

## Polymorphism of T-cell Receptor $\gamma$ Short Tandem Repeats as a Susceptibility Risk Factor of Hepatocellular Carcinoma

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**Abstract.** *Background:* T-cells play a critical role in the immunological surveillance network against cancer formation. The activation of T-cells is initiated by binding of T-cell receptors (TCR) with antigen epitopes. Polymorphisms of TCR- $\gamma$  microsatellite (short tandem repeats, STR) marker has been associated with early-onset colorectal cancer. The aim of this study was to test the relationship of TCR- $\gamma$  STR genetic polymorphisms and hepatocellular carcinoma (HCC). *Materials and Methods:* A total of 225 chronic hepatitis B- or C-related HCC and liver cirrhosis patients were enrolled in this study. The other 225 sex-matched cirrhotic patients without HCC were recruited as controls. Their TCR- $\gamma$  STR polymorphisms at loci D7S1818 and D7S2206 were measured by polymerase chain reaction. Dietary habits and other possible risk factors for HCC were also assessed by a structured questionnaire. *Results:* Compared to controls, the HCC patients were older in age ( $64.9 \pm 10.3$  vs.  $53.5 \pm 10.1$  years,  $p < 0.001$ ) and had a higher percentage of family history of HCC (13.3% vs. 7.6%,  $p = 0.045$ ) and habitual alcohol use (23.1% vs. 15.6%,  $p = 0.042$ ). A total of 20 genotypes of TCR- $\gamma$  D7S1818 STR were detected. Of these, the genotype 16 (13/14 of repeat number of GATA) had a higher percentage in the HCC groups than in the controls (13.8% vs. 6.7%,  $p = 0.013$ ). After adjustment for age, family history of HCC and habitual alcohol use, the TCR- $\gamma$  genotype 16 remained a significant risk factor for HCC (OR: 2.18, 95% CI: 1.02-4.65,  $p = 0.045$ ). *Conclusion:* The TCR- $\gamma$  STR polymorphism may be associated with HCC susceptibility.

The intact human immune system is effective in preventing cancer formation. Within our immune system, T-cells play a crucial role in the immunological surveillance network and cytotoxicity. For example, T-cells are activated by the binding of T-cell antigen receptor (TCR) with antigen epitopes. Two chain combinations of the TCR are used for antigen recognition: TCR  $\alpha/\beta$  and TCR  $\gamma/\delta$  (1, 2). Transcription of the  $\gamma$  gene results in protein biosynthesis of the  $\gamma$  chain, which is expressed along with the  $\delta$  chain on the surface of T-cells (3, 4). The  $\gamma/\delta$ -presenting T-cells can recognize both allogeneic- and self-major histocompatibility complex and, in some cases, unprocessed, native antigens (1).

The tissue distribution of  $\gamma/\delta$  T-cells varies in healthy and diseased conditions. Gamma and  $\delta$  T-cells comprise variable percentages of T-cells in inflammatory infiltrates of chronic liver diseases (5, 6). A significant increase of  $\gamma/\delta$  T-cells in the capsular region of hepatocellular carcinoma (HCC) was noted (7). These tumor-infiltrating lymphocytes bearing the  $\gamma/\delta$  T-cell receptor may exhibit an anti-tumor effect (8). It was demonstrated that an insufficient T-cell response to tumor antigens may be liable for carcinogenesis (9).

The TCR subunit  $\gamma$  is assigned to human chromosome 7p15-14. Two microsatellite (short tandem repeats, STR) loci, D7S1818 and D7S2206, were identified (10). These microsatellite markers are repeated (GATA)<sub>n</sub> tetranucleotide sequences in the vicinity of the TCRG gene on chromosome 7p (10). Uthoff *et al.* first pointed out that TCR- $\gamma$  genotype BC of D7S1818 and haplotype AC of D7S1818/D7S2206 were associated with colorectal cancer in patients younger than 60 years old (10). These novel markers may be of use in surveilling young patients at risk for colorectal cancer.

