# Polymorphisms in RAS Guanyl-releasing Protein 3 are Associated with Chronic Liver Disease and Hepatocellular Carcinoma in a Korean Population

**Ah-Reum Oh[1], Seung Ku Lee[1], Min-Ho Kim[1], Jae Youn Cheong[2], Sung Won Cho[2], Kap-Seok Yang[3] and KyuBum Kwack[1]\***

[1]Medical Genomics Laboratory, Graduate School of Life Science and Biotechnology, Pochon CHA University, Seongnam 463-836, Korea, [2]Department of Gastroenterology, Genomic Research Center for Gastroenterology, Ajou University School of Medicine, Suwon 442-721, Korea, [3]Macrogen Inc. 60-24, Seoul 153-781, Korea

## Abstract

RAS guanyl-releasing protein 3 (RasGRP3), a member of the Ras subfamily of GTPases, functions as a guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-regulated switch that cycles between inactive GDP- and active GTP-bound states during signal transduction. Various growth factors enhance hepatocellular carcinoma (HCC) proliferation via activation of the Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathway, which depends on RasGRP3 activation. We investigated the relationship between polymorphisms in RasGRP3 and progression of hepatitis B virus (HBV)-infected HCC in a Korean population. Nineteen RasGRP3 SNPs were genotyped in 206 patients with chronic liver disease (CLD) and 86 patients with HCC. Our results revealed that the T allele of the rs7597095 SNP and the C allele of the rs7592762 SNP increased susceptibility to HCC (OR=1.55, p=0.04 and OR=1.81~2.61, p=0.01~0.03, respectively). Moreover, patients who possessed the haplotype (ht) 1 (*A-T-C-G*) or diplotype (dt) 1 (*ht1/ht1*) variations had increased susceptibility to HCC (OR=1.79 ~2.78, p=0.01~0.03). In addition, we identified an association between haplotype1 (ht1) and the age of HCC onset; the age of HCC onset are earlier in ht1 +/+ than ht1 +/- or ht1 -/- (HR=0.42~0.66, p=0.006~0.015). Thus, our data suggest that RasGRP3 SNPs are significantly associated with an increased risk of developing HCC.

## Introduction

Hepatocellular carcinoma (HCC) is the fifth-most prevalent cancer worldwide and the third-most lethal malignancy (Bosch, Ribes *et al.*, 2004; Bruix, Boix *et al.*, 2004). On average, approximately 398,000 males are diagnosed with HCC each year in the world (Parkin, Bray *et al.*, 2001). It is well known that hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin B1 intake, and metabolic disorders such as hemochromatiosis and alpha-antitrypsin deficiency are major risk factors for liver cirrhosis and HCC (Arbuthnot and Kew, 2001). The incidence of HCC that is coincident with HBV infection is high in East Asian and African countries (Caselmann, 1998). More than 90% of HCC tumors are found in patients with chronic hepatitis or cirrhosis (Laurent-Puig and Zucman-Rossi, 2006). Recent studies have compared healthy patients with those who suffer from liver disease (including chronic hepatitis, cirrhosis, and HCC). However, few studies have studied the progression of chronic liver disease with regard to HCC.

Genetic variations are thought to influence the risk of developing HCC (Edmondson, Henderson *et al.*, 1976; Cha and Dematteo, 2005), particularly those that involve the activation of cellular oncogenes or the inactivation of tumor suppressor genes in various signaling pathways (e.g., mutation of beta-catenin-related Wnt/beta-catenin signals (Pang, Yuen *et al.*, 2004) and overexpression of Ras signaling (Mitin, Rossman *et al.*, 2005)). Also, single nucleotide polymorphsims (SNPs) of many famous genes, such as p53 (Kirk, Lesi *et al.*, 2005), HDAC10 (Park, Kim *et al.*, 2007) and MMP2 (Wu, Zhang *et al.*, 2008), have been significantly associated with HCC. The significant SNPs of these genes may represent genetic markers that are in linkage disequilibrium (LD) with other causative variations. Also recently, many SNP studies that are related to various human diseases have been reported (Lee, Kim *et al.*, 2007).

Ras signaling transduction pathways influence cell proliferation, survival, differentiation, vesicular trafficking, and gene expression in B cells (Mitin, Rossman *et al.*, 2005). In previous studies, various growth factors were found to enhance HCC cell proliferation, as well as tu-

*Corresponding author: E-mail kbkwack@cha.ac.kr,
                                           kbkwack@gmail.com
Tel +82-31-725-8376, Fax +82-31-725-8350

mor invasion and metastasis, via the activation of the Ras/Raf-1/extracellular signal-regulated kinase (ERK) pathway (Nonomura, Ohta *et al.*, 1987; Pang, Yuen *et al.*, 2004; Tsuboi, Ichida *et al.*, 2004). Also, over-expression of Ras genes is common in human HCC tissue. Active Ras stimulates the Raf1-Mek-1/ERK effectors by binding to RassF1A and appears to induce apoptosis (Ito, Sasaki *et al.*, 1998). RassF1A hyper-methylation has been observed in various cancer cell lines, including an HCC cell line (Schmidt, McKillop *et al.*, 1997).

RasGRP3 (Ras guanyl-releasing protein 3) is a member of the Ras subfamily of GTPases as a guanosine di-phosphate (GDP)/guanosine triphosphate (GTP) nucleotide exchange factor (GEF) and functions in signal trans-duction as a GTP/GDP-regulated switch that cycles between inactive GDP- and active GTP-bound states (Rebhun, Castro *et al.*, 2000). These factors are key links between cell surface receptors and Ras activation. RasGRP3 is located from chromosomes 2p24.1 to p25.1 and comprises 19 exons. RasGRP3 consists of 4 domains that contain the Ras exchange motif (REM), a Ras-GEF domain, EF-hands and a C1 domain. The C1 domain of RasGRP3 binds directly to phorbol esters, acts as a potent tumor-promoting operator, and is ex-pressed in skeletal muscle and liver (Lorenzo, Kung *et al.*, 2001). In addition, the activation of RasGRP3 by Thr-133 phosphorylation induces the B cell receptor sig-naling and activates small GTPases of the Ras, Rho, and Ra1 families, which play important roles in tumor biology (Aiba, Oh-hora *et al.*, 2004). RasGRP3 also is thought to be associated with developing blood vessels via its involvement in vascular endothelial growth factor (VEGF) signaling, which plays an important role in angio-genesis (Roberts, Anderson *et al.*, 2004).

While RasGRP3 plays important roles in the regulation of Ras signaling, studies have not examined the poly-morphisms that are present within this protein. There-fore, we explored RasGRP3 polymorphisms in a Korean population and explored the link between these poly-morphisms and the risk of developing HCC during HBV infection.

