Effect of RECK Gene Polymorphisms on Hepatocellular Carcinoma Susceptibility and Clinicopathologic Features

Tsung-Te Chung1,2,9, Chao-Bin Yeh3,4,9, Yi-Ching Li5, Shih-Chi Su6, Ming-Hsien Chien7,8, Shun-Fa Yang1, Yi-Hsien Hsieh9,10,11*

1 Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan, 2 Department of Otolaryngology, Show Chwan Memorial Hospital, Changhua, Taiwan, 3 Department of Emergency Medicine, School of Medicine, Chung Shan Medical University, Taichung, Taiwan, 4 Department of Emergency Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan, 5 Department of Pharmacology, Chung Shan Medical University, Taichung, Taiwan, 6 Department of Urology, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States of America, 7 Taipei Medical University-Wan Fang Hospital, Taipei, Taiwan, 8 Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, 9 Department of Biochemistry, School of Medicine, Chung Shan Medical University Hospital, Taichung, Taiwan, 10 Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, 11 Clinical Laboratory, Chung Shan Medical University Hospital, Taichung, Taiwan

Abstract

Background: The reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) down-regulation has been confirmed in numerous human cancers and is clinically associated with metastasis. This study investigates the potential associations of RECK single-nucleotide polymorphisms (SNPs) with hepatocellular carcinoma (HCC) susceptibility and clinical-pathologic characteristics.

Methodology/Principal Findings: A total of 135 HCC cancer patients and 501 cancer-free controls were analyzed for four RECK SNPs (rs10814325, rs16932912, rs11788747, and rs10972727) using real-time PCR and PCR-RFLP genotyping analysis. After adjusting for other co-variants, the individuals carrying RECK promoter rs10814325 inheriting at least one C allele had a 1.85-fold (95% confidence interval (CI), 1.03–3.36) risk of developing HCC compared to TT wild type carriers. The HCC patients, who carried rs11788747 with at least one G allele, had a higher distant metastasis risk than wild type probands.

Conclusions: RECK gene polymorphisms might be a risk factor increasing HCC susceptibility and distant metastasis in Taiwan.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide, with 750,000 newly diagnosed cases in 2008. HCC is the third most common cause of cancer mortality [1]. Taiwan has the third highest incidence area in the world, with a 33.7/105 age-standardized rate in 2008. HCC was the leading cause of cancer death among men, with a mortality of 39.3/105, and the second among women, with mortality of 14.7/105 in 2008 [2]. The development of HCC is a multistep and complex process. Multiple environmental risk factors, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, cirrhosis, carcinogen exposure, and a variety of genetic factors contribute to hepatocarcinogenesis [3,4]. Cumulative studies have suggested the associations between HCC cancer risk and SNPs in selected candidate genes. For example, insulin-like growth factor (IGF)-2, IGF-2R, plasminogen activator inhibitor (PAI)-1, and matrix metalloproteinase (MMP) 14 [5–7] are HCC risk factors.

The reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) gene is a novel transformation suppressor gene against activated ras oncogenes, and induces flat reversion in v-K-ras-transformed NIH/3T3 cells [8,9]. The RECK gene encodes a membrane-anchored glycoprotein that can negatively regulate matrix metalloproteinases (MMPs) and inhibit tumor invasion, angiogenesis, and metastasis. RECK-expressing tumors show a significant reduction in microvessel density and branching, and result in tumor tissue death in mice [8,10]. RECK down-regulation or promoter hypermethylation have been confirmed in many human cancers, including pancreatic cancer, breast cancer, lung cancer, colorectal cancer, cholangiocarcinoma, gastric cancer, prostate cancer, oral cancer, esophageal cancer, and osteosarcoma, and correlated with tumor metastasis or poor prognosis [11–23].

