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An insertion/deletion polymorphism in the 3′ untranslated region of β-transducin repeat-containing protein (*βTrCP*) is associated with susceptibility for hepatocellular carcinoma in Chinese

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ABSTRACT

Hepatocellular carcinoma (HCC) is an epithelial cancer which originates from hepatocytes or their progenitors. As a positive regulator of NFκB signaling pathway, β-transducin repeat-containing protein (*βTrCP*) is overexpressed and oncogenic in epithelial cancers, suggesting a potential role of βTrCP in HCC susceptibility. We carried out a case-control study in a Chinese population (256 cases and 367 controls) to estimate the susceptibility to HCC associated with a 9 bp insertion/deletion polymorphism (rs16405) in 3′ untranslated region of *βTrCP*. Using unconditional logistic regression, we found that 9N del/del and 9N ins/del genotypes were significantly associated with decreased HCC risk: OR = 0.44 (0.24–0.83) ($p = 0.004$) and OR = 0.56 (0.31–1.00) ($p = 0.034$), respectively. Furthermore, *in vivo* experiments showed that mRNA levels of βTrCP from HCC tumor tissues were correlated with rs16405 genotypes. HCC tumor tissues with homozygous for 9N ins/ins has the highest level of βTrCP, which are 3.99 and 7.04-fold higher than heterozygous 9N ins/del and homozygous 9N del/del, respectively. Based on bioinformatics prediction, we found that the risk allele for rs16405 disrupted a binding site for human microRNA-920 which would negatively regulate βTrCP. We propose a microRNA-920 mediated βTrCP regulation model depending on rs16405 genotype, which warrants further replication association studies and follow-up functional experiments.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with the highest incidence in Southeast Asia and sub-Saharan Africa [1]. Apart from genetic factors, the most prominent etiological factors associated with HCC are chronic viral hepatitis B and C infections (HBV and HCV), exposure to environmental chemicals or alcohol, and metabolic liver diseases [2]. Molecular biology of carcinogenesis and tumor progression of HCC has been increasingly understood with intense research in recent years. However, the molecular and cellular mechanisms of HCC pathogenesis are still poorly understood. As in many cancers, variants of the genes involved in multistage of hepatocarcinogenesis may determine individual's susceptibility to the development of HCC [3,4]. Identification of susceptibility genes related to HCC is important as it may help to predict individual and population risk and clarify pathophysiologic mechanisms relevant to HCC [5].

β-Transducin repeat-containing protein (*βTrCP*) gene encodes a member of the F-box protein family, which is characterized by an approximately 40 amino acid motif, the F-box. The F-box proteins constitute one of the four subunits of ubiquitin protein ligase complex called SCFs (SKP1-cullin-F-box), which function in phosphorylation-dependent ubiquitination [6]. The maintenance and preservation of distinct phases during the cell cycle is a highly complex and coordinated process. βTrCP has emerged as a key player in the S and G2 DNA-damage response checkpoint, the main function of which is to mediate cell cycle arrest to allow time to repair DNA lesions [7]. Therefore, given the crucial function of the cell cycle machinery in regulating cell cycle progression, the altered proteolysis of cell cycle regulators is clearly a contributing factor in the unrestrained proliferation that is typical in cancer cells. There is accumulating evidence that βTrCP possesses mainly oncogenic characteristics, and overexpression of βTrCP has been reported in multiple cancers [8,9]. Specifically, elevated expressed βTrCP level has been observed in hepatoblastoma, which is the most frequent malignant type of pediatric liver tumors [10]. Thus far, the genetic contribution of βTrCP to HCC susceptibility has not yet to be investigated. Here, we select a 9 bp (AACAGTGGA)

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insertion/deletion polymorphism (rs16405) in the 3′UTR of *βTrCP* for a case-control study. The aim of present study is to investigate whether a particular allele or genotype of rs16405 would modify the occurrence of HCC in a Chinese population and the potential pathogenesis mechanisms of HCC mediated by the insertion/deletion polymorphism.

Materials and methods

Study populations. The case-control study was performed on genomic DNA extracted from peripheral blood of newly diagnosed incident HCC cases together with controls after obtaining informed consent. All subjects recruited were unrelated ethnic Han Chinese. The case series comprised 256 HCC patients diagnosed, hospitalized and treated in the affiliated hospitals of Soochow University from 2003 to 2006. All the patients had not been given any medical treatments. Patients were excluded who were suffering from: (a) autoimmune hepatitis or toxic hepatitis; (b) primary or secondary biliary cirrhosis or Budd–Chiari syndrome; (c) other tumors except HCC; (d) recurrence of HCC and (e) liver disease due to parasitosis, diabetes, fatty liver, metabolism disorders and severe cardiovascular diseases. The diagnosis of these patients was confirmed by a pathological examination combined with positive imaging (magnetic resonance imaging and/or computerized tomography). Tumor stages were determined according to a modified American Joint Committee on Cancer (AJCC) and international union against cancer (UICC) standard. Three hundred and sixty-seven controls were cancer-free individuals selected from a community nutritional survey which was conducted in the same region during the same period as recruitment of cancer patients. Controls without clinical evidence of liver disease were matched for age and sex to each set of HCC individuals. Each subject was personally face-to-face interviewed by trained interviewers, with a pretested questionnaire to obtain information on demographic data and related risk factors, including tobacco smoking and alcohol drinking. The subjects who smoked more than one cigarette per day for more than one year were classified as smokers. Others were defined as non-smokers. Subjects were considered as alcohol drinkers, if they drank at least once per week. All participants were negative for antibodies to hepatitis C virus, hepatitis D virus or HIV. Liver tissue samples from patients with a diagnosis of HCC were collected from the affiliated hospital of Soochow University on the availability of frozen stored tissue from HCC resections from 2006 through 2008. All cases had histological confirmation of their tumor diagnosis. The design of the study was approved by the Ethical Committee of Soochow University.