## Methods

### Subjects

The study population contained a total of 302 patients, including 86 patients with HCC patients (*i.e.*, cases) and 206 patients with CLD (*i.e.*, controls). A total of 302 Korean subjects with present or past evidence of HBV infection were recruited from the Ajou Genomic Research Center for Gastroenterology at the Ajou

University Medical Center in Suwon, South Korea be-tween March 2002 and February 2006. The study proto-col was approved by the Institutional Review Boards (IRBs) of Ajou University Hospital and Pochon CHA University. Serological markers were identified using commercially available assays for hepatitis B surface antigen (HBsAg), antibodies to hepatitis B core antigen (Anti-HBc; HBcAb), antibodies to hepatitis B surface an-tigen (Anti-HBs; HBsAb), hepatitis B 'e' antigen (HBeAg), antibodies to hepatitis B 'e' antigen (Anti-HBe; HBeAb), HBV-DNA, albumin, alpha-fetoprotein (AFP), glutamyl transpeptidase, platelets, aspartate amino-transferase (AST), alanine aminotransferase (ALT), bilir-ubin, cholesterol, total protein, and white blood cells. Patients were divided into 2 groups. The first group (i.e., n=206) experienced CLD for more than 7 months with-out HCC. This group consisted of patients with chronic hepatitis and liver cirrhosis who were positive for HBsAg and negative for HBsAb (HBcAb (+), HBeAg (+), or HBV-DNA (+)). Patients with chronic hepatitis exhibited elevated ALT levels (*i.e.*, $\geq 2$ times the upper limit of normal) at least once during the follow-up period and were diagnosed via ultrasonography. The second group (*i.e.*, n=86) consisted of patients with HCC who tested positive for HBsAg and negative for HBsAb (HBcAb (+), HBeAg (+), or HBV-DNA (+)). These patients were diag-nosed using standard criteria (*i.e.*, serum AFP levels greater than 400 ng/ml) via ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI). Patients who tested positive for anti-HCV or anti-HIV antibodies were excluded from the analysis.

### Selection of SNPs in the RasGRP3 gene

We selected the RasGRP3 gene, which regulates ras signaling; its signaling is important for carcinogenic mechanisms and is especially important for the develop-ment of hepatocellular carcinoma. The basic hypothesis that underlies our approach is that there is more than 1 common SNP in the genes of interest that are asso-ciated with an altered risk of HCC. To identify a set of tagging SNPs (tSNPs), that efficiently tags all the known common variants (MAF; minor allele frequency $>0.05$) and is likely to tag of the unknown common variants, coding SNPs, 5'-UTR, and 3'-UTR regulatory SNPs were selected based on the JPT (Japanese in Tokyo) and CHB (Chinese in Beijing) data that were down-loaded from the International HapMap site (http://www.hapmap.org) and a public SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). To cover the whole RasGRP3 gene by a series of LD blocks, intronic SNPs at regular intervals were selected.

## Genotyping analysis

Genomic DNA (gDNA) was extracted from whole blood using a commercial gDNA extraction kit (*i.e.*, G-DEX™ blood genomic DNA purification kit, Intron Biotechnology Inc. Seongnam, Korea). The amount of gDNA was determined using the Picogreen double-strand DNA quantification reagent, according to a standard protocol (Molecular Probes, Eugene, OR). The gDNA was analyzed using a Victor™ 3 multilabel counter (with excitation at 480 nm and emission at 520 nm; PerkinElmer Inc. Waltham, MA), and a standard curve for the concentration of gDNA was created using known concentrations of $\lambda$ DNA. Genotyping was performed using the Golden Gate genotyping assay according to a standard protocol (Illumina, Inc., San Diego, CA, USA). In brief, polymerase chain reactions (PCRs) were performed in reaction mixtures that contained allele-specific extension oligomers and 250 ng of genomic DNA by ramping the temperature from $70^{o}C$ to $30^{o}C$ over a period of 16 hours (Supplementary Table 1). The specific extension products were then used in PCRs that consisted of 34 cycles of 35 seconds at $95^{o}C$, 35 seconds at $56^{o}C$, and 2 minutes at $72^{o}C$. The PCR products were placed in 96-well filter plates (Millipore, Billerica, MA, USA) for purification. Samples were then placed in 384-well microplates and hybridized at $60^{o}C$ for 30 minutes and at $45^{o}C$ for 16 hours. Finally, the 384-well microplates were washed and scanned at a resolution of 0.8 um using a BeadArray Reader (Illumina, Inc.). Genotyping analysis was performed using Illumina's Beadstudio software (Version 3). Other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

## Statistical analysis

The $\chi^2$ test was used to assess Hardy-Weinberg equilibrium (HWE). Three genetic models (*i.e.*, codominant, dominant, and recessive), proposed by Lewis (Lewis, Cathryn *et al*. 2002), were used to test for associations between CLD and HCC. LD blocks of 7 SNPs were confirmed using HAPLOVIEW software (version 4.0; http//www.broad.mit.edu/mpg/haploview), according to the confidence interval method. The SNPs were included in an LD block if the MAF exceeded 0.1 and exhibited HWE (p > 0.05). Each individual haplotype was inferred using the SAS haplotype procedure. Using a single SNP association test, a haplotype association test, and a diplotype association test, multiple logistic regression models were used to calculate odds ratios (ORs), 95% confidence intervals (CIs), and corresponding p-values, while controlling for age and gender as covariables. Cox regression models were used to calcu-

late hazard ratios (HRs), 95% CIs, and corresponding p-values, while controlling for gender in the haplotype association test. All statistical tests were performed using SAS software (SAS Enterprise Guide 4.1; SAS Institute, Cary, NC, USA) and HapAnalyzer (Jung, Pack *et al*., 2004), and results were considered statistically significant if p-values were greater than 0.05.

## Results

The clinical characteristics of the controls (CLD) and cases (HCC) are shown in Table 1. The association between RASGRP3 polymorphisms, CLD, and HCC was evaluated. Two SNPs were identified in the 5'-flanking region (rs597593, rs583942), 2 SNPs were identified in the coding region (rs11680495, rs13388394), 14 SNPs were identified in the intronic region (rs17012996, rs7597095, rs2305577, rs7565568, rs2305573, rs10189430, rs17013236, rs7592762, rs4670191, rs10182807, rs17013337, rs12470399, rs13415927), and 1 SNP was identified in the 3'-untranslated region (UTR; rs1801894) (Supplementary Table 2). Twelve of 19 SNPs were monomorphic or not polymorphic and did not exhibit MAF values less than 0.05 (data not shown). Seven SNPs were identified in the Korean population (Fig. 1A). The LD block was constructed using the confidence interval method in the HAPLOVIEW program (*i.e.*, HWE > 0.05 and MAF > 0.1, CLD=206, HCC=86) (Fig. 1B). The genotype frequencies of each polymorphism were analyzed in the CLD and HCC groups using logistic regression models (Table 2), while controlling for age and sex as covariates. We identified 2 SNPs (rs7597095, rs7592762)

**Table 1.** Clinical characteristics of patients with chronic liver disease and hepatocellular carcinoma

|  | Chronic liver disease (CLD) | Hepatocellular carcinoma (HCC) |
|---|---|---|
| n | 206 | 86 |
| Gender (M/F) | 158/48 | 69/17 |
| Age (mean±SD) | 42.86±9.62 | 53.88±10.58 |
| HBsAb (Anti-HBs; positive rate, %) | 0 | 0 |
| HBeAg (positive rate, %) | 61.98 | 37.04 |
| HBeAb (Anti-Hbe; positive rate, %) | 42.55 | 64.15 |
| HBsAg (positive rate, %) | 100 | 100 |
| AST (U/L, mean±SD) | 93.67±161.74 | 117.93±137.78 |
| ALT (U/L, mean±SD) | 107.36±170.25 | 52.22±38.80 |
| Albumin (g/dL, mean±SD) | 3.96±0.61 | 3.39±0.69 |
| Bilirubin (mg/dL, mean±SD) | 1.71±2.88 | 2.63±4.23 |

