Biochemical and Biophysical Research Communications 391 (2010) 552–556

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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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ARTICLE INFO

Article history:
Received 14 November 2009
Available online 24 November 2009

Keywords:
Hepatocellular carcinoma
βTrCP
Insertion/deletion polymorphism
MicroRNA-920

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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doi:10.1016/j.bbrc.2009.11.096

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

Table 1
Demographic characteristics among HCC cases and controls.

Characteristics	Case		Control		*P*-value
	n (=256)	Frequencies (%)	*n* (=367)	Frequencies (%)	
Age (mean ± SD)	52.0 ± 10.5		51.7 ± 11.2		0.74[a]
Gender					
Male	167	65.2	249	67.8	0.49[b]
Female	89	34.8	118	32.2	
Smoking Status					
Smokers	85	33.2	108	29.4	0.31[b]
Non-smokers	171	66.8	259	70.6	
Drinking status					
Drinker	114	44.5	154	42.0	0.52[b]
Nondrinker	142	55.5	213	58.0	
Tumor stages					
Ia + Ib	191	74.6			
IIa + IIb	57	22.3			
IIIa + IIIb	8	3.1			
HBsAg, N (%)					
Positive	180	70.3	36	9.8	**<0.0001**[b]
Negative	76	29.7	331	90.2	

Bold *P* values indicate statistically significant.
[a] Two-sided two-sample *t*-test between cases and controls.
[b] Chi-square test for differences between cases and controls.

Table 2
Genotype and allele frequencies of rs16405 among cases and controls, and risk of HCC.

Genotype/Allele	Cases, *n* (%)		Control, *n* (%)		OR[a] (95% C.I.)	*P*-value
9N ins/ins	38	14.8	30	8.2	1.00 (Reference)	
9N ins/del	118	46.1	164	44.7	0.56 (0.31–1.00)	0.034
9N del/del	100	39.1	173	47.1	0.44 (0.24–0.83)	0.004
P_{trend}						0.005
9N ins/del + 9N del/del	218	85.2	337	91.8	0.49 (0.29–0.85)	0.008
9N ins	194	37.9	224	30.5	1.00 (Reference)	
9N del	318	62.1	510	69.5	0.72 (0.56–0.92)	0.007

[a] Adjusted for sex, age, smoking status, drinking status and HBV infection.

risk of HCC compared with its homozygote 9N ins/ins (OR 0.56, [95% C.I. 0.31–1.00] and OR 0.44, [95% C.I. 0.24–0.83], respectively) (Table 2). Meanwhile, frequency of 9 bp deletion or insertion allele was significantly different between HCC and control groups. Carriage of the 9 bp deletion allele was associated with a greatly decreased risk of developing the disease (OR 0.72, [95% C.I. 0.56–0.92], $P = 0.007$).

Association of rs16405 genotypes with βTrCP mRNA expression

We investigated whether the transcription of βTrCP could be controlled by rs16405 polymorphism. βTrCP mRNA expression levels of HCC tumor tissues with different rs16405 genotypes were examined using real-time RT-PCR. Melting curve analysis showed that PCR products of βTrCP and beta-actin were specific. The results of the *in vivo* experiment showed that differences among three genotypic groups were significant ($P < 0.01$) and mRNA levels of βTrCP from HCC tumor tissues were correlated with different rs16405 genotypes. HCC tumor tissues with homozygous for 9N ins/ins had the highest level of βTrCP, which were 3.99 and 7.04-fold higher than heterozygous 9N ins/del and homozygous 9N del/del, respectively (Fig. 1).