DNA extraction and genotyping. A Chelex method was used for extracting genomic DNA of blood samples [11]. DNA fragments containing the polymorphism were amplified with the forward primer 5′-CCAGATCAGCCAGAAAATGCAA-3′ and reverse primer 5′-CATTGATGGAGCCCAGGAAACT-3′. PCR was performed in a total volume of 37.5 μL, including 3.75 μL 10× PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 mM each primer,100 ng of genomic DNA, and 1.5 U of Taq DNA polymerase. The PCR conditions were 94 °C for 5 min, followed by 35 cycles of 40 s at 94 °C, 40 s at 60 °C, and 40 s at 72 °C, with a final elongation at 72 °C for 5 min. The PCR products were analyzed by 7% non-denaturing polyacrylamide gel electrophoresis and visualized by silver staining [12]. The genotypes were determined by the numbers and positions of the band on the gels. The 9 bp deletion allele yielded a 221 bp band and the insertion allele yielded a 230 bp band. To validate the genotyping method, we analyzed 20 randomly selected DNA samples by both direct sequencing and PCR method; the concurrence rate of these two methods was 100%, suggesting that the PCR method was reliable. Genotyping was performed without knowledge of the case or control status. A 10% random sample was tested in duplicate by different persons, and the reproducibility was 100%.

Real-time RT-PCR analysis of mRNA levels of βTrCP in different genotypic HCC tumor tissues. Total RNA was isolated from tumor tissue specimens with different genotypes using RNA isolation kit of Ambion and then converted to cDNA using an oligo$(dT)_{15}$ primer and Superscript II (Invitrogen). A SYBR® Green gene expression assay was performed using ABI 7500 to quantify relative βTrCP expression in these samples. Beta-actin was chosen as the internal control. Primer sequences used for βTrCP and beta-actin were designed using Primer Express software (version 2.0, Applied Biosystems) and purchased from Invitrogen. The primer sequences were as follows: βTrCP-F: 5′-TGTGGCCAAAACAAAACTTGCC-3′, βTrCP-R: 5′-ATCTGACTCTGACCACTGCTC-3′, beta-actin-F: 5′-CATGTACGTTG CTATCCAGGC-3′, beta-actin-R: 5′-CTCCTTAATGTCACGCACGAT-3′. The 25 μl total volume final reaction mixture consisted of 1 μM of each primer, 12.5 μl of Master Mix (Applied Biosystems, Foster City, CA, USA), and 2.5 μl of cDNA. Negative controls consisted of distilled H_2O. PCR was performed using the following conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. PCR efficiencies were calculated with relative standard curves derived from a cDNA mixture (a 10-fold dilution series with five measuring points in triplicates) and gave regression coefficients >0.98. A melting curve analysis was performed for the PCR products of βTrCP and beta-actin to evaluate primer specificity. The expression levels of βTrCP were normalized with beta-actin with an arbitrary unit.

Bioinformatics prediction of microRNA-binding. The human *βTrCP* 3′UTR containing rs16405 was identified according to the UCSC genome browser (http://genome.ucsc.edu). The mature human microRNA sequences were obtained from the microRNA database, miRBase (http://microrna.sanger.ac.uk).

The hybridization of putative microRNA and the *βTrCP* 3′UTR harboring either the 9 bp insertion or deletion allele were predicted by miRanda (java edition) with default parameters [13].

Statistical analysis. The genotype distribution was analyzed for Hardy–Weinberg equilibrium using chi-square test. Unconditional logistic regression was used to analyze the association between rs16405 and cancer risk, adjusted for sex, age, smoking status, drinking status and HBV infection. The normalized expression values of βTrCP were analyzed using one way ANOVA. These statistical analyses were implemented in Statistic Analysis System software (version 8.0, SAS Institute). Probability values of 0.05 or less were used as the criterion of statistical significance, and all statistical tests were two sided.

Results

Association of HCC with rs16405 polymorphism

The demographic characteristics of the 256 HCC patients and 367 controls included in the analysis were summarized in Table 1. There were no statistically significant differences between cases and controls in terms of the frequency distribution of sex, age, smoking and drinking status. As expected, HBV infection was a significant risk factor for HCC. About 70.3% of the cases were HBsAg positive, which were significantly higher than that of the controls (9.8%, $P < 0.0001$). Genotype distributions had no deviation from Hardy–Weinberg equilibrium in both case and control groups. Our results showed that rs16405 was significantly associated with HCC, at both the allele and genotype levels (Table 2). After adjustment for sex, age, smoking status, drinking status and HBV infection, we found that the heterozygote 9N ins/del and homozygote 9N del/del of rs16405 was associated with a significantly reduced