(A)

(B)
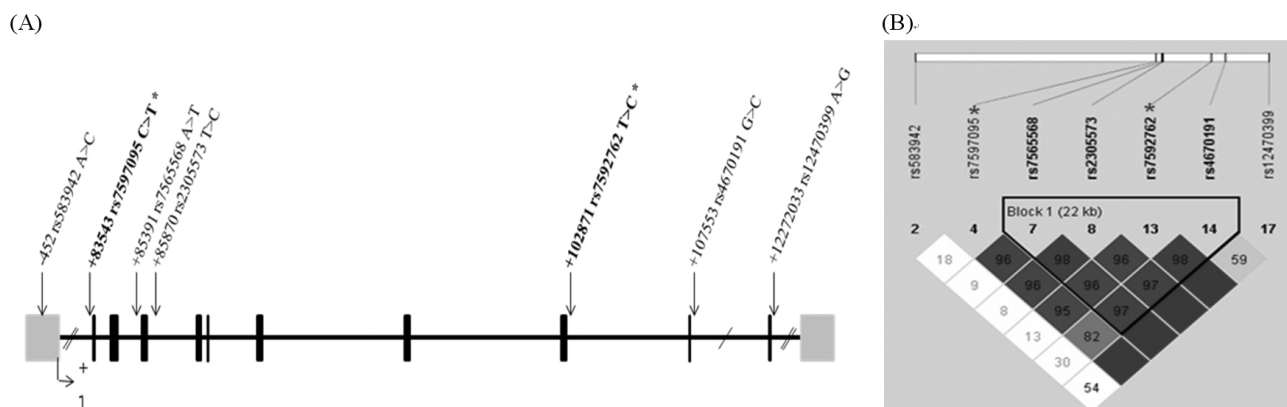


**Fig. 1.** RasGRP3 gene map, SNPs, and LD. (A) RasGRP3 gene map, indicating the location of 7 SNPs. The 3'- and 5'-UTR are shown as gray boxes, exons are shown as black boxes, and the introns are indicated by lines. The first base of the transcriptional site is denoted as nucleotide +1. Asterisks indicate significantly associated polymorphisms in CLD and HCC. (B) The 4 SNPs (*i.e.*, rs75655688, rs2305573, rs7592762, rs4670191) were used in the haplotype estimation, and the minor allele frequencies of those SNPs were greater than 0.05. Asterisks indicate significantly associated polymorphisms in CLD and HCC.

**Table 2.** Logistic analysis of RasGRP3 gene polymorphisms in the Korean population

| SNP | Geno-type | Region | CLD | HCC | Codominant | | Dominant | | Recessive | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | OR (95%CI) | p | OR (95%CI) | p | OR (95%CI) | p |
| rs583942 | AA | Flanking_5UTR | 112 (54.4) | 42 (48.8) | 1.204 (0.746~1.946) | 0.447 | 1.305 (0.733~2.323) | 0.366 | 1.006 (0.279~3.618) | 0.993 |
| | AC | | 82 (39.8) | 39 (45.3) | | | | | | |
| | CC | | 12 (5.8) | 5 (5.8) | | | | | | |
| rs7597095 | CC | intron | 87 (42.2) | 31 (36.0) | **1.547 (1.012~2.365)** | **0.044** | 1.790 (0.980~3.271) | 0.058 | 1.727 (0.759~3.933) | 0.193 |
| | CT | | 95 (46.1) | 42 (48.8) | | | | | | |
| | TT | | 24 (11.7) | 13 (15.1) | | | | | | |
| rs7565568 | AA | intron | 110 (57.6) | 48 (55.8) | 0.867 (0.541~1.391) | 0.554 | 0.890 (0.499~1.587) | 0.693 | 0.646 (0.184~2.269) | 0.495 |
| | AT | | 81 (38.9) | 34 (39.5) | | | | | | |
| | TT | | 15 (7.2) | 4 (4.7) | | | | | | |
| rs2305573 | TT | intron | 112 (53.8) | 48 (55.8) | 0.941 (0.588~1.504) | 0.799 | 1.004 (0.563~1.790) | 0.990 | 0.652 (0.18~2.299) | 0.506 |
| | TC | | 80 (26.8) | 34 (39.5) | | | | | | |
| | CC | | 14 (4.7) | 4 (4.7) | | | | | | |
| rs7592762 | TT | intron | 102 (54.3) | 33 (38.4) | **1.806 (1.171~2.786)** | **0.007** | **1.972 (1.091~3.564)** | **0.024** | **2.611 (1.086~6.274)** | **0.032** |
| | TC | | 86 (45.7) | 41 (47.7) | | | | | | |
| | CC | | 18 (9.5) | 12 (14.0) | | | | | | |
| rs4670191 | GG | intron | 69 (33.5) | 35 (40.7) | 0.759 (0.501~1.151) | 0.195 | 0.590 (0.325~1.070) | 0.082 | 0.923 (0.425~2.003) | 0.840 |
| | GC | | 102 (49.5) | 37 (43.0) | | | | | | |
| | CC | | 35 (17.0) | 14 (16.3) | | | | | | |
| rs12470399 | AA | intron | 161 (78.2) | 64 (74.4) | 0.846 (0.461~1.550) | 0.588 | 0.890 (0.449~1.765) | 0.739 | 0.412 (0.051~3.354) | 0.407 |
| | AG | | 41 (19.9) | 20 (23.3) | | | | | | |
| | GG | | 4 (1.9) | 2 (2.3) | | | | | | |

Logistic regression models were used to calculate odds ratios (*i.e.*, 95% confidence intervals) and corresponding p-values for each SNP. p-values obtained using the codominant, dominant and recessive, models are also shown. Age and sex were adjusted in the logistic analysis as covariates. Abbreviations: Freq., frequency; CLD, chronic liver disease; HCC, hepatocellular carcinoma; 95% CI, 95% confidence interval; LCL, lower confidence limit; UCL, upper confidence limit; OR, odds ratio. Bold indicates p<0.05.

that were significantly associated with CLD and HCC. The T allele of the rs7597095 SNP was associated with an increased risk of developing HCC, according to the

codominant model (*i.e.*, OR=1.55, 95% CI=1.01~2.37, p=0.044). The C allele of the rs7592762 SNP was associated with an increased risk of developing HCC, ac-

cording to the codominant, dominant, and recessive models (*i.e.*, OR=1.81; 95% CI=1.17~2.79; p=0.007, OR=1.97; 95% CI=1.09~3.56; p=0.024, OR=2.61; 95% CI=1.09~6.27; p=0.032, respectively) (Table 2).

The haplotype frequencies and association test are summarized in Table 3. The 4 RasGRP3 polymorphisms were in an LD (|D'| value, ranging from 0.96 to 0.98) block, and we observed 9 haplotypes of a total of 16 haplotypes. Among these haplotypes, 5 had a frequency of less than 1% and therefore analyzed, except for its haplotypes (data not shown). Of the 4 remaining haplotypes, RasGRP3 *ht1* [A-T-C-G] was significantly associated with a risk of developing HCC in the codominant, dominant, and recessive models (OR=1.79; 95% CI=1.16~2.78; p=0.008, OR=2.78; 95% CI=1.15~6.67; p=0.024, OR=1.89; 95% CI=1.05~3.45; p=0.033, re-

spectively).