A previous study showed high RECK mRNA expression in HCC tumor tissues from patients with better survival and less invasive clinicopathologic features [24]. However, to our knowledge, no study has investigated the role of RECK gene variants in HCC development and clinical factors. This study performs a case-control study for four SNPs located in the promoter or exon regions of the RECK gene (Fig. 1) to analyze the associations
between RECK gene SNPs and HCC susceptibility and clinicopathologic characteristics

Materials and Methods

Study subjects and specimen collection

The present hospital based case-control study recruited 135 HCC patients between 2007 to 2010 in Chung Shan Medical University Hospital, Taiwan. The diagnosis of HCC was according to the characteristic criteria of the national guidelines for HCC, such as liver tumor tissue diagnosed by histology or cytology irrespective of α-fetoprotein (AFP) titer where imaging data, either computed tomography or magnetic resonance imaging, showed one of following three cases: (1) One or more liver masses ≥2 cm in diameter; (2) One imaging data with early enhancement and a high level of AFP ≥400 ng/mL; (3) One imaging data with early arterial phase contrast enhancement plus early venous phase contrast washout. Meanwhile, 501 race- and ethnic group-matched non-cancer evidence community residents who entered the hospital for health check-ups were enrolled as the control group.

HCC patients were staged clinically at the time of diagnosis according to the TNM staging system of the American Joint Committee on Cancer (AJCC) (2002) [25]. Liver cirrhosis was diagnosed with liver biopsy, abdominal sonography, or biochemical evidence of liver parenchymal damage with endoscopic esophageal or gastric varices. The patients’ clinicopathological characteristics, such as clinical staging, tumor size, lymph node metastasis, distant metastasis, hepatitis B surface antigen (HBsAg), antibody to HCV (anti-HCV), liver cirrhosis, AFP, aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were verified by chart review. The whole blood specimens collected from healthy controls and HCC patients, were placed in tubes containing ethylenediaminetetraacetic acid (EDTA) and immediately centrifuged and stored at 20°C. Before the conduction of this study, approval from the Institutional Review Board of Chung Shan Medical University Hospital, Taiwan. The diagnosis of HCC was HCC patients between 2007 to 2010 in Chung Shan Medical University Hospital, Taiwan.

Polymerase chain reaction-restriction fragment length polymorphism

The RECK rs16932912, rs11788747 and rs10972727 gene polymorphisms were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay as previously described [21]. The primer sequences and restriction enzyme for analysis of the RECK gene polymorphisms are described in Table 1. The PCR was performed in a 10 μL volume containing 100 ng DNA template, 1.0 μL of 10× PCR buffer (Invitrogen, Carlsbad, CA), 0.25 μL of Taq DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 mM dNTPs (Promega, Madison, WI), and 200 nM of each primer (MDBio Inc, Taipei, Taiwan). The PCR cycling conditions were 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, with a final step at 72°C for 20 min.

Table 1. Primer sequences and PCR-RFLP conditions for amplification of RECK SNPs.

| SNP | Sequences | Product | Enzyme |
|-----|-----------|---------|--------|
| rs16932912 | 5’-TGGAGATGGTGTAGTGCTG-3’ | 103 bp | Tfl |
| rs11788747 | 5’-CGGTACACCAATGCTCAATAC-3’ | 242 bp | RsaI |
| rs10972727 | 5’-ATCTCATCCGGAAGATAACC-3’ | 140 bp | HpyCIV |

Real-time PCR

The allelic discrimination of the RECK rs10814325 gene polymorphisms was assessed with the ABI StepOne™ Real-Time PCR System (Applied Biosystems) and analyzed using SDS v3.0 software (Applied Biosystems), using the TaqMan assay (assay IDs: C_27084758_10). The final volume for each reaction was 5 μL, containing 2.5 μL TaqMan Genotyping Master Mix, 0.125 μL DNA sequence analysis.

Figure 1. The location of human RECK gene single-nucleotide polymorphisms (SNPs) and functional amino acid.
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Genomic DNA extraction

Genomic DNA was extracted by QIAamp DNA blood mini kits (Qiagen, Valencia, CA) according to the instructions of manufacturer. DNA was dissolved in TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA] and then quantified by a measurement of OD260. Final preparation was stored at −20°C and used as templates for polymerase chain reaction (PCR).
**Results**

