1.75 |

[a] Adjusted for age.

## 4. Discussion

In this study we evaluated the significant association between SNPs in the promoter region of the AFP gene and partial AFP locus with the risk of developing HCC and cirrhosis after adjustment for age and gender. Numerous studies suggest a role for AFP in hepatocarcinogenesis [9–20]. However little is known about the role that sequence variation within the AFP gene plays in HCC or cirrhosis risk. This is the first genetic study that explores the impact that DNA sequence variation within the AFP promoter and partial AFP locus has in liver disease risk in the Indonesian population. Using SNPs and haplotypes for association study on liver disease risk we identified 6 individual SNPs, which were correlated with the presence of HCC and cirrhosis. Among these SNPs we observed strong linkage disequilibrium between four SNPs located in close proximity in intron 1. In addition genetic variations in intron 7 and 3′UTR of AFP gene, rs2298839 and rs10020432, were significantly associated with increased risk of cirrhosis albeit not with the presence of HCC in our study population.

Previously Chen et al. reported the association of 3 novel SNPs located at positions −330, −401 and −692 in relation to the transcriptional start site within the AFP promoter region with increased concentrations of serum AFP among a Chinese study population including 83 HCC patients and 28 controls recruited in Hong Kong. The CG genotype of 692 SNP was associated with the high risk of HCC development [24]. Interestingly despite a careful examination the polymorphisms at the positions reported in the publication were not detected in our study population; which suggests that the association of the reported SNPs with serum AFP concentration or the risk of HCC might be specific for Chinese population in Hong Kong, but not for ethnic Indonesians. Several disease-associated SNPs do show evidence of positive local selection. Regardless of whether the observed differences are due to drift or selection, worldwide variation in risk allele frequencies might be quite significant. Substantial variation in risk allele frequencies between populations has been reported, and this may account for differences in disease prevalence between human populations [27]. Additional studies in other populations might therefore be necessary for further clarification.

Among the 5 identified SNPs, which were located within a 1.5 kb region upstream of the *AFP* gene transcriptional start site [Table 3], C>G nucleotide change in rs6834059 at position −205 was associated with the reduced risk of HCC or cirrhosis and lower mean serum AFP concentration. DNA sequences upstream of the *AFP* translation initiation site possess multiple putative transcription factor binding sites. The polymorphism was located in the immediate vicinity of a 600 bp repressor region upstream of *AFP* promoter (position −863 to

−250) [28]. Sequence prediction analysis using PATCH™ public 1.0 indicated that C/G polymorphism at position −205 might potentially lead to the creation of novel transcription binding site for p53 [29,30]. Interestingly, p53 has been reported to facilitate AFP repression by competing with Hepatocyte Nuclear Factor 3 (HNF3) to bind DNA in the *AFP* repressor region. p53 recognizes a binding site in the repressor region, which results in chromatin remodeling and *AFP* suppression [31–35]. It is therefore tempting to suggest that a nucleotide change at position −205 might promote p53 binding, which might result in a low serum AFP concentration in GG genotype and hence be negatively associated with liver disease risk. However, further detailed investigation is clearly required to explore the functional and expression consequences of those SNPs located within the *AFP* promoter.

Genetic variations in the intron 1 of the *AFP* gene were found to be significantly associated with HCC and cirrhosis. rs3796678, rs3796677, rs3796676 and rs2853218 were located in close proximity within intron 1, and were correlated with LD $r^2$-values of 0.874 and $D'$ of 0.937 (Supplementary data). Since these SNPs showed a similar genotype distribution (Tables 3 and 4), it is tempting to speculate that the four SNPs were not independent of each other and likely carry similar information about risk. Besides transcription factor binding, polymorphisms located in non-coding regions may have consequences for gene splicing or mRNA stabilization. rs3796678 is located near the exon intron junction; sequence analysis using splice site predictor databases such as NNSplice, SpliceView, GeneSplicer and NetGene2 predicts that a T to A nucleotide change at this position could create a potential splice site; which might suggest the functional significance of this genetic variation [36–40]. Additionally a polymorphism in *AFP* intron 2 was found to be significantly associated with the presence of HCC both in cirrhosis and control subjects. A nucleotide change in this position resulted in GG, GA and AA genotypes, and the GA genotype was significantly associated with increased risk of HCC in cirrhosis subjects with adjusted $P = 0.0107$, OR (95%CI) = 2.02 (1.178–3.48) and adjusted $P = 0.0122$, OR = 2.26 (95%CI: 1.19–4.27) for control subjects. The mean serum AFP concentration for the GA genotype was higher than the AA or GG genotype, however the $P$-value was statistically less significant (data not shown).

Among the SNPs identified in our study population, 2 SNPs showed a significant association exclusively with the presence of cirrhosis. rs2298839 is located in intron 7 of AFP gene. As in the case of rs3796678, sequence analysis from splice predictor databases also suggested that a G to A nucleotide change in rs2298839 could create a potential splice site [36–40]. SNPs in splicing regulatory sites may interfere with splicing regulation, resulting in unintentional exon skipping or intron retention. Indeed, in an effort to propose a new integrative scoring system for prioritizing SNPs based on their possible deleterious effects in a probabilistic framework, Lee et al. suggested that despite the relatively smaller number of SNPs on splice sites and on coding regions, these regions are enriched for putative deleterious SNPs [41].

A SNP at 3′UTR of AFP, rs10020432, was significantly associated with increased risk of cirrhosis. Near gene 3′ UTR was linked with miRNA involvement in gene expression regulation. The miRNAs are a large class of small, regulatory non-coding RNAs in plants and animals that inhibit gene expression by base pairing with target mRNAs at the 3′ UTR of a gene, leading to mRNA cleavage or translational repression [42]. The aberrant expression of miRNA promotes tumorigenesis, metastasis, and other features of cancer [43,44]. Besides association of polymorphism in miRNA with cancer, nucleotide change in the miRNA binding site has been reported to be associated with the risk of sporadic colorectal cancer, non-small cell lung cancer, oral cancer and breast cancer [45–51]. Further analysis of the genomic region of *AFP* exon 15 using miRBase, miRanda and PicTar databases [49–53] revealed putative binding sites for a number of miRNAs (miR-7, miR-488, miR-942, miR-220, miR-924, miR-620, miR-583, and miR-561).

We explored the possibility of miRNA binding in the 3′UTR of *AFP* where rs10020432 is located by sequence analysis using TargetScan [54–58]. A G to A nucleotide change in rs10020432 might potentially result in a binding site for miR-374 and subsequent gene expression alteration, however this warrants further investigation.

In conclusion, we reported genetic polymorphisms in the *AFP* gene, which are associated with HCC and cirrhosis in the Indonesian population. Additional case control study with larger subject numbers or in other populations, as well as further investigation into the biological functions of these polymorphisms may provide new clues for HCC development.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.cca.2009.11.030.

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