We next analyzed the associations between diplotypes of the 4 RasGRP3 haplotypes (*i.e.*, A-T-C-G, A-T-T-C, A-T-T-G, T-C-T-C) and the risk of developing HCC (Table 4). Eighteen distinct diplotypes were identified. Among them, 8 exhibited low frequencies and were not analyzed. When we compared dt1 with dt6 and dt1 with dt8, an increased risk of developing HCC was associated with dt1 (*i.e.*, for dt1 vs. dt6, OR=8.318, p=0.009; and for dt1 vs. dt8, OR=3.821, p=0.045). However, dt6 was associated with a decreased risk of developing HCC (*i.e.*, for dt3 vs. dt6, OR=4.890, p=0.039; and for dt6 vs. dt7, OR=0.189, p=0.046).

A Cox regression model that compared age at disease onset with HCC revealed that RasGRP3 ht1 conferred susceptibility to HCC (*i.e.*, for the codominant

**Table 3.** Association of the RasGRP3 haplotype with HCC, as determined via logistic regression analysis

| Haplotype | | CLD | HCC | Codominant | | Dominant | | Recessive | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | OR (95% CI) | p | OR (95% CI) | p | OR (95% CI) | p |
| ht1 (A-T-C-G) | ht1/ht1 | 17 (8.25) | 12 (13.95) | **1.79 (1.16~2.78)** | **0.008** | **2.78 (1.15~6.67)** | **0.024** | **1.89 (1.05~3.45)** | **0.033** |
| | ht1/- | 87 (42.23) | 40 (46.51) | | | | | | |
| | -/- | 102 (49.51) | 34 (39.53) | | | | | | |
| ht2 (A-T-T-C) | ht2/ht2 | 3 (1.46) | 1 (1.16) | 0.73 (0.40~1.35) | 0.312 | 0.32 (0.02~4.76) | 0.406 | 0.75 (0.39~1.45) | 0.386 |
| | ht2/- | 54 (26.21) | 21 (24.42) | | | | | | |
| | -/- | 149 (72.33) | 64 (74.42) | | | | | | |
| ht3 (A-T-T-G) | ht3/ht3 | 19 (9.22) | 7 (8.14) | 0.73 (0.47~1.15) | 0.170 | 0.65 (0.23~1.79) | 0.402 | 0.68 (0.38~1.20) | 0.184 |
| | ht3/- | 80 (38.83) | 28 (32.56) | | | | | | |
| | -/- | 107 (51.94) | 51 (59.30) | | | | | | |
| ht4 (T-C-T-C) | ht4/ht4 | 14 (6.80) | 3 (3.49) | 0.93 (0.58~1.49) | 0.777 | 0.47 (0.11~1.89) | 0.285 | 1.05 (0.59~1.89) | 0.868 |
| | ht4/- | 77 (37.38) | 35 (40.70) | | | | | | |
| | -/- | 115 (55.83) | 48 (55.81) | | | | | | |

Haplotype association testing was conducted using SAS. Abbreviations: OR, odds ratio; CLD, chronic liver disease; HCC, hepatocellular carcinoma. Bold indicates p-values＜0.05.

**Table 4.** Frequencies and association of RasGRP3 diplotype on logistic regression analysis

| Diplotype | Number (%) | | OR | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | CLD | HCC | dt1 | dt2 | dt3 | dt4 | dt5 | dt6 | dt7 | dt8 | dt9 | dt10 |
| dt1 (ht1/ht1) | 17 (8.25) | 12 (13.95) | | 2.743 | 1.701 | 2.188 | 8.163 | **8.318** | 1.573 | **3.821** | 3.223 | **5.244** |
| dt2 (ht1/ht2) | 17 (8.25) | 8 (9.30) | 0.136 | | 0.62 | 0.798 | 2.976 | 3.033 | 0.573 | 1.393 | 1.175 | 1.912 |
| dt3 (ht1/ht3) | 32 (15.53) | 16 (18.60) | 0.335 | 0.449 | | 1.286 | 4.799 | **4.89** | 0.925 | 2.246 | 1.895 | 3.083 |
| dt4 (ht1/ht4) | 34 (16.50) | 16 (18.60) | 0.164 | 0.723 | 0.624 | | 3.731 | 3.802 | 0.719 | 1.747 | 1.473 | 2.397 |
| dt5 (ht2/ht2) | 3 (1.46) | 1 (1.16) | 0.153 | 0.463 | 0.277 | 0.363 | | 1.019 | 0.193 | 0.468 | 0.395 | 0.642 |
| dt6 (ht2/ht3) | 20 (9.71) | 3 (3.49) | **0.009** | 0.194 | **0.039** | 0.084 | 0.99 | | **0.189** | 0.459 | 0.388 | 0.631 |
| dt7 (ht2/ht4) | 16 (7.77) | 9 (10.47) | 0.478 | 0.432 | 0.894 | 0.586 | 0.265 | **0.046** | | 2.429 | 2.049 | 3.335 |
| dt8 (ht3/ht3) | 19 (9.22) | 7 (8.14) | **0.045** | 0.648 | 0.194 | 0.374 | 0.608 | 0.358 | 0.205 | | 0.844 | 1.373 |
| dt9 (ht3/ht4) | 26 (12.62) | 9 (10.47) | 0.061 | 0.815 | 0.27 | 0.507 | 0.527 | 0.246 | 0.28 | 0.803 | | 1.627 |
| dt10 (ht4/ht4) | 14 (6.80) | 3 (3.49) | **0.046** | 0.461 | 0.156 | 0.275 | 0.777 | 0.639 | 0.16 | 0.716 | 0.564 | |

p-value

Abbreviations: OR, odds ratio. Bold indicates p-values＜0.05.

**Table 5.** Cox regression analysis for age of HCC occurrence as a function of haplotype of RasGRP3

| Type | Haplotype | Frequency | Mean | SD | Model | HR | 95% CI | | p |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | | | | | | LCL | UCL | |
| ht1 | ht1/ht1 | 12 | 48.08 | 11.13 | Co-dominant | **0.662** | **0.474** | **0.924** | **0.015** |
| | ht1/- | 40 | 54.68 | 10.69 | Dominant | **0.416** | **0.224** | **0.775** | **0.006** |
| | -/- | 34 | 55.00 | 9.89 | Recessive | 0.704 | 0.454 | 1.093 | 0.118 |
| ht2 | ht2/ht2 | 1 | 69.00 | | Co-dominant | 1.258 | 0.810 | 1.954 | 0.307 |
| | ht2/- | 21 | 55.86 | 11.17 | Dominant | 2.479 | 0.342 | 17.998 | 0.369 |
| | -/- | 64 | 53.00 | 10.29 | Recessive | 1.232 | 0.757 | 2.008 | 0.401 |
| ht3 | ht3/ht3 | 7 | 55.57 | 10.21 | Co-dominant | 1.189 | 0.853 | 1.657 | 0.308 |
| | ht3/- | 28 | 51.64 | 11.06 | Dominant | 1.361 | 0.626 | 2.960 | 0.436 |
| | -/- | 51 | 54.88 | 10.37 | Recessive | 1.226 | 0.790 | 1.903 | 0.363 |
| ht4 | ht4/ht4 | 3 | 56.33 | 9.50 | Co-dominant | 1.059 | 0.732 | 1.533 | 0.760 |
| | ht4/- | 35 | 55.60 | 8.95 | Dominant | 1.283 | 0.404 | 4.074 | 0.673 |
| | -/- | 48 | 52.48 | 11.67 | Recessive | 1.041 | 0.678 | 1.598 | 0.855 |