This study analyzes the demographic characteristics of sample specimens and found that 41.9% (210 of 501) and 34.8% (47 of 135) of healthy control subjects and patients with HCC, respectively, had consumed alcohol; 41.1% (206 of 501) and 40.7% (55 of 135) of healthy controls and patients with HCC, respectively, had smoked. The distributional differences of alcohol (p = .136) and tobacco consumption (p = .937) between healthy controls and patients with HCC were insignificant, whereas age distribution (control: 52.9 ± 14.48; HCC: 64.24 ± 11.08) (p < .001) and gender distribution (p < .001) between the 2 subgroups were significantly different (Table 2). To reduce the possible interference of the confounding variables, we used adjusted odds ratios (AORs) with 95% confidence intervals (CIs) were estimated by multiple logistic regression models after controlling for age and gender in each comparison. Table 3 shows the genotype distributions and the association between HCC and gene polymorphisms of RECK. The alleles with the highest distribution frequency for rs10814325, rs16932912, rs11788747, and rs10972727 genes of RECK in both recruited HCC patients and healthy controls were heterozygous T/C, homozygous G/G, homozygous A/A, and homozygous T/T, respectively. After adjusting variables, people with rs10814325 TC, CC, TC+CC showed a 2.112-fold (95% CI: 1.108–4.020); 2.104-fold (95% CI: 1.211–3.939), or 1.85-fold (95% CI: 1.027–3.361) higher risk of HCC compared to wild type individuals. People with rs16932912, rs11788747, and rs10972727 genes of RECK polymorphism showed no higher risk of HCC compared to wild type individuals.

The distribution frequency of clinical statuses and RECK genotypes frequencies in HCC patients were estimated to clarify the role of RECK gene polymorphisms in the clinicopathological states of HCC patients, including TNM clinical staging, primary tumor size, lymph node involvement, distant metastasis, hepatitis B surface antigen (HBsAg), antibody to HCV (anti-HCV), and liver cirrhosis. There was no observed significant association between the rs10814325, rs16932912, and rs10972727 gene polymorphisms and clinicopathologic states. However, among HCC patients, those who had rs11788747 SNPs also had a higher risk of distant metastasis (p < .003) than wild type patients (Table 4), but no difference existed in HBsAg, anti-HCV, Child-Pugh grade or liver cirrhosis (Table 5).

AFP, AST, and ALT are common clinical pathological markers of HCC. To clarify the relationship between the progress of clinical status and the level of clinical pathological markers in HCC patients, this study also analyzes the levels of these pathological markers associated with RECK genotypic frequencies. Table 6 shows that the levels of ALT were significantly different between the rs11788747 AA and AG/GG genotypes (p < .05).

**Discussion**

This study provides novel information on the effects of single-nucleotide polymorphisms of RECK on HCC susceptibility and clinicopathologic status association. The genomic structure for the RECK gene has been identified on the chromosome region 9p13–p12. The RECK gene includes 21 exons and 20 introns, and 13 SNPs were identified [26]. Among 13 SNPs, rs16932912, rs11788747, and rs10972727 were found within the coding sequence in exons 9, 13, and 15, respectively [26]. These polymorphisms are non-synonymous and changed amino acid sequence as well as RECK protein structure. For example, Valine changes to Lysine in rs16932912 SNP. Rs10814325 SNP was located at promoter sites and was predicted to change transcription factor binding sites by TRANSFAC [12]. Although the functional importance of rs10814325 SNPs has not been tested experimentally, clinical research from Germany shows that breast cancer patients carrying rs10814325 -402 T/C SNP have a greater chance of survival than those carrying T/T wild type [12]. In the present study, individuals carrying the RECK promoter rs10814325 inheriting at least one C allele had a 1.85-fold higher risk of HCC compared to TT wild type carriers. However, no significant association was present between the rs10814325 gene polymorphisms and clinicopathologic states. The data are apparently contradictory in relation to carcinogenesis and probably survival. However, the potential mechanism and functional consequences of the promoter sequence change should be elucidated in the laboratory and clinically as well. Meanwhile, in this study, the frequencies of rs10814325 T/T, T/C and C/C were 28.5%, 44.9% and 26.5%, respectively. However, in

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**Table 2. The distributions of demographical characteristics in 501 healthy controls and 135 patients with HCC.**

| Variable              | Controls (N = 501) | Patients (N = 135) | p value |
|-----------------------|-------------------|--------------------|---------|
| **Age (yrs)**         | Mean ± S.D.       | Mean ± S.D.        |         |
|                       | 52.9 ± 14.48      | 64.24 ± 11.08      | < .001* |
| **Gender**            | n (%)             | n (%)              |         |
| Male                  | 415 (82.8%)       | 92 (68.1%)         |         |
| Female                | 86 (17.2%)        | 43 (31.9%)         | < .001* |
| **Alcohol consumption**|                   |                    |         |
| No                    | 291 (58.1%)       | 88 (65.2%)         |         |
| Yes                   | 210 (41.9%)       | 47 (34.8%)         | p = 0.136 |
| **Tobacco consumption**|                   |                    |         |
| No                    | 295 (58.9%)       | 80 (59.3%)         |         |
| Yes                   | 206 (41.1%)       | 55 (40.7%)         | p = 0.937 |

Mann-Whitney U test or Fisher’s exact test was used between healthy controls and patients with HCC.

