RESEARCH COMMUNICATION

No Association of the Exonuclease 1 T439M Polymorphism and Risk of Hepatocellular Carcinoma Development in the Turkish Population: a Case-control Study

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Abstract

Exonuclease 1 (Exo 1) is an important nuclease involved in the mismatch repair system that contributes to maintaining genomic stability, modulating DNA recombination and mediatingcell cycle arrest. A cytosine (C)/thymine (T) common single nucleotide polymorphism (SNP) at second position of codon 439 in exon 10 of Exo 1 determines a threonine (Thr, T) to methionine (Met, M) (T439M) aminoacidic substitution which may alter cancer risk by influencing the activity of Exo 1 protein. The association of Exo 1 T439M polymorphism with hepatocellular carcinoma (HCC) susceptibility has yet to be investigated. To assess this possibility in a Turkish population, a hospital-based case-control study was designed consisting of 224 subjects with HCC and 224 cancer-free control subjects matched for age, gender, smoking and alcohol status. The genotype frequency of the Exo 1 T439M polymorphism was determined by using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. No statistically significant differences were found in the allele or genotype distributions of the Exo 1 T439M polymorphism among HCC and cancer-free control subjects (P>0.05). Our result demonstrates for the first time that the Exo 1 T439M polymorphism does not have a major role in genetic susceptibility to hepatocarcinogenesis, at least in the population studied here. Independent studies are need to validate our findings in a larger series, as well as in patients of different ethnic origins.

Key words: Hepatocellular carcinoma - exo 1 T439M polymorphism - case-control study - genetic susceptibility

Asian Pacific J Cancer Prev, **12**, 2455-2460

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer death. Because of its high fatality rates, the incidence and mortality ratios are approximately equal (Parkin et al., 2005). It is now well established that multiple risk factors contribute to hepatocarcinogenesis, including chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections, cirrhosis, carcinogen exposure (such as aflatoxin B1), excessive alcohol drinking (Farazi and DePinho, 2006; El-Serag and Rudolph, 2007). Although many individuals exposed to these risk factors, HCC develops only in a small group of exposed people, implying that genetic factors might contribute to the carcinogenic mechanism. So the search for genetic factors that could help to select patients at higher risk and thus to modulate the indications of screening procedures is necessary (Llovet et al., 2004). Moreover, identification of predictive factors could lead to a better diagnosis and planning of new prevention strategies in these patients (Lodato et al., 2006).

Human cells are exposed to constant endogenous (e.g. reactive oxygen species) and exogenous (e.g. UV-radiation) stresses that threaten DNA. In addition, DNA damage can arise from spontaneous replication errors (Shimada and Nakanishi, 2006). Human DNA repair mechanisms are thought to prevent or delay genetic instability and tumorigenesis, thus acting as a barrier against cancer development (Bartkova et al., 2005; Gorgoulis et al., 2005). It is thought that individuals with defects in their DNA repair mechanisms lose their natural protection against tumorigenesis and are more susceptible to cell transformation and cancer. One of the major DNA repair pathways in human cells is the mismatch repair (MMR), which maintains genomic stability, modulates DNA recombination, and mediates cell cycle arrest (Iyer et al., 2006). The gene Exonuclease 1 (Exo 1;

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MIM # 606063) is a member of the MMR system, and also belongs to the RAD2 nuclease family. It locates at chromosome 1q42-q43, contains one untranslated exon followed by 13 coding exons and encodes an 846 amino acid protein (Schmutte et al., 1998; Tishkoff et al.; 1998; Wilson III et al., 1998). Exo 1 can interact physically with the MMR protiens MSH2 and MLH1 in both yeast and human cells, and with MSH3 in human cells (Jager et al., 2001; Rasmussen et al., 2000; Schmutte et al., 2001; Tishkoff et al., 1997; Tran et al., 2001). Exo 1 functions in DNA replication, repair, recombination, mutation avoidance and is essential for male and female meiosis (Tran et al., 2004; Wei et al., 2003). A cytosine (C)/ thymine (T) common single nucleotide polymorphism (SNP) at second position of codon 439 in exon 10 of Exo 1 gene (dbSNP ID:rs rs4149963), resulting in the substitution of an threonine (Thr, T) residue (ACG) by methionine (Met, M) residue (ATG) (also designated Exo 1 T439M) in the MLH1 interaction domain, has been suggested to influence the MMR function of Exo 1 (Yamamoto et al., 2005). Recently, Yamamoto et al (2005) explored the association between T439M polymorphism of Exo 1 with development of colorectal cancer in Japanese population, and found that Exo 1 T439M was associated with altered risk of colorectal cancer.