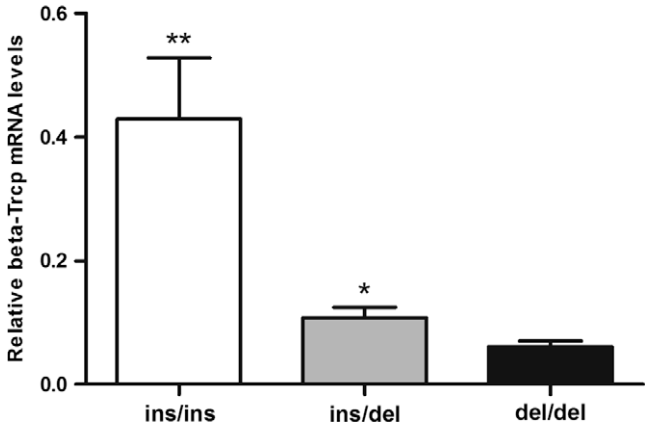

Fig. 1. Real-time RT-PCR analysis of mRNA levels of βTrCP in different genotypic HCC tumor tissues. Sample numbers for different genotype (9N ins/ins, 9N del/del and 9N ins/del) were 8, 10 and 13, respectively. Data are represented as mean ± SE. The normalized expression values of βTrCP were analyzed using one way ANOVA. $^{*}P < 0.05$ compared with 9N del/del genotype, $^{**}P < 0.01$ compared with 9N ins/del and 9N del/del genotypes.

rs16405 is located within the hsa-miR-920 potential target sequence in βTrCP 3′UTR

Using miRanda software, we have determined that the *βTrCP* 3′UTR harbors a putative mir-920 microRNA-binding site. Computer alignment demonstrates that this polymorphism occurs in the middle of the miR-920 complementarily seed binding sequence (Fig. 2). Computational modeling suggests that the thermodynamics of binding between miR-920 and the *βTrCP* 3′UTR harboring either the 9 bp insertion or deletion allele is distinct, the presence of the 9 bp insertion allele would disrupt the binding of miR-920 and *βTrCP* 3′UTR, which would result in a destabilized *βTrCP* 3′UTR hybrid.

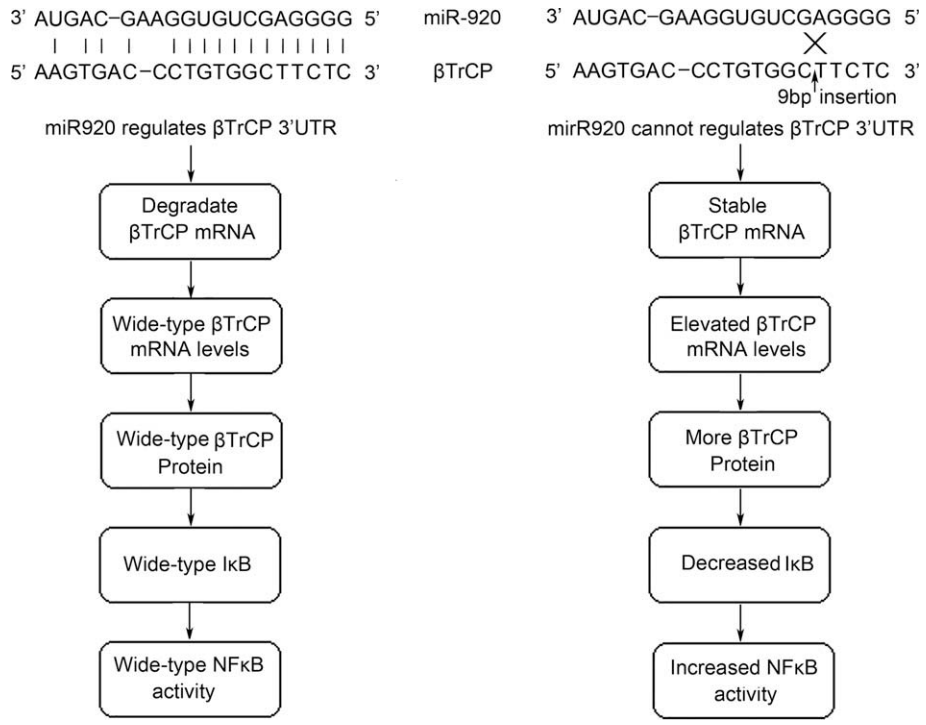

Fig. 2. Characterization of miR-920 targeting at the 3′UTR of *βTrCP* gene and the model for molecular mechanism of rs16405 mediated miR-920 regulation of βTrCP expression. The 9 bp insertion allele in the 3′UTR of *βTrCP* disrupts miR-920 binding, leading to relative higher βTrCP levels, which would consequently resulting decreased IκB level and increased NFκB activity.