Cox regression analysis was to calculate hazard ratios, 95% confidence intervals, and corresponding p-values for haplotypes, while controlling for sex. Abbreviations: 95% CI, 95% confidence interval; HR, hazard ratios. Bold indicate p-values $<0.05$.

model, mean$\pm$STD=48.08$\pm$11.13, HR=0.66, 95% CI= 0.47~0.92, and p=0.015; in the dominant model, mean$\pm$STD=54.68$\pm$10.69, HR=0.42, 95% CI=0.22~0.78, and p=0.006). Patients who possessed RasGRP3 ht1 developed HCC more quickly than patients who possessed ht-/+ or ht1-/- patients, as indicated by Kaplan-Meier survival curve analysis (Table 5 and Fig. 2). Also, a Cox regression model that compared age at disease onset with HCC revealed that each SNP of RasGRP3 conferred susceptibility to HCC. Patients who had the C allele of rs7592762 (*i.e.*, for the codominant, mean$\pm$STD=54.91$\pm$10.03, HR=1.53, 95% CI=1.09~2.14, and p=0.013; in the recessive model, mean$\pm$ STD=48.03$\pm$11.13, HR=2.38, 95% CI=1.28~4.44, and p=0.006) and/or the C allele of rs4670191 (*i.e.*, for the dominant model, mean$\pm$STD=57.38$\pm$9.38, HR=0.64, 95% CI= 0.41~0.99, and p=0.045) developed HCC more quickly than other patients (Supplementary Table 3).

## Discussion

We identified genetic markers that were associated with HCC susceptibility in a Korean population. HCC is the most common type of malignant tumor and the third-most common cause of cancer-related deaths (Bruix, Boix *et al.*, 2004). The progression of human cancers to malignant tumors involves the mutation of genes, such as p53, breast cancer 1 (BRCA1), and Ras, which contribute to cell proliferation, cell cycle progression, apoptosis, and metastasis (Greenblatt, Bennett *et al.*, 1994; Levy-Lahad and Friedman, 2007). Other genes that are involved in Ras signaling regulate various cellular functions, including cell growth, survival, and mi-
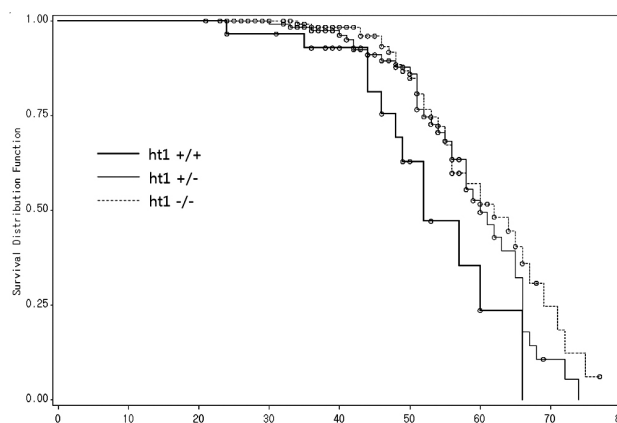
**Fig. 2.** Kaplan-Meier survival curves for haplotype 1. Plots represent mean-censored data (*i.e.*, in the CLD group).

gration (Vojtek and Der, 1998). Ras genes enhance the metastatic phenotype of the human HCC cell line (Wang, Lin *et al.*, 2001), and Ras proteins are known to be overexpressed in human cirrhotic liver and HCC (Nonomura, Ohta *et al.*, 1987).

Activation of Ras signal transduction pathways depends on RasGRP3, which functions as a GTP/GDP switch. Via the actions of RasGRP3, activated GTP-Ras proteins bind and regulate functionally diverse downstream effectors, such as Ras, Raf1, MEK, and ERK. The RasGRP family is directly associated with the Ras and GTPase molecules (Bar-Sagi and Hall, 2000; Shields, Pruitt *et al.*, 2000), and RasGRP deregulation has been linked to carcinogenesis and tumor progression (Khosravi-Far and Der, 1994).

The C1 domain of RasGRP3 binds directly to phorbol esters that are potent tumor-promoting operator, thereby increasing the levels of ERK1/ERK2 (Lorenzo, Kung *et al.*, 2001). Also, Rap1 has been reported to be activated by phorbol esters through RasGRP3. RasGRP3-/- B cells exhibit undetectable basal levels of Ras-GTP, as well as defective BCR-induced Ras activation, and BCR-induced proliferation in vitro depends on RasGRP3 (Coughlin, Stang *et al.*, 2005; Tangye and Hodgkin, 2004). In addition, RasGRP3 interacts with dynein light chain 1 (DLC1), contributes to cytoskeleton-mediated motility and intracellular transport, and interacts with various proteins that are associated with cell survival and apoptosis (e.g., BimL and IKB$\alpha$) (Crepieux, Kwon *et al.*, 1997; Puthalakath, Huang *et al.*, 1999). Thus, RasGRP3 may interact with significant SNPs (*i.e.*, ht1 and dt1), thereby regulating GDP/GTP switches and converting inactive forms to active forms upstream of Ras. These actions also may enhance the expression of Ras and downstream effectors molecules. Our data revealed that patients who possessed the SNPs ht1 or dt1 progressed more quickly to HCC than did other patients (Table 5, Fig. 2).

To our knowledge, this is the first case-control study that demonstrates a significant association between RasGRP3 SNPs and progression from CLD to HCC in a Korean population. Our results show that the T allele of the rs7565568 SNP and the C allele of the rs7592762 SNP increase susceptibility to HCC. Moreover, patients with CLD who possessed ht1 (*i.e.*, A-T-C-G) or dt1 (*i.e.*, A-T-C-G/A-T-C-G) were at increased risk for developing HCC. Finally, we identified an association between ht1/ht1 and the age of HCC onset; a highly significant acceleration in HCC progress was apparent among the RasGRP3 ht1-bearing patients.

Thus, our findings suggest significant associations between RasGRP3 SNPs and the development of HCC during HBV infection. In summary, we identified 2 RasGRP3 SNPs (rs7597095, rs7592762), 1 haplotype (ht1; A-T-C-G), and 1 diplotype (dt1; A-T-C-G/A-T-C-G) that were significantly associated with the development of HCC-infected HBV.

## References

Aiba, Y., Oh-hora, M., Kiyonaka, S., Kimura, Y., Hijikata, A., Mori, Y., and Kurosaki, T. (2004). Activation of RasGRP3 by phosphorylation of Thr-133 is required for B cell receptor-mediated Ras activation. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16612-16617.

Arbuthnot, P., and Kew, M. (2001). Hepatitis B virus and hepatocellular carcinoma. *Int. J. Exp. Pathol.* 82, 77-100.

Bar-Sagi, D., and Hall, A. (2000). Ras and Rho GTPases: a family reunion. *Cell* 103, 227-38.

Bosch, F.X., Ribe, J., Diaz, M., and Cleries, R. (2004). Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 127, S5-S16.

Bruix, J., Boix, L., Sala, M., and Llovet, J.M. (2004). Focus on hepatocellular carcinoma. *Cancer Cell* 5, 215-219.