HCC is one of the most common malignancies in Asia and sub-Saharan Africa (11, 12). It is the leading cause of cancer death in Taiwan (13). The risk factors of HCC include chronic viral hepatitis, liver cirrhosis, aflatoxin exposure, alcohol consumption, smoking, the male gender, hemochromatosis and genetic factors (14-16). Among these risk factors, chronic viral hepatitis is the major cause of

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HCC (17). However, only a fraction of chronic viral hepatitis carriers finally progress to HCC in their lifetime (18). It is obvious that other genetic and environmental factors additionally result in HCC. Our previous study suggested that the polymorphism of *N*-acetyltransferase gene may increase the risk for HCC, under the influence of the environmental factor high red meat consumption (19).

To date no report concerning the association of TCR- $\gamma$  and the susceptibility of HCC has been published. The genetic polymorphism of TCR- $\gamma$  STR may influence the host response to carcinogenesis and affect the development of HCC. The purpose of this study was to examine whether the TCR- $\gamma$  microsatellite marker is a risk factor of HCC. The gene-environment interaction between TCR- $\gamma$  STR and dietary habits was also evaluated.

## Materials and Methods

**Subjects studied.** All the chronic hepatitis B virus (HBV)- or hepatitis C virus (HCV)-related cirrhotic patients with HCC, successively admitted to Taipei Veterans General Hospital between 1999 and 2002, were considered for enrollment in this study. A total of 225 patients agreed to participate in this study and were finally recruited. The diagnosis of HCC was based on either histological findings or elevated serum alpha-fetoprotein levels ( $>400$  ng/ml) combined with at least two positive imaging studies from ultrasonography, computed tomography or angiography. The diagnosis of cirrhosis was based on the results of a liver biopsy, or the typically clinical stigmata of cirrhosis including ascites, esophageal varices, hepatic encephalopathy, hypoalbuminemia and the imaging presence of an uneven surface on the liver, decreased hepatic size, collateral circulation and splenomegaly.

In addition, a total of 225 HBV- or HCV-related cirrhotic patients without HCC were recruited as controls. This cohort had been followed-up regularly at the gastroenterology outpatient clinic of Taipei Veterans General Hospital since 1996. They were enrolled voluntarily. Controls were selected by matching gender to the HCC patients.

After obtaining written informed consent, subjects were interviewed. They completed an abbreviated food frequency questionnaire. Patients who declined to give consent or who failed to answer the questionnaire were excluded. This study protocol was approved by the Institutional Review Board of Taipei Veterans General Hospital.

The dietary habits of the subjects were assessed using a semi-quantitative food frequency questionnaire (19). Habitual alcohol drinking was defined as daily consumption of at least 20 g of alcohol for more than 10 years.

**Analysis of microsatellite marker.** Ten milliliters of venous blood were collected from each subject studied. Genomic DNA was isolated from the peripheral blood leukocytes. The microsatellite loci D7S1818 and D7S2206 are repetitive (GATA)<sub>n</sub> tetranucleotide sequences in the vicinity of the *TCRG* gene on chromosome 7p. Oligonucleotide primers, forward 5'-(FAM)CCCTAACTCCCCATGTTGATG-3' and reverse 5'-CACCCAGGATTGTGCTAACCT-3' specific for D7S1818, and forward 5'-(HEX)TTACAAATGTCAGAGCAG-3'

Table I. Characteristics and risk factors of controls and hepatocellular carcinoma (HCC) patients.

	Control N (%)	HCC N (%)	P value
Age (years)	64.9±10.3	53.5±10.1	<0.001
Gender			
M	183 (81.3)	183 (81.3)	1.000
F	42 (18.7)	42 (18.7)	
Chronic viral hepatitis			
Hepatitis B	184 (81.8)	184 (81.8)	0.750
Hepatitis C	32 (14.2)	29 (12.9)	
Hepatitis B + C	9 (4.0)	12 (5.3)	
Child-Pugh classification			
A	116 (51.6)	99 (44.0)	0.402
B	75 (33.3)	87 (38.7)	
C	34 (15.1)	39 (17.3)	
Family history of HCC			
Yes	17 (7.6)	30 (13.3)	0.045
No	208 (92.4)	195 (86.7)	
Smoking			
Yes	94 (41.8)	95 (42.2)	0.924
No	131 (58.2)	130 (57.8)	
Habitual alcohol use			
Yes	35 (15.6)	52 (23.1)	0.042
No	190 (84.4)	173 (76.9)	
Red meat intake (serving/day)			
Low ( $\leq 0.5$ )	73 (32.4)	72 (32.0)	0.946
Intermediate ( $>0.5-1$ )	100 (44.4)	98 (43.6)	
High ( $>1$ )	52 (23.1)	55 (24.4)	
White meat intake (serving/day)			
Low ( $<1$ )	84 (37.3)	76 (33.8)	0.431
High ( $\geq 1$ )	141 (62.7)	149 (66.2)	
Salted food intake (serving/wk)			
Low ( $<1$ )	174 (77.3)	171 (76.0)	0.738
High ( $\geq 1$ )	51 (22.7)	54 (24.0)	
Vegetable consumption (serving/day)			
Low ( $<1$ )	60 (26.7)	69 (30.7)	0.348
High ( $\geq 1$ )	165 (73.3)	156 (69.3)	
Fruit intake (serving/day)			
Low ( $<1$ )	51 (22.7)	62 (27.6)	0.232
High ( $\geq 1$ )	174 (77.3)	163 (72.4)	