Sequence variants in DNA repair genes also are thought to modulate DNA repair capacity and consequently may be associated with altered cancer risk (Hung et al., 2005). Since SNP is the most frequent and subtle genetic variation in the human genome and has great potential for application to association studies of complex disease (Kirk et al., 2002). We hypothesized that T439M polymorphism in Exo 1 gene may act as a genetic modifier in individual susceptibility to HCC. According to our recent knowledge, no research has been conducted to evaluate Exo 1 T439M polymorphism and risk of HCC development. To test the hypothesis that the polymorphism of Exo 1 T439M is associated with risk of developing HCC, we performed genotyping analysis using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay in a hospital-based case-control study of 224 HCC patients and 224 age, gender, smoking and alcohol consumption matched cancer-free controls in Turkish population.

Materials and Methods

Study Population

The study population and subject characteristics were previously described elsewhere (Akkız et al., 2010; Akkız et al., 2011). This is an ongoing molecular epidemiologic study of HCC conducted in Adana, Turkey and the subject recruitment was approved by the Committee for Ethics of Medical Experiment on Human Subjects, Faculty of Medicine, Çukurova University. Briefly, all subjects were genetically unrelated Turkish and were from Çukurova and the surrounding regions of southern Turkey. Submission of the individuals to the study was conditioned by an obtained written informed consent form regarding the use of their blood samples for research studies. The study proceeded in agreement with the Helsinki declaration approved on the World Medical Association meeting in Edinburgh.

Blood samples were collected from 224 consecutive patients with HCC seen in the department of gastroenterology and general surgery between September 2005 and June 2011. During the same time, 224 unrelated community residents with no evidence of hepatocellular or other cancer who entered the hospital for health check-ups were enrolled as the control group. The 224 cancer-free control subjects did not have a history of liver disease and had no serological evidence of hepatitis B or C virus infection. Each control was pair-matched by sex, age (±3 years), smoking and alcohol consumption to a patient with HCC. These characteristics allowed us the choice of a control population without any possible risk bias for HCC. The HCC diagnostic criteria was based on the guideline proposed by European Association for the Study of the Liver (EASL) (Bruix et al., 2001). We gave a diagnosis of HCC when a patient had one or more risk factors (i.e., HBV or HCV infection, or cirrhosis) and one of the following: >400 ng/mL α-fetoprotein (AFP) and at least one positive finding following examination using spiral computed tomography (CT), contrast-enhanced dynamic MRI, or hepatic angiography; or <400 ng/mL α-fetoprotein and at least two findings following CT, MR, or hepatic angiography. A positive HCC finding using dynamic CT or MRI is indicative of arterial enhancement followed by venous washout in the delayed portal/venous phase. In addition; we performed histopathological diagnosis for cases that did not fulfill all of the clinical non-invasive diagnostic criteria of HCC.

Cirrhosis was diagnosed with liver biopsy, abdominal sonography, and biochemical evidence of parenchymal damage plus endoscopic esophageal or gastric varices (Tsai et al., 1994). Patients with cirrhosis were classified into three Child-Pugh grades based on their clinical status (Pugh et al., 1973). Serum HBsAg and Anti-HCV were assessed using an immunoassay (Abbott Laboratories, Abbott Park, IL, USA). Serum AFP concentration was measured by microparticle enzyme immunoassay (Abbott Laboratories, AXSYM, USA). Heavy alcohol intake was defined as a daily minimum consumption of 160 g alcohol for at least eight years.