Caselmann, W.H. (1998). Pathogenesis of hepatocellular carcinoma. *Digestion* 59 Suppl 2, 60-63.

Cha, C., and Dematteo, R.P. (2005). Molecular mechanisms in hepatocellular carcinoma development. *Best Pract. Res. Clin Gastroenterol* 19, 25-37.

Coughlin, J.J., Stang, S.L., Dower, N.A., and Stone, J.C. (2005). RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. *J. Immunol.* 175, 7179-84.

Crepieux, P., Kwon, H., Leclerc, N., Spencer, W., Richard, S., Lin, R., and Hiscott, J. (1997). I kappaB alpha physically interacts with a cytoskeleton-associated protein through its signal response domain. *Mol. Cell. Biol.* 17, 7375-7385.

Edmondson, H.A., Henderson, B., and Benton, B. (1976). Liver-cell adenomas associated with use of oral contraceptives. *N. Engl. J. Med.* 294, 470-472.

Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.* 54, 4855-4878.

Ito, Y., Sasaki, Y., Horimoto, M., Wada, S., Tanaka, Y., Kasahara, A., Ueki, T., Hirano, T., Yamamoto, H., Fujimoto, J., Okamoto, E., Hayashi, N., and Hori, M. (1998). Activation of mitogen-activated protein kinases/extracellular signal-regulated kinases in human hepatocellular carcinoma. *Hepatology* 27, 951-958.

Jung, H.Y., Park, J.S., Park, Y.J., Kim, Y.J., Kimm, K.C., and Koh, I.S. (2004). HapAnalyzer: minimum haplotype analysis system for association studies. *Genomics & Informatics* 2, 107-109.

Khosravi-Far, R., and Der, C.J. (1994). The Ras signal transduction pathway. *Cancer Metastasis Rev.* 13, 67-89.

Kirk, G.D., Lesi, O.A., Mendy, M., Szymanska, K., Whittle, H., Goedert, J.J., Hainaut, P., and Montesano, R. (2005). 249(ser) TP53 mutation in plasma DNA, hepatitis B viral infection, and risk of hepatocellular carcinoma. *Oncogene* 24, 5858-5867.

Laurent-Puig, P., and Zucman-Rossi, J. (2006). Genetics of hepatocellular tumors. *Oncogene* 25, 3778-3786.

Levy-Lahad, E., and Friedman, E. (2007). Cancer risks among BRCA1 and BRCA2 mutation carriers. *Br. J. Cancer* 96, 11-15.

Lewis, C.M. (2002). Genetic association studies: design, analysis and interpretation. *Brief Bioinform* 3, 146-153.

Lee, S.K., Kim, H.G., Kang, J.J., Oh, W., Oh, B.E., and Kwack, K. (2007). Characterization of single nucleotide polymorphisms in 55disease-associated genes in a Korean population. *Genomics & informatics* 5, 152-160.

Lorenzo, P.S., Kung, J.W., Bottorff, D.A., Garfield, S.H., Stone, J.C., and Blumberg, P.M. (2001). Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res.* 61, 943-9.

Mitin, N., Rossman, K.L., and Der, C.J. (2005). Signaling interplay in Ras superfamily function. *Curr. Biol.* 15(14), R563-R574.

Nonomura, A., Ohta, G., Hayashi, M., Izumi, R., Watanabe, K., Takayanagi, N., Mizukami, Y., and Matsubara, F. (1987). Immunohistochemical detection of ras oncogene p21 product in liver cirrhosis and hepatocellular carcinoma. *Am. J. Gastroenterol.* 82, 512-518.

Pang, R., Yuen, J., Yuen, M.F., Lai, C.L., Lee, T.K., Man, K., Poon, R.T., Fan, S.T., Wong, C.M., Ng, I.O., Kwong, Y.L., and Tse, E. (2004). PIN1 overexpression and beta-catenin gene mutations are distinct oncogenic events in human hepatocellular carcinoma. *Oncogene* 23, 4182-4186.

Park, B.L., Kim, Y.J., Cheong, H.S., Lee, S.O., Han, C.S., Yoon, J.H., Park, J.H., Chang, H.S., Park, C.S., Lee, H.S., and Shin, H.D. (2007). HDAC10 promoter polymorphism associated with development of HCC among chronic HBV patients. *Biochem. Biophys. Res. Commun.* 363, 776-781.

Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. (2001). Estimating the world cancer burden: Globocan 2000. *Int. J. Cancer* 94, 153-156.

Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M., and Strasser, A. (1999). The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol. Cell.* 3, 287-296.

Rebhun, J.F., Castro, A.F., and Quilliam, L.A. (2000). Identification of guanine nucleotide exchange factors (GEFs) for the Rap1 GTPase. Regulation of MR-GEF by M-Ras-GTP interaction. *J. Biol. Chem.* 275, 34901-34908.

Roberts, D.M., Anderson, A.L., Hidaka, M., Swetenburg, R.L., Patterson, C., Stanford, W.L., and Bautch, V.L. (2004). A vascular gene trap screen defines RasGRP3 as an angiogenesis-regulated gene required for the endothelial response to phorbol esters. *Mol. Cell. Biol.* 24, 10515-10528.

Schmidt, C.M., McKillop, I.H., Cahill, P.A., and Sitzmann, J.V. (1997). Increased MAPK expression and activity in primary human hepatocellular carcinoma. *Biochem Biophys. Res. Commun.* 236, 54-58.

Shields, J.M., Pruitt, K., McFall, A., Shaub, A., and Der, C.J. (2000). Understanding Ras: 'it ain't over 'til it's over'. *Trends Cell Biol.* 10, 147-154.

Tangye, S.G., and Hodgkin, P.D. (2004). Divide and conquer: the importance of cell division in regulating B-cell responses. *Immunology* 112, 509-520.

Tsuboi, Y., Ichida, T., Sugitani, S., Genda, T., Inayoshi, J., Takamura, M., Matsuda, Y., Nomoto, M., and Aoyagi, Y. (2004). Overexpression of extracellular signal-regulated protein kinase and its correlation with proliferation in human hepatocellular carcinoma. *Liver Int.* 24, 432-436.

Vojtek, A.B., and Der, C.J. (1998). Increasing complexity of the Ras signaling pathway. *J. Biol. Chem.* 273, 19925-19928.

Wang, Q., Lin, Z.Y., and Feng, X.L. (2001). Alterations in metastatic properties of hepatocellular carcinoma cell following H-ras oncogene transfection. *World J. Gastroenterol.* 7, 335-339.

Wu, L.M., Zhang, F., Xie, H.Y., Xu, X., Chen, Q.X., Yin, S.Y., Liu, X.C., Zhou, L., Xu, X.B., Sun, Y.L., and Zheng, S.S. (2008). MMP2 promoter polymorphism (C-1306T) and risk of recurrence in patients with hepatocellular carcinoma after transplantation. *Clin. Genet.* 73, 273-278.