\* Data expressed as mean±SD or number (%).

and reverse 5'-CAGAACCAAAAGAAGAG-3' specific for D7S2206, respectively, were custom synthesized (MWG-Biotech, High Point, NC, USA). D7S1818 and D7S2206 were amplified by polymerase chain reaction (PCR; PTC-200, Bio-Rad Laboratories, Hercules, CA, USA). The PCR mixture consisted of a 30- $\mu$ L reaction volume, including 50 ng of genomic DNA, 2.5  $\mu$ M of dNTPs, 3  $\mu$ M of MgCl<sub>2</sub> and 1 U of fast-start Taq DNA polymerase. The PCR conditions were an initial denaturation step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 55°C for 30 seconds, with an extension at 72°C for 60 sec and a final extension at 72°C for 10 min. PCR products were subsequently subjected to electrophoresis in the ABI377 machine. The electrophoresis condition was resolved at 40 mW for 4 h at 50°C in 10% Long Ranger™ polyacrylamide gels (BioWhittaker Molecular Applications, Rockland, ME, USA) and in 1X TBE buffer. Allelic

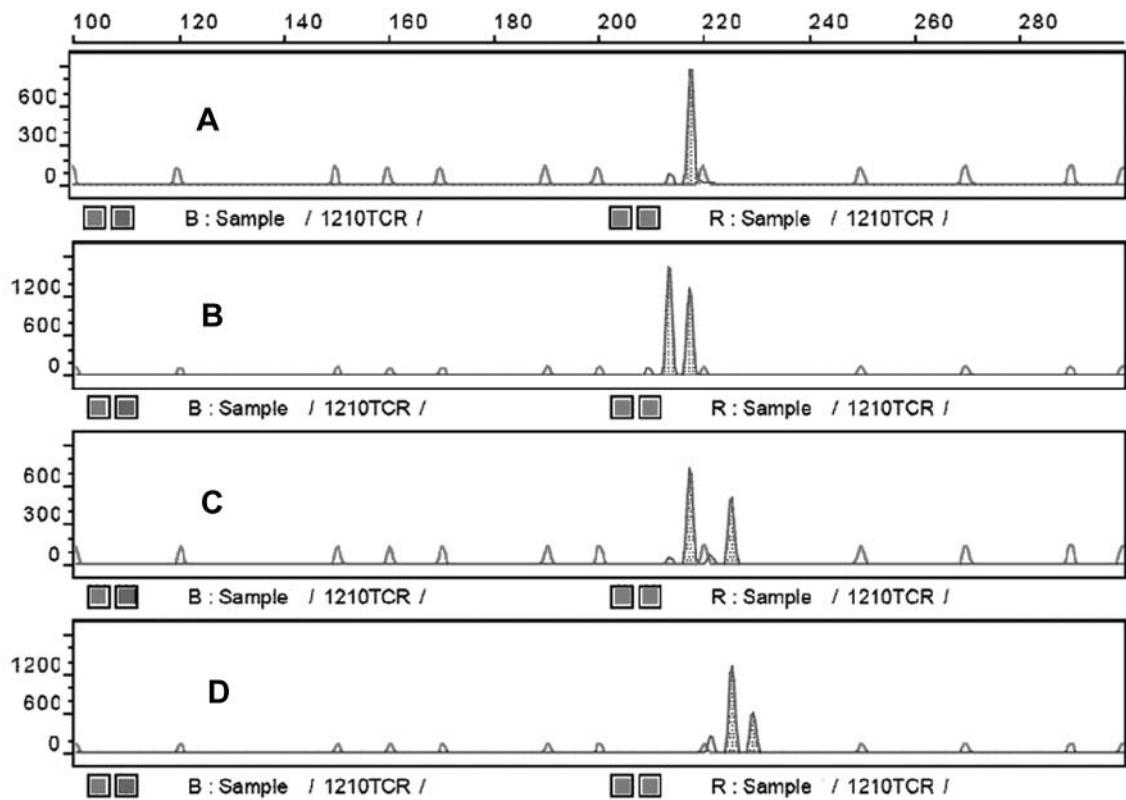


Figure 1. Polymorphism of the short tandem repeat locus D7S1818 is shown by GeneScan. Polymorphisms of D7S1818 short tandem repeats were determined by PCR. The resulting sizes of PCR products represent numbers of repeats. The 213 bp, 217 bp, 221 bp, 225 bp and 229 bp represent repeats of 11, 12, 13, 14 and 15, respectively. The corresponding genotypes of samples A, B, C and D are 12/12, 11/12, 12/14 and 14/15, respectively.

bands were called and determined by computer software (GeneScan® Analysis Software 3.1.2, Applied Biosystems, Foster City, CA, USA) (Figure 1).