All subjects were interviewed using a structured questionnaire to obtain information on demographic factors and health characteristics. Technicians who performed the blood tests were blinded to the identity and disease status of participants. Peripheral blood samples taken from patients and controls, and blood specimens, including white blood cells and serum, were frozen at -20 oC until analysis.

DNA Extraction

A 5 mL sample of venous blood was collected

from each subject into a test tube containing EDTA as anticoagulant. Genomic DNA was extracted from peripheral whole blood using High Pure PCR Template Preperation Kit (Roche Diagnostics. GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism Analysis

PCR-RFLP analysis was performed to determine the genotype of the C/T polymorphism of Exo 1 gene, as described previously (Yamamoto et al., 2005). The 125 base pair (bp) fragment encompassing the C to T polymorphic site in Exo 1 region (T439M) was amplified using specific primers 5'- TCT CTA AGT ACA GGT GAA ACA AAG -3' and 5'- GAG CTA TTT TTC TTG GTC TTC TAC -3'. Amplification was performed in GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Singapore). The 20 µl PCR mixture contained approximately 250 ng DNA, with 0.25 µM of both primer, 0.1 mM of each dNTP, 1X PCR buffer, 1.5 mM MgCI2 and 1U Taq polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: 94 oC for 5 min, followed by 35 cycles of 94 oC for 30 s, 58 oC for 30 s and 72 oC for 30 s, with a final extension at 72 oC for 10 min. As a negative control, PCR mix without DNA sample was used to ensure contamination free PCR product. After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was digested overnight with 5 units BsaAI (from an Escherichia coli strain that carries the BsaAI gene from Bacillus stearothermophilus G668, recognizing the sequence 5'-YAC-GTR-3') enzyme at 37 ˚C (New England Biolabs Inc., Beverly, MA) and electrophoresed on 3% agarose gel containing 0.5 µg/ml ethidium bromide and visualized under UV illumination. PCR products with Thr at the polymorphic site were digested into two fragments, 100 bp and 25 bp, while those with Met were not because of the absence of a BsaAI restriction site. Samples yielding 100 bp and 25 bp fragments were scored as Thr/Thr, those with single 125 bp fragments as Met/Met, and 125 bp, 100 bp and 25 bp as Thr/Met. This assay was illustrated in Figure 1. To ensure quality control, genotyping was performed without knowledge of the subjects' case/control status and a 15% random sample of cases and controls was genotyped twice by different persons; reproducibility was 100%.

Statistical Analysis

The sample size was calculated using the QUANTO 1.1 program (hydra.usc.edu/gxe). The desired power of our study was set at 80%. Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS) for Windows (version 10.0). Differences in the distributions of demographic characteristics between the cases and controls were evaluated using the Student's t-test (for continuous variables) and $\chi 2$ test (for categorical variables). The

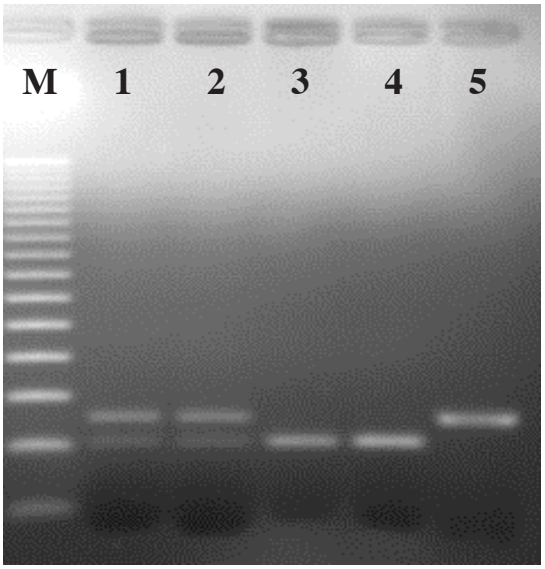

Figure 1. Analysis of the Exo 1 T439M Polymorphism. A representative agarose gel picture showing PCR-RFLP analysis of Exo 1 T439M genotypes in genomic DNAs of study subjects with restriction endonuclease enzyme BsaAI. M: 50-bp DNA ladder, Lanes 1 and 2: Thr/Met heterozygous (125 bp, 100 bp and 25 bp), Lanes 3 and 4: Thr/Thr homozygous (100 bp and 25 bp), Lane 5: Met/Met homozygous (125 bp)