**Supplementary Table 1.** Primer sequence information of each SNP for genotyping

| SNP | Universal PCR sequence 1 | Universal PCR sequence 2 | Universal PCR sequence 3 |
| --- | --- | --- | --- |
| rs597593 | ACTTCGTCAGTAACGGACAAAATCTACTCTTAAATTCCTTTTTGAAGA | GAGTCGAGGTCATATCGTAAAATCTACTCTTAAATTCCTTTTTGAAGG | ATATTAATAAACATGCACACAAAGTATCAGTCGTGCGATGTTCCAAGCGTCTGCCTATAGTGAGTC |
| rs583942 | ACTTCGTCAGTAACGGACGCAAATATTATACTGTGTATGCCATGCTTTA | GAGTCGAGGTCATATCGTGCAAATATTATACTGTGTATGCCATGCTTTC | AAACATTAATTTTAAAGACAATTTTTGCCCGGCATAATACAGTCCTACGTCTGCCTATAGTGAGTC |
| rs17012996 | ACTTCGTCAGTAACGGACTCTCCTCACCTTCTGGTCAATGAA | GAGTCGAGGTCATATCGTTCTCCTCACCTTCTGGTCAATGAG | GCATCCTTCTTCAATGTCTTCTCGCCCAGTTGATGGAGAGCGTAGTCTGCCTATAGTGAGTC |
| rs7597095 | ACTTCGTCAGTAACGGACGCTTTGCACACCCCTGCAGCTA | GAGTCGAGGTCATATCGTGCTTTGCACACCCCTGCAGCTG | GCCCTCAATGTATACCAATTCCATGTATGACGGCTCCTTATGTGGGTCTGCCTATAGTGAGTC |
| rs2305577 | ACTTCGTCAGTAACGGACGGGTGGGAGGGGTATCAGAGTAGAGT | GAGTCGAGGTCATATCGTGGGTGGGAGGGGTATCAGAGTAGAGC | TGTTGTCAAACTAGGTCTGCATTTCTGTAGCATCGAATCAGTGGAGTCTGCCTATAGTGAGTC |
| rs11680495 | ACTTCGTCAGTAACGGACGCGTATGACTGAGGAATTTCGGGAAGT | GAGTCGAGGTCATATCGTGCGTATGACTGAGGAATTTCGGGAAGC | GCTAGTCAACTAGGATATGAAAAATGACCTCAAGGAAGCGGCGATTGTCTGCCTATAGTGAGTC |
| rs7565568 | ACTTCGTCAGTAACGGACGCAGACAGGGTAGGTTTTGCACTGAA | GAGTCGAGGTCATATCGTGCAGACAGGGTAGGTTTTGCACTGAT | CATCCAGAATTTCTTACTTAGTGTCATCAACCTATTCTGCGGCAAAGGGGTCTGCCTATAGTGAGTC |
| rs2305573 | ACTTCGTCAGTAACGGACCTTCTATCACTGTGCTCTGCTGGAA | GAGTCGAGGTCATATCGTCTTCTATCACTGTGCTCTGCTGGAG | AGTGCCCTTAATTCACAGGAGACAGCAACTTGGGCTCCGGTTGTCTGCCTATAGTGAGTC |
| rs10189430 | ACTTCGTCAGTAACGGACGCAAACAACTTTCCCTCCTGCACAA | GAGTCGAGGTCATATCGTGCAAACAACTTTCCCTCCTGCACAC | CAATGTGTGGCTAGGAAATTCCGTAAACTGTAGACCGCCTTGTCGTCTGCCTATAGTGAGTC |
| rs7605981 | ACTTCGTCAGTAACGGACAGCAGCTCCCAGGTGCTCAGT | GAGTCGAGGTCATATCGTAGCAGCTCCCAGGTGCTCAGG | CTGAGTCACGGATGAGGGATATCGACTTTGTAGCAGCCGAGGTTGTCTGCCTATAGTGAGTC |
| rs17013236 | ACTTCGTCAGTAACGGACGGGAAAGCTACAACAAAATTAGAAGGG | GAGTCGAGGTCATATCGTGGGAAAGCTACAACAAAATTAGAAGGC | TAAAAATGTCGGATTACATTTATTTGCGAAACCACGTCATACTTGCACGTCTGCCTATAGTGAGTC |
| rs13388394 | ACTTCGTCAGTAACGGACGACAGGCTTGTTGGGCGTCGT | GAGTCGAGGTCATATCGTGACAGGCTTGTTGGGCGTCGC | GTAGGCTGCTGATAAACAAAAATACACGTTGCCCTTAGGTATCCGGTCTGCCTATAGTGAGTC |
| rs7592762 | ACTTCGTCAGTAACGGACTGCCCTCTCTTTATTCTCCCTCAA | GAGTCGAGGTCATATCGTTGCCCTCTCTTTATTCTCCCTCAG | CACTTACCTCCACTAATTTCCTTGCTGTATTCACCACCGTGGCTAGTCTGCCTATAGTGAGTC |
| rs4670191 | ACTTCGTCAGTAACGGACGTGGTGGAGATTTTTTGAGGTTGAGAC | GAGTCGAGGTCATATCGTGTGGTGGAGATTTTTTGAGGTTGAGAG | TAAGCAATAGAAGCAATATAGCAATCCGTTGCAGTAGGTGACATATCCGTCTGCCTATAGTGAGTC |
| rs10182807 | ACTTCGTCAGTAACGGACGTGGCTCTGAGAAAGAAAAGGGAAAC | GAGTCGAGGTCATATCGTGTGGCTCTGAGAAAGAAAAGGGAAAG | AAACTCATGTTATCACCACAGCGGCATTTCGTGTACCTACCACCGTCTGCCTATAGTGAGTC |
| rs17013337 | ACTTCGTCAGTAACGGACGTGCACTTGCCTTCCTGAGGTTCT | GAGTCGAGGTCATATCGTGTGCACTTGCCTTCCTGAGGTTCC | AAAATTCTCTTAGATTTGCTCAGACTCTGTGAGCGTTATCGACCCGTCTGCCTATAGTGAGTC |
| rs12470399 | ACTTCGTCAGTAACGGACGCAACCCACAAAGAAAACCAGAAGAA | GAGTCGAGGTCATATCGTGCAACCCACAAAGAAAACCAGAAGAG | GAGGCTGAAAGTGCTGAGATACAAACCGTGTAGGATTGGCCTCGTCTGCCTATAGTGAGTC |
| rs13415927 | ACTTCGTCAGTAACGGACGTGTTATATGAGATCCAGCCATGCAA | GAGTCGAGGTCATATCGTGTGTTATATGAGATCCAGCCATGCAG | AGGTTCCAATTTGTGTTTCAGTGGACGTGCATGATAAGCTCCCGTCTGCCTATAGTGAGTC |
| rs1801894 | ACTTCGTCAGTAACGGACGGAAAAATAGAGCTGGGACTGAGCCTT | GAGTCGAGGTCATATCGTGGAAAAATAGAGCTGGGACTGAGCCTG | GAATGACAGGAATGATCTATTACCATCAAATGCGGTCTCTAGTTACGGGTCTGCCTATAGTGAGTC |