**Statistical analysis.** The Chi-square test was applied to evaluate the association of risk factors, genotypes and the susceptibility of HCC. The Student's *t*-test was used to compare the mean age between cases and controls. An odds ratio (OR) with a 95% confidence interval (CI) of the risk factor and TCR- $\gamma$  genotype for HCC was calculated by logistic regression. Statistical tests were based on two-tailed probability. A *p*-value below 0.05 was considered significant. All analyses were performed using SPSS 10.0 software (SPSS Inc., Chicago, IL, USA).

## Results

Compared to controls, the HCC patients were older in age ( $64.9 \pm 10.3$  vs.  $53.5 \pm 10.1$  years,  $p < 0.001$ ) and had a higher percentage of family history of HCC (13.3% vs. 7.6%,  $p = 0.045$ ) and habitual alcohol drinking (23.1% vs. 15.6%,  $p = 0.042$ , Table I).

A total of 20 genotypes of the TCR- $\gamma$  D7S1818 STR polymorphism were detected (Table II and Figure 1). Genotype 12 (12/13 in repeated number of GATA) had the highest frequency in both controls and HCC patients. The

Table II. Genotypes of TCR- $\gamma$  D7S1818 short tandem repeat polymorphisms in controls and HCC patients.

Geno-type	Repeats of (GATA) in two alleles	Control		HCC		Total
		N	(%)	N	(%)	
1	10/11	3	(1.3)	2	(0.9)	5 (1.1)
2	10/12	5	(2.2)	2	(0.9)	7 (1.6)
3	10/13	2	(0.9)	4	(1.8)	6 (1.3)
4	10/14	1	(0.4)	1	(0.4)	2 (0.4)
5	10/15	2	(0.9)	1	(0.4)	3 (0.7)
6	11/11	3	(1.3)	2	(0.9)	5 (1.1)
7	11/12	18	(8.0)	11	(4.9)	29 (6.4)
8	11/13	12	(5.3)	4	(1.8)	16 (3.6)
9	11/14	2	(0.9)	3	(1.3)	5 (1.1)
10	11/15	3	(1.3)	1	(0.4)	4 (0.9)
11	12/12	36	(16.0)	31	(13.8)	67 (14.9)
12	12/13	58	(58.8)	69	(30.7)	127 (28.2)
13	12/14	26	(11.6)	25	(11.1)	51 (11.3)
14	12/15	4	(1.8)	5	(2.2)	9 (2.0)
15	13/13	18	(8.0)	20	(8.9)	38 (8.4)
16	13/14	15	(6.7)*	31	(13.8)*	46 (10.2)
17	13/15	3	(1.3)	3	(1.3)	6 (1.3)
18	13/16	5	(2.2)	1	(0.4)	6 (1.3)
19	14/14	5	(2.2)	6	(2.7)	11 (2.4)
20	14/15	4	(1.8)	3	(1.3)	7 (1.6)

\**p*=0.013, compared to other genotypes.