observed genotype frequencies were compared with expected values calculated from Hardy–Weinberg equilibrium theory ($p2 + 2pq + q2 = 1$; where p is the frequency of the wild-type allele and q is the frequency of the variant allele) by using a $\chi 2$ test with degree of freedom equal to 1 among cases and controls, respectively. Pearson's $\chi 2$ test was used to determine whether there was any significant difference in allele and genotype frequencies between patients and controls. The associations between Exo 1 T439M genotypes and the risk of HCC were estimated by computing the odds ratios (ORs) and their 95% confidence intervals (CIs) from binary logistic regression analysis. The homozygous genotype for the Thr allele of Exo 1 was used as the reference in calculating ORs and 95% CIs. Statistical modeling was performed on the relative risk of the Met/Met genotype or the Thr/Met genotype against the Thr/Thr genotype independently. Furthermore, to estimate the recessive or dominant effect of Exo1 T439M genotype on risk, statistical modeling was performed on the relative risk of the Met/Met genotype against the Thr/Met + Thr/Thr genotype (recessive model) or the Thr/Met + Met/Met genotype against the Thr/Thr genotype (dominant model). Probability levels less than 0.05 were used as a criterion of significance.

Results

General Characteristic of the Subjects

A total of 448 Turkish subjects were enrolled in our study. As expected, no significant difference was found between case patients and control subjects with regard to age and sex (P = 0.68 and P = 1.00, respectively) which

Table 1. Allele and Genotype Frequency Distributions of Exo1 T439M Polymorphism

	Cases	Controls	P value[a]	OR (95% CI)
Allele frequency				
Thr	409 (91.3%)	410 (91.5%)		1.00 (Reference)
Met	39 (8.7%)	38 (8.5%)	0.91	1.03 (0.67-1.57)
General genotype				
Thr/Thr	186 (83.0 %)	186 (83.0%)		1.00 (Reference)
Thr/Met	37 (16.5%)	38 (17.0%)	0.92	1.09 (0.59-1.60)
Met/Met	1 (0.5%)	0 (0%)	--	Not calculated
Dominant genotype				
Thr/Thr	186 (83.0%)	186 (83.0%)		1.00 (Reference)
All Met	38 (17.0%)	38 (17.0%)	1.00	1.00 (0.61-1.64)
Recessive genotype				
All Thr	223 (99.5%)	224 (100.0%)		1.00 (Reference)
Met/Met	1 (0.5%)	0 (0.0%)	--	Not calculated

[a]Data were calculated by logistic regression analysis

Table 2. Allele and Genotype Frequency Distributions by Gender and Viral Etiology

	Cases	Controls	P value[a]	OR (95% CI)
Male	180	180		
Thr	326 (90.6%)	330 (91.7%)		1.00 (Reference)
Met	34 (9.4%)	30 (8.3%)	0.60	1.13 (0.71-1.81)
Thr/Thr	147 (81.7%)	150 (83.3%)		1.00 (Reference)
Thr/Met	32 (17.8%)	30 (16.7%)	0.69	0.91 (0.63-1.88)
Met/Met	1 (0.5%)	0 (0%)--	Not calculated	
Female	44	44		
Thr	83 (94.3%)	80 (90.9%)		1.00 (Reference)
Met	5 (5.7%)	8 (9.1%)	0.39	0.63 (0.21-1.84)
Thr/Thr	39 (88.6%)	36 (81.8%)		1.00 (Reference)
Thr/Met	5 (11.4%)	8 (18.2%)	0.37	0.58 (0.17-1.93)
Met/Met		0 (0%)	0 (0%)	--
Not calculated				
HBV-related	133	224		
Thr	238 (89.5%)	410 (91.5%)		1.00 (Reference)
Met	28 (10.5%)	38 (8.5%)	0.36[b]	1.24 (0.78-1.97)
Thr/Thr	106 (79.7%)	186 (83.0%)		1.00 (Reference)
Thr/Met	26 (19.5%)	38 (17.0%)	0.52[b]	1.20 (0.69-2.10)
Met/Met	1 (0.8%)	0 (0%)	--	Not calculated
HCV-related	57	224		
Glu	106 (93.0%)	410 (91.5%)		1.00 (Reference)
Lys	8 (7.0%)	38 (8.5%)	0.61[b]	0.83 (0.40-1.72)
Glu/Glu	49 (86.0%)	186 (83.0%)		1.00 (Reference)
Glu/Lys	8 (14.0%)	38 (17.0%)	0.59[b]	0.80 (0.35-1.82)
Lys/Lys	0 (0%)	0 (0%)	--	Not calculated
Non-viral-related			34	224
Glu	65 (95.6%)	410 (91.5%)		1.00 (Reference)
Lys	3 (4.4%)	38 (8.5%)	0.25[b]	0.52 (0.16-1.64)
Glu/Glu	31 (91.2%)	186 (83.0%)		1.00 (Reference)
Glu/Lys	3 (8.8%)	38 (17.0%)	0.24[b]	0.47 (0.14-1.63)
Lys/Lys	0 (0%)	0 (0%)	--	Not calculated