Discussion

It is increasingly clear that genetic factors play a critical role in determining risk of HCC, and many polymorphisms associated with HCC in various genes have been reported [14]. βTrCP has been previously suggested to be important in cancer biogenesis owing to the deregulated proteolysis of its substrates [6]. Considering the close functional relationship between βTrCP and multiple cancers, it is conceivable that mutations and polymorphisms in *βTrCP* may account for a proportion of HCC susceptibility. We investigate the association between HCC and an indel polymorphism of *βTrCP* gene in a Chinese population in which we found that presence of 9 bp deletion allele of *βTrCP* seems to confer lower risk for HCC when compared with noncarriers, independent of age, sex, and HBV infection status. Furthermore, we evaluated the actual effects of the polymorphism on βTrCP expression in different genotypic HCC tumor tissues. The differential βTrCP expression presumably caused by genetic variants of *βTrCP* in HCC tissues was observed, suggesting a possible role of *βTrCP* polymorphisms in HCC carcinogenesis. To our knowledge, this is the first study which not only evaluates the association between genetic variants in *βTrCP* and HCC, but also provides potential molecular mechanism of this association.

Mammals express two distinct paralogues of βTrCP with biochemical properties that are indistinguishable. Work by several groups has demonstrated the versatility of βTrCP in regulating various cellular processes through mediating the degradation of a variety of targets [7,15,16]. Owing to the diversity in its substrates, βTrCP might be expected both to be oncogenic and display tumor suppressor activity. However, overwhelming evidence indicates that βTrCP possesses mainly oncogenic characteristics. Indeed, overexpression of βTrCP has been reported in many cases [8–10,17]. Being a negative regulator of NFκB, IκB functions as a tumor suppressor, and the aberrant activation of NFκB due to defective IκB activity has been demonstrated in HCC [18]. As one of the substrates of βTrCP, IκB activity can be lost which might occur in the context of βTrCP overexpression. In addition, βTrCP also plays a critical role in the inducible processing that converts NFκB into an active form, leading to the development of HCC. Based on our data, it is logical to assume that the risk allele of βTrCP may cause aberrantly increased βTrCP levels, resulting increased IκB ubiquitination, which in turn results in the translocation of NFκB to the nucleus and the transactivation of antiapoptosis genes important for cell survival.

MicroRNAs (miRNAs) are a class of short non-coding RNAs with posttranscriptional regulatory functions. They participate in diverse biological pathways and function as gene regulators [19]. The binding of miRNA to mRNA is critical for regulating the mRNA level and protein expression. However, this binding can be affected by polymorphisms residing in the miRNA target site, which can either abolish existing binding sites or create illegitimate binding sites. Thus, polymorphisms in 3′UTR targeted by miRNAs can alter the strength of miRNA binding, with consequences on regulation of target genes thereby affecting the individual's cancer risk [20–21]. Based on bioinformatics analysis, rs16405 lies within a predicted binding site (seed region) for human miR-920 (Fig. 2). Therefore, we hypothesized that miR-920 would bind tightly to *βTrCP* mRNA transcripts containing the 9 bp deletion allele, negatively regulating βTrCP expression. Conversely, the binding with mRNA transcripts containing the 9 bp insertion allele would be disrupted, allowing upregulated βTrCP expression. Although the majority of reported miRNA-binding sites lie within 3′UTRs, it is recently demonstrated that degradation of mRNA of βTrCP1 (one of βTrCP isoforms) is miRNA dependent and miR-183 can interact with the coding region of βTrCP1mRNA [22], which suggest that miRNAs may not be restricted to targeting only 3′UTRs of mammalian mRNAs. Thus, our results indicate different miRNAs might target same mRNA within different regions which in line with the bioinformatics prediction that target sites for miRNA can be identified in open reading frames and 5′UTRs [23].

Since the phenomenon of linkage disequilibrium generally means that there will be multiple variants nearby that have a similar probability of conferring risk and driving the association. It is worthy to note that the presence of this HCC-associated polymorphism in a miRNA target site does not immediately imply that this polymorphism is causative. However, it certainly warrants further replication association studies and follow-up functional experiments. Taken together, our data suggest that common genetic changes in *βTrCP* may influence HCC risk, likely through at least in part by hsa-miR-920-mediated regulation, which is possibly involved in the pathogenesis of HCC.

Acknowledgments

This study is supported by grants from National Science Foundation of China (No. 30800621) and China Postdoctoral Science Foundation (Nos. 20080431121 and 200902530).

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