Table III. Logistic regression analysis of the TCR- $\gamma$  STR genotype 16 and other risk factors for HCC.

	Odds ratio	95% Confidence interval	P
<b>Univariate</b>			
TCR- $\gamma$ STR genotype 16	2.24	1.17-4.27	0.015
<b>Multivariate</b>			
TCR- $\gamma$ STR genotype 16	2.18	1.02-4.65	0.045
Age	1.11	1.09-1.14	0.001
Family history of HCC	1.88	0.93-3.82	0.080
Habitual alcohol use	1.52	0.87-2.63	0.141

genotype 16 (13/14 in repeat number of GATA) had a higher percentage in the HCC group than in the controls (13.8% vs. 6.7%,  $p=0.013$ ). After adjustment for age, family history of HCC and habitual alcohol use, the TCR- $\gamma$  genotype 16 remained a significant risk factor of HCC (OR: 2.18, 95% CI:1.02-4.65,  $p=0.045$ , Table III).

When analyzed on TCR- $\gamma$  D7S2206 STR, there was no significant polymorphism in our patients.

## Discussion

Although it is generally accepted that host T-cell responses play a pivotal role in the immunological surveillance of tumor formation, little is known about the TCR genetic polymorphism and its susceptibility to HCC. To the best of our knowledge, the present study first demonstrated that TCR- $\gamma$  STR polymorphism was associated with HCC risk for viral hepatitis-related cirrhotic patients.

Uthoff *et al.* indicated that individuals with the TCR- $\gamma$  STR genotype BC of D7S1818 and haplotype AC of D7S1818/D7S2206 have a higher risk of developing colon cancer at a younger age (10). Similarly, the present study revealed that patients with the TCR- $\gamma$  genotype 16 (13/14 in repeat number of GATA) had higher risk of HCC. Why this genotype increases the susceptibility of HCC is unknown. Genotype 16 has high STR repeats. The high STR repeats may be unstable, which was shown to be the underlying cause of many human disorders when repeats extended beyond a critical number (20, 21). One of the typical examples is Huntington's disease, a "trinucleotide repeat" disorder, caused by an increase in the number of CAG repeats in the *HD* gene on chromosome 4. Repeats of 36 or larger are associated with this disease expression, whereas repeats of 35 or smaller are normal (20). Other diseases linked to increased STR number are fragile X syndrome, dystrophia myotonica, spinocerebellar ataxia,

etc. Our finding was similar to Uthoff's results that patients with the high TCR- $\gamma$  STR number may have increased susceptibility of cancer (10).

A total of 20 genotypes of TCR- $\gamma$  STR were detected in our study. The genotypes 17 to 20 have higher STR repeat numbers in alleles than the genotype 16 (13/14 in GATA repeat number). If the above hypothesis that high STR repeat numbers may increase the risk of HCC is possible, why was there no statistical significance of genotypes 17 to 20 and the susceptibility of HCC in this study? The probable explanation is that the genetic frequencies in these genotypes were low and thus a statistical difference was hard to achieve. The other possibility is that under some unknown mechanism, only the genotype 17 is associated with HCC susceptibility.

Many risk factors for hepatocarcinogenesis have been recommended, including chronic viral hepatitis B and C, alcohol, smoking, low vegetable consumption, gender, cirrhosis and genetic factors (13). In the present study, we matched control and HCC patients in terms of gender and viral hepatitis-related cirrhosis status in order to critically assess the influence of TCR- $\gamma$  STR genotype and dietary habit on the HCC susceptibility. After adjusting these factors, the results of our study seem more reliable.

Why the high TCR- $\gamma$  STR number result in predisposition to the development of HCC is unknown. We speculate that the high TCR- $\gamma$  STR number may encode abnormal  $\gamma$  protein, which in turn hinders the combination of T-cell and TCR, rendering an inadequate T-cell response and finally resulting in HCC formation. However, further study to correlate the TCR- $\gamma$  STR genotype and phenotype is warranted. In addition, the association of TCR- $\gamma$  STR genetic polymorphism and susceptibility of HCC need to be verified in other ethnic groups. Nevertheless, the present study revealed the possibility of TCR- $\gamma$  STR polymorphism and the susceptibility of HCC.

In conclusion, the STR polymorphism of TCR- $\gamma$  may be associated with HCC occurrence in viral hepatitis-related cirrhotic patients.

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