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Short Communication

Lack of an association of programmed cell death-1 PD1.3 polymorphism with risk of hepatocellular carcinoma susceptibility in Turkish population: A case–control study

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ABSTRACT

Aim: The programmed cell death-1 (PD-1) is a potent immunoregulatory molecule which is responsible for the negative regulation of T-cell activation and peripheral tolerance. Recently, overexpression of PD-1 has been reported to contribute to immune system evasion and poor survival of hepatocellular carcinoma (HCC). A common single nucleotide polymorphism in intron 4 of PD-1 gene called PD-1.3 has been reported to influence PD-1 expression, but its association with HCC has yet to be investigated. The aim of the present study was to investigate whether this polymorphism could be involved in the risk of HCC susceptibility.
Methods: The genotype frequency of PD-1.3 polymorphism was determined by using a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method in 236 subjects with HCC and 236 cancer-free control subjects matched on age, gender, smoking and alcohol status.
Results: No statistically significant differences were found in the genotype distributions of the PD-1.3 polymorphism among HCC and cancer-free control subjects ($P = 0.22$).
Conclusion: Our results demonstrate for the first time that the PD-1.3 polymorphism has not been in any major role in genetic susceptibility to hepatocellular carcinogenesis, at least in the population studied here. Independent studies are needed to validate our findings in a larger series, as well as in patients of different ethnic origins.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth 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 (Jemal et al., 2011). Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are the major cause of HCC (El-Serag and Rudolph, 2007; Farazi and DePinho, 2006). Nevertheless, only a fraction of infected patients develop HCC during their lifetime suggesting that genetic factors might modulate HCC development.

Programmed cell death-1 (PD-1; also called CD279) is a member of the CD28/B7 superfamily of costimulatory molecules that regulates T cell tolerance and is expressed on activated $CD4^+$ T cells, $CD8^+$ T cells, natural killer T cells, B cells, and activated monocytes (Sharpe et al., 2007). The human gene encoding PD-1 is located on chromosome 2q37.3. PD-1 is a 55 kDa (228 amino acid) type I transmembrane receptor protein composed of an IgV-like extracellular domain, and a cytoplasmic domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) (Dinesh et al., 2010; Sharpe et al., 2007). Interaction between PD-1 and its ligands PD-L1 (B7-H1; CD274) or PD-L2 (B7-DC; CD273) can activate the cytoplasmic ITIM of PD-1 and induce the inhibitory signal to attenuate T-lymphocyte activation and proliferation, suppress cytokine secretion and T cell apoptosis, as well as maintain peripheral tolerance (Freeman et al., 2000; Latchman et al., 2001; Nishimura and Honjo, 2001). Moreover, PD-Ls have been widely reported to be expressed on the cell lines or tissues of a variety of tumors including HCC (Gao et al., 2009; Karim et al., 2009). The increase of the expression of PD-Ls in HCC can contribute to tumor evasion (Shi et al., 2011), and cause poor clinical prognosis of HCC (Hsu et al., 2010; Shi et al., 2011; Zeng et al., 2011). The expression of PD-Ls on tumor cells and PD-1 on tumor infiltrating lymphocytes suggests the presence of an immune inhibitory milieu for tumor progression (Haghshenas et al., 2011). In addition, blockade of PD-L1 enhances tumor-specific T-cell responses and it can promote anti-tumor immunity responses and inhibit tumor growth (Blank et al., 2006; Iwai et al., 2002).

Based on inhibitory role of PD-1 in anti-tumor responses, we considered the *PD-1* gene (Gene bank ID: 5133) as a power candidate for genetic susceptibility of individuals to HCC. A guanine (G) to adenine (A) single nucleotide polymorphism (SNP) at nucleotide +7146 was recently described in intron 4 of PD-1 gene and called PD-1.3 (dbSNP ID: rs11568821) (Prokunina et al., 2002). A region of *PD-1* intron 4 was described as an enhancer-like structure containing binding sites

Abbreviations: SNP, Single nucleotide polymorphism; HCC, Hepatocellular carcinoma; CI, Confidence interval; OR, Odds ratio; PCR–RFLP, polymerase chain reaction–restriction fragment length polymorphism.

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for several transcription factors (Prokunina et al., 2002). PD-1.3 polymorphism in this region affected the binding of the runt-related transcription factor 1 (RUNX1, also called AML1) and associated it with several autoimmune diseases (Kroner et al., 2005; Prokunina et al., 2002; Prokunina et al., 2004). However, to our recent knowledge, no research has been conducted to evaluate PD-1.3 polymorphism and risk of HCC development. To test the hypothesis that the polymorphism of PD-1.3 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 236 HCC patients and 236 age, gender, smoking and alcohol consumption matched cancer-free controls in Turkish population.

2. Materials and methods

2.1. Study population

The study population and subject characteristics were previously described elsewhere (Bayram, 2012; Bayram et al., 2012). 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 236 consecutive patients with HCC seen in the department of gastroenterology and general surgery between September 2005 and June 2012. During the same time, 236 unrelated community residents with no evidence of hepatocellular or other cancers who entered the hospital for health check-ups were enrolled as the control group. The 236 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 were based on the guideline proposed by the 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 a histopathological examination 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 a 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 °C until analysis.

2.2. 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 Preparation Kit (Roche Diagnostics, GmbH, Mannheim, Germany) according to the manufacturer's protocol.

2.3. Polymerase chain reaction–restriction fragment length polymorphism analysis

PCR–RFLP analysis was performed to determine the genotype of the G/A polymorphism of *PD-1* gene, as described previously (Hoffmann et al., 2010). The 331 base pair (bp) fragment encompassing the G to A polymorphic site in *PD-1* region was amplified using specific primers 5′-CCA GGC AGC AAC CTC AAT C-3′ and 5′-GTC CCC CTC TGA AAT GTC C-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, 1× PCR buffer, 1.5 mM MgCl2 and 1 U Taq polymerase (Promega, Madison, WI, USA). The following cycling conditions were used: 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 5 min. As a negative control, PCR mix without DNA sample was used to ensure a contamination free PCR product. After confirmation of successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was digested overnight with 10 units of PstI (from an *Escherichia coli strain* that carries the *PstI* gene from *Providencia stuartii 