* p value < .05 as statistically significant. doi:10.1371/journal.pone.0033517.t002
Sweden, the genotypes distribution of RECK rs10814325 T/T, T/C and C/C were 83.7%, 15.8% and 0.5%, respectively [12]. Obviously, there is an ethnicity-related variation in the frequency of rs10814325 polymorphisms. The contradictory findings of previous research may be the result of the different ethnic backgrounds of patients.

This study shows that individuals carrying the RECK promoter rs10814325 polymorphism have a 1.85-fold risk of developing HCC associated with Table 3.

### Table 3. Adj usted odds ratio (AOR) and 95% confidence interval (CI) of HCC associated with RECK genotypic frequencies.

| Variable     | Controls (N = 501) n (%) | Patients (N = 135) n (%) | OR (95% CI) | AOR (95% CI) |
|--------------|--------------------------|--------------------------|-------------|--------------|
| rs10814325   |                          |                          |             |              |
| TT           | 143 (28.5%)              | 21 (15.6%)               | 1.00        | 1.00         |
| TC           | 225 (44.9%)              | 72 (53.3%)               | **2.179 (1.283–3.700)** | **2.11 (1.11–4.03)** |
| CC           | 133 (26.5%)              | 42 (31.1%)               | **2.150 (1.211–3.820)** | **2.18 (1.21–3.94)** |
| TC+CC        | 358 (71.5%)              | 114 (84.4%)              | **2.168 (1.310–3.590)** | **1.85 (1.03–3.36)** |

| rs16932912   |                          |                          |             |              |
| GG           | 272 (54.3%)              | 64 (47.4%)               | 1.00        | 1.00         |
| GA           | 192 (38.3%)              | 57 (42.2%)               | 1.262 (0.844–1.886) | 1.41 (0.88–2.27) |
| AA           | 37 (7.4%)                | 14 (10.4%)               | 1.608 (0.821–3.151) | 1.81 (0.75–4.37) |
| GA+AA        | 229 (45.7%)              | 71 (52.6%)               | 1.318 (0.900–2.018) | 1.32 (0.94–2.33) |

| rs11788747   |                          |                          |             |              |
| AA           | 281 (56.1%)              | 83 (61.5%)               | 1.00        | 1.00         |
| AG           | 192 (38.3%)              | 41 (30.4%)               | 0.723 (0.477–1.097) | 0.66 (0.40–1.07) |
| GG           | 28 (5.6%)                | 11 (8.1%)                | 1.330 (0.635–2.785) | 1.27 (0.51–3.16) |
| AG+GG        | 220 (45.7%)              | 52 (38.5%)               | 0.800 (0.542–1.181) | 0.74 (0.47–1.17) |

| rs10972727   |                          |                          |             |              |
| TT           | 279 (55.7%)              | 76 (56.3%)               | 1.00        | 1.00         |
| TA           | 191 (38.1%)              | 54 (40.0%)               | 1.038 (0.700–1.540) | 1.03 (0.64–1.64) |
| AA           | 31 (6.2%)                | 5 (3.7%)                 | 0.723 (0.477–1.097) | 0.66 (0.40–1.07) |
| TA+AA        | 222 (44.3%)              | 59 (43.7%)               | 0.976 (0.665–1.431) | 0.96 (0.61–1.51) |

The odds ratios (ORs) with their 95% confidence intervals were estimated by logistic regression models. The adjusted odds ratios (AORs) with their 95% confidence intervals were estimated by multiple logistic regression models after controlling for age, gender, alcohol and tobacco consumption.

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Sweden, the genotypes distribution of RECK rs10814325 T/T, T/C and C/C were 83.7%, 15.8% and 0.5%, respectively [12]. Obviously, there is an ethnicity-related variation in the frequency of rs10814325 polymorphisms. The contradictory findings of previous research may be the result of the different ethnic backgrounds of patients.