[a]Data were calculated by logistic regression analysis; [b]Adjusted for age, sex, smoking and drinking status

implied that age and sex matched adequately. Similarly, there were no significant differences in smoking status and alcohol consumption between case and control group.

Genotype Frequency Distribution of Exo 1 T439M

The frequency distributions of the different genotypes for Exo 1 T439M polymorphism are shown in Table 1. The genotypic frequencies of the control (n = 224; $\chi 2$ =0.142 df = 1, P =0.71) were in Hardy–Weinberg equilibrium, suggesting that there was no population stratification and no sampling bias. The patients' frequencies were also in Hardy–Weinberg equilibrium (n = 224; $\chi 2$ = 0.056 df = 1, P = 0.81). The allelic frequencies of case subjects (Thr, 0.91; Met, 0.09) were not significantly different from those of the control subjects (Thr, 0.92; Met, 0.08) (p=0.91 Thus, genotypic frequencies in the cases were similar to that of the controls ($\chi 2 = 1.01$, df = 2, P = 0.60).

Exo 1 T439M Polymorphism Polymorphism and Risk of Hepatocellular Carcinoma

To evaluate the risk of HCC according to the Exo 1 T439M genotype, logistic regression analysis was conducted (Table 1). Using the Thr/Thr genotype as the reference genotype, Thr/Met genotype increased risk but not significantly associated with the risk of HCC (OR = 1.09; 95% CI = 0.59-1.60, P = 0.92). An OR for the Met/Met genotype could not be calculated. With the Thr/Thr genotype as reference, the OR for combined Thr/Met and Met/Met genotypes (dominant genetic model) together was 1.00 (95% CI= 0.61-1.64, P=1.00). When we used Thr/Thr and Thr/Met genotypes as a reference, we could not calculate the OR of the Met/Met genotype (recessive genetic model).

Stratified Analyses

To observe whether the effect of genetic variation was modified by epidemiologic factors, HCC patients and controls were stratified on the basis of various host characteristics including sex, hepatocellular carcinoma etiology (viral infection status) and age. Subgroup analysis revealed that the effect of gender and viral infection status were not significantly different among Exo 1 T439M genotypes (Table 2). Also, age at diagnosis with HCC (mean±standard deviation) was not significantly different among Exo 1 T439M genotypes (Thr/Thr: 60.19±11.00, Thr/Met: 63.12±12.00 and Met/Met: 56.00±0.0, p = 0.32). In addition to this, we did not find any significant association between different genotypes of Exo 1 T439M polymorphism and demographical variables like liver cirrhosis, child Pugh grade of cases and AFP levels (data not given).