**Supplementary Table 2.** Logistic analysis of 19 SNPs of the RasGRP3 gene in a Korean population

| rs | Genotype | Function | Co | | Do | | Re | |
|---|---|---|---|---|---|---|---|---|
| | | | OR (95%CI) | p | OR (95%CI) | p | OR (95%CI) | p |
| rs597593 | TT | Flanking_5UTR | 1.616 (0.822~3.178) | 0.164 | 1.509 (0.694~3.279) | 0.299 | 1.651 (0.731~3.729) | 0.228 |
| | TC | | | | | | | |
| | CC | | | | | | | |
| rs583942 | AA | Flanking_5UTR | 1.204 (0.746~1.946) | 0.491 | 1.305 (0.733~2.323) | 0.447 | 1.006 (0.279~3.618) | 0.366 |
| | AC | | | | | | | |
| | CC | | | | | | | |
| rs17012996 | AA | Intron | 4.231 (0.702~25.520) | 0.116 | 4.231 (0.702~25.520) | 0.116 | | |
| | AG | | | | | | | |
| rs7597095 | CC | Intron | 1.547 (1.012~2.365) | 0.044 | 1.790 (0.980~3.271) | 0.058 | 1.727 (0.759~3.933) | 0.193 |
| | CT | | | | | | | |
| | TT | | | | | | | |
| rs2305577 | TT | Intron | 0.746 (0.280~1.986) | 0.558 | 0.746 (0.280~1.986) | 0.558 | | |
| | TC | | | | | | | |
| | CC | | | | | | | |
| rs11680495 | TT | Coding | | | | | | |
| rs7565568 | AA | Intron | 0.867 (0.541~1.391) | 0.554 | 0.890 (0.499~1.587) | 0.693 | 0.646 (0.184~2.269) | 0.495 |
| | AT | | | | | | | |
| | TT | | | | | | | |
| rs2305573 | TT | Intron | 0.941 (0.588~1.504) | 0.799 | 1.004 (0.563~1.790) | 0.990 | 0.652 (0.185~2.299) | 0.506 |
| | TC | | | | | | | |
| | CC | | | | | | | |
| rs10189430 | GG | Intron | | | | | | |
| | GT | | | | | | | |
| rs7605981 | AA | Intron | | | | | | |
| | AC | | | | | | | |
| rs17013236 | CC | Intron | | | | | | |
| rs13388394 | AA | Coding | | | | | | |
| | AG | | | | | | | |
| rs7592762 | TT | Intron | 1.806 (1.171~2.786) | 0.007 | 1.972 (1.091~3.564) | 0.024 | 2.611 (1.086~6.274) | **0.032** |
| | TC | | | | | | | |
| | CC | | | | | | | |
| rs4670191 | GG | Intron | 0.759 (0.501~1.151) | 0.195 | 0.590 (0.325~1.070) | 0.082 | 0.923 (0.425~2.003) | 0.840 |
| | GC | | | | | | | |
| | CC | | | | | | | |
| rs10182807 | GG | Intron | | | | | | |
| rs17013337 | GG | Intron | 0.286 (0.034~2.427) | 0.252 | 0.286 (0.034~2.427) | 0.252 | | |
| | GA | | | | | | | |
| rs12470399 | AA | Intron | 0.846 (0.461~1.550) | 0.588 | 0.890 (0.449~1.765) | 0.739 | 0.412 (0.051~3.354) | 0.407 |
| | AG | | | | | | | |
| | GG | | | | | | | |
| rs13415927 | AA | Intron | 3.934 (1.746~8.864) | 0.001 | 3.784 (1.606~8.920) | 0.002 | | |
| | AG | | | | | | | |
| | GG | | | | | | | |
| rs1801894 | GG | 3UTR | | | | | | |

Logistic regression models were used to calculate odds ratios (*i.e.*, 95% confidence intervals) and corresponding p-values for each SNP. p-values obtained using the codominant, dominant,and recessive models are also shown. Age and sex were adjusted in the logistic analysis as covariates. Abbreviations: Freq., frequency; CLD, chronic liver disease; HCC, hepatocellular carcinoma; 95% CI, 95% confidence interval; LCL, lower confidence limit; UCL, upper confidence limit OR, odds ratio. Boldindicates $p < 0.05$.

**Supplementary Table 3.** Cox regression analysis for age of HCC occurrence as a function of each SNP of RasGRP3

| SNP | Region | Genotype | Frequency | Mean | SD | Model | HR | 95% CI | | p-val |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | LCL | UCL | |
| rs583942 | Flanking_5UTR | AA | 42 | 53.93 | 11.65 | Co-dominant | 1.077 | 0.763 | 1.52 | 0.674 |
| | | AC | 39 | 53.51 | 9.96 | Dominant | 1.134 | 0.739 | 1.739 | 0.565 |
| | | CC | 5 | 56.4 | 6.19 | Recessive | 0.943 | 0.38 | 2.34 | 0.900 |
| rs7597095 | Intron | CC | 31 | 55.74 | 9.7 | Co-dominant | 1.327 | 0.978 | 1.801 | 0.069 |
| | | CT | 42 | 53.52 | 10.48 | Dominant | 1.494 | 0.954 | 2.34 | 0.080 |
| | | TT | 13 | 50.62 | 12.72 | Recessive | 1.398 | 0.771 | 2.537 | 0.270 |
| rs7565568 | Intron | AA | 48 | 52.48 | 11.67 | Co-dominant | 0.917 | 0.634 | 1.325 | 0.644 |
| | | AT | 34 | 55.76 | 9.03 | Dominant | 0.889 | 0.58 | 1.364 | 0.590 |
| | | TT | 4 | 54.75 | 8.38 | Recessive | 0.995 | 0.363 | 2.726 | 0.992 |
| rs2305573 | Intron | TT | 48 | 52.48 | 11.67 | Co-dominant | 0.965 | 0.67 | 1.391 | 0.849 |
| | | TC | 34 | 55.76 | 9.03 | Dominant | 0.953 | 0.621 | 1.463 | 0.825 |
| | | CC | 4 | 54.75 | 8.38 | Recessive | 0.996 | 0.363 | 2.729 | 0.994 |
| rs7592762 | Intron | TT | 33 | 54.91 | 10.03 | Co-dominant | 1.527 | 1.092 | 2.136 | **0.013** |
| | | TC | 41 | 54.76 | 10.57 | Dominant | 1.445 | 0.929 | 2.249 | 0.103 |
| | | CC | 12 | 48.08 | 11.13 | Recessive | 2.383 | 1.28 | 4.437 | **0.006** |
| rs4670191 | Intron | GG | 35 | 49.89 | 10.87 | Co-dominant | 0.804 | 0.581 | 1.114 | 0.190 |
| | | GC | 37 | 57.38 | 9.38 | Dominant | 0.64 | 0.414 | 0.99 | **0.045** |
| | | CC | 14 | 54.64 | 10.07 | Recessive | 1.05 | 0.587 | 1.878 | 0.870 |
| rs12470399 | Intron | AA | 64 | 52.55 | 10.64 | Co-dominant | 0.708 | 0.462 | 1.085 | 0.113 |
| | | AG | 20 | 56.35 | 8.83 | Dominant | 0.711 | 0.429 | 1.177 | 0.184 |
| | | GG | 2 | 72 | 4.24 | Recessive | 0.383 | 0.092 | 1.598 | 0.188 |
| rs13415927 | Intron | AA | 69 | 54.3 | 10.08 | Co-dominant | 1.564 | 0.924 | 2.648 | 0.096 |
| | | AG | 16 | 53.25 | 12.23 | Dominant | 1.459 | 0.851 | 2.502 | 0.170 |
| | | GG | 1 | 35 | | Recessive | 60.667 | 6.771 | 543.573 | **0.0002** |

Cox regression analysis was to calculate hazard ratios, 95% confidence intervals and corresponding p-values for SNPs, while controlling for sex. Abbreviations: 95% CI, 95% confidence interval; HR, hazard ratios. Bold indicates p-values $< 0.05$.