This study shows that individuals carrying the RECK promoter rs10014325 polymorphism have a 1.85-fold risk of developing HCC associated with Table 3.

### Table 4. Clinical TNM staging and RECK genotypic frequencies in 135 HCC patients.

| Variable     | rs10814325 | rs16932912 | rs11788747 | rs10972727 |
|--------------|------------|------------|------------|------------|
|              | TT (N = 21) | TC+CC (N = 144) | p value | GG (N = 64) | GA+AA (N = 71) | p value | AA (N = 83) | AG+GG (N = 52) | p value |
|              | n (%)      | n (%)      | p value    | n (%)      | n (%)      | p value    | n (%)      | n (%)      | p value    |
| Clinical stage |            |            |            |            |            |            |            |            |            |
| Stage I/II   | 12 (57.1%) | 72 (63.2%) | 0.601      | 38 (59.4%) | 46 (64.8%) | 0.517      | 54 (65.1%) | 30 (57.7%) | 0.390      |
| Stage III/IV | 9 (49.2%)  | 42 (36.8%) |            | 26 (40.6%) | 25 (35.2%) |            | 29 (34.9%) | 22 (42.3%) |            |
| Tumor size   |            |            |            |            |            |            |            |            |            |
| ≤T2          | 13 (61.9%) | 72 (64.9%) | 0.791      | 41 (64.1%) | 46 (64.8%) | 0.930      | 55 (66.3%) | 32 (61.5%) | 0.577      |
| > T2         | 8 (38.1%)  | 42 (35.1%) |            | 23 (35.9%) | 25 (35.2%) |            | 28 (33.7%) | 20 (38.5%) |            |
| Lymph node metastasis | No | Yes | No | Yes | No | Yes | No | Yes | No | Yes |
| No           | 19 (90.5%) | 2 (9.5%)   | 60 (93.8%) | 4 (4.4%) | 68 (95.8%) | 3 (4.2%) | 79 (95.2%) | 4 (4.8%) | 72 (94.7%) | 2 (5.3%) |
| Yes          | 19 (90.5%) | 2 (9.5%)   | 110 (96.5%)| 4 (3.5%) | 59 (92.2%) | 5 (7.8%) | 70 (98.6%) | 1 (1.4%) | 46 (88.5%) | 6 (11.5%) |

| Distant metastasis | No | Yes | No | Yes | No | Yes |
|--------------------|----|-----|----|-----|----|-----|
| No                 | 19 (90.5%) | 2 (9.5%) | 109 (95.6%) | 110 (96.5%) | 59 (92.2%) | 5 (7.8%) |
| Yes                | 2 (9.5%) | 4 (3.5%) | 70 (98.6%) | 6 (11.5%) | 7 (100%) | 6 (11.5%) |

* p value < 0.05.

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HCC compared to wild type carriers. In an animal model, the administration of black-tea polyphenon-B significantly reduces the incidence of dimethylaminooazobenzene (DAB)-induced hepatomas as evidenced by modulation of RECK, matrix metalloproteinase (MMP)-2, MMP-9, and the tissue inhibitor of matrix metalloproteinase-2 [27]. The alternation of RECK promoter methylation and RECK expression is likely the result of activating the extracellular signal-regulated kinase (ERK) pathway [28,29]. In a human model, up-regulation of the mitogen-activated protein kinase (MAPK) pathway and genes is associated with an activated cell cycle and may be of critical importance to the formation and maintenance of hepatocellular carcinoma [30,31]. These findings might be the molecular hepatocarcinogenesis of RECK, but the mechanism should be elucidated in laboratory and clinical settings.

This study shows that HCC patients carrying rs11788747 polymorphisms had a higher risk of distant metastasis (p = .003) than wild type carriers. Previous research confirms the relationships of RECK expression and gene status with tumor metastasis. Using Matrigel invasion chamber assay in vitro and in experimental and spontaneous metastasis assays in vivo, the experiments in this study artificially restored RECK expression in tumor cells (such as HT1080 fibrosarcoma or B16 melanoma), in which endogenous RECK was undetectable. This approach greatly suppresses their invasive and metastatic potentials [8]. Low RECK expression colorectal cancer and esophageal cancer patients exhibited more lymph node metastasis [14,32]. In non-small cell lung cancer patients, RECK promoter methylation has a higher incidence of lymph node metastasis [33]. In betel quid-chewing oral cancer patients, those who have the RECK polymorphisms with risk of HCC. Results showed RECK promoter SNP polymorphisms with risk of HCC. Further collection of samples should investigate the RECK sequence variants and their biological function in Taiwanese HCC patients.