Discussion

This molecular epidemiological study investigated whether the Exo 1 T439M polymorphism could have an impact on susceptibility to HCC. Exo 1 T439M polymorphism was selected as the candidate polymorphism because recent evidences indicated that Exo 1 can play a role of mediator in a wide spectrum of biological processes (Tran et al., 2004). Exo 1 playing an essential role as both 5'-3' and 3'-5' nucleases and contributing to the overall stabilility of MMR complex (Tran et al., 2004; Liberti and Rasmussen, 2004). The

MMR system corrects base-base mispairs and small insertion/deletion loops, responsible for maintaining the genome integrity (Tran et al., 2004; Marti et al., 2002; Modrich and Lahue, 1996]. In addition to the unique function in MMR system, Exo 1 was also linked to carcinogenesis through its role in recombinational events, such as repairing of DNA double-strand breaks and maintaining of telomere stabilization (Tran et al., 2004; Wei et al., 2003; Liberti and Rasmussen, 2004). Furthermore, Exo 1 has recently been shown to contribute to DNA damage-induced apoptosis (Bolderson et al., 2009; Klapacz et al., 2009). Recent findings indicated that mammalian Exo1 is responsible for mutation prevention and the mice with Exo 1 inactivation have reduced survival time and increased risk for tumor development (Wei et al., 2003). The Exo 1 T439M polymorphism is non-synonymous SNP that result in replacement of amino acids, in turn, possibly affecting the protein functions. Exo 1 deficiency would reduce the MMR capacity. In contrary to our expectation, in the present case-control study in Turkish population, distribution of Exo 1 T439M genotype was not different between HCC cases and controls. No significant association emerged between risk of HCC and Exo 1 T439M polymorphism in overall statistical analyses.

The findings of our study are not similar to those reported by Yamamoto et al. (2005), who found that Thr/Met genotype (OR = 2.03; 95% CI = 1.04-3.98, P = 0.04) and Thr/Met and Met/Met genotypes combined (OR = 2.37; 95% CI = 1.23-4.56, P = 0.01) demonstrated significant association with the development of colorectal cancer after adjusting for age, gender and smoking status in Japanese population. A rational explanation for this cancer-dependent difference in risk conferred by the examined Exo 1 T439M polymorphism may be attributable to differences in the pathways of carcinogenesis among the various types of human cancers. These discrepancies may also be due to the differences of the ethnic variation as well as on several environmental and other factors that influence that population. Geographic or ethnic differences have been reported regarding the genotype frequency of several polymorphisms (International HapMap Project). For instance, Thr allele frequency of Exo 1 T439M polymorphism among the different ethnicities is as follows: 0.912 in Caucasians, 0.860 in Japanese, 0.907 in Chinese and 1.00 in Africans (International HapMap Project). This study found that the Thr allele frequency of Exo 1 T439M polymorphism was 0.920 among our Turkish control subjects, similar to the reported allele frequencies in Caucasians. Further investigations of Exo 1 T439M polymorphism in various types of cancer and different populations are in needed and Exo 1 T439M may be a promising biomarker for specific types of cancers.

The limitations of our study are as follows. First limitation of the present study is that it was hospital-based case–control study, and patients were selected at a single institution (Çukurova University, Balcalı Hospital) and thus may have been unrepresentative of HCC patients in the general population. In addition, it should be noted that the control subjects were recruited at the same hospital. However, in the control group, the agreement between the observed distributions of Exo 1 T439M genotype frequencies with the expected according to the Hardy–Weinberg equilibrium model suggested no selection bias. Second, this study is limited by the relatively small number of cases and controls. Therefore further studies with a larger number of subjects are needed to clarify this issue. Third, we also limited our study to Turkish population due to variation in allele frequency between different ethnic groups has been observed for Exo 1 T439M polymorphism. Fourth, due to the lack of data on Exo 1 expression according to T439M genotypes in our HCC group, future work need to be done in order to explore the correlation between levels of Exo 1 in normal liver and HCC tissues in the context of different genotypes of Exo 1 T439M polymorphism.

In conclusion; our results demonstrate for the first time that the Exo 1 T439M polymorphism have not been any major role in genetic susceptibilty to hepatocellular carcinogenesis within the studied population. Beacuse this is the first report concerning the Exo 1 T439M polymorphism and the risk of HCC in the literature, further independent studies are required to validate our findings in a larger series, as well as in patients of different ethnic origins and to better understand Exo 1 T439M polymorphism and susceptibility to HCC.

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