Author Contributions
Conceived and designed the experiments: TC SY. Performed the experiments: MC YH SY. Analyzed the data: SS CY. Contributed reagents/materials/analysis tools: TC YL SY. Wrote the paper: YH SY.

**Table 5.** Clinical status and RECK genotypic frequencies in 135 HCC patients.

| Variable       | rs10814325 (N = 21) | rs16932912 (N = 144) | rs11788747 (N = 64) | rs10972727 (N = 71) |
|----------------|---------------------|----------------------|---------------------|---------------------|
|                | TT                  | TC/CC                | GG                  | GA/AA               |
|                | (p value)           | (p value)            | (p value)           | (p value)           |
| RECK           |                     |                      |                     |                     |
| Negative       | 10 (47.6%)          | 71 (62.3%)           | 37 (57.8%)          | 44 (62.0%)          |
|                | 0.208               | 0.280                | 0.622               | 0.665               |
| Positive       | 11 (52.4%)          | 43 (37.7%)           | 27 (42.2%)          | 27 (38.0%)          |
|                | 0.677               | 0.199                | 0.130               | 0.142               |
| Child-Pugh grade |                   |                      |                     |                     |
| A              | 5 (71.4%)           | 84 (73.7%)           | 49 (76.6%)          | 50 (70.4%)          |
|                | 0.830               | 0.421                | 0.957               | 0.873               |
| B or C         | 6 (28.6%)           | 15 (23.4%)           | 21 (29.6%)          | 22 (26.5%)          |
|                | 0.100               | 0.083                | 0.041               | 0.154               |
| Liver cirrhosis |                   |                      |                     |                     |
| Negative       | 5 (23.8%)           | 20 (29.2%)           | 17 (26.6%)          | 14 (26.9%)          |
|                | 0.010               | 0.044                | 0.055               | 0.060               |
| Positive       | 16 (76.2%)          | 84 (73.7%)           | 47 (73.4%)          | 60 (72.3%)          |
|                | 0.130               | 0.060                | 0.055               | 0.040               |

**Table 6.** Association of RECK genotypic frequencies with HCC laboratory status.

| Characteristic | rs10814325 (TT) (N = 21) | rs16932912 (TC/CC) (N = 144) | rs11788747 (GG) (N = 64) | rs10972727 (GA/AA) (N = 71) |
|----------------|---------------------------|-------------------------------|--------------------------|-----------------------------|
| a-Fetoprotein* | 165.8 ± 50.6              | 191.4 ± 70.3                 | 128.5 ± 24.5             | 134.0 ± 0.16                |
| ALT*          | 0.289                     | 0.880                        | 0.980                     | 0.465                       |
| AST            | 3999.3 ± 1542              | 178.7 ± 33.6                 | 144.1 ± 34.4             | 153.0 ± 0.11                |
| p value        | 0.130                      | 0.060                        | 0.055                     | 0.125                       |
| rs16932912     |                           |                               |                          |                             |
| G              | 3582.8 ± 2169.5            | 155.4 ± 30.7                 | 136.1 ± 23.0             | 135.0 ± 0.10                |
| p value        | 0.897                      | 0.431                        | 0.777                     | 0.125                       |
| rs11788747     |                           |                               |                          |                             |
| A              | 3261.3 ± 1376.1            | 220.9 ± 46.7                 | 179.4 ± 35.2             | 147.0 ± 0.10                |
| p value        | 0.992                      | 0.093                        | 0.040*                    | 0.659                       |
| rs10972727     |                           |                               |                          |                             |
| T              | 3317.6 ± 1491.4            | 225.2 ± 50.7                 | 182.1 ± 42.5             | 151.0 ± 0.12                |
| p value        | 0.941                      | 0.095                        | 0.052                     | 0.919                       |

Mann-Whitney U test was used between two groups.

*p value < 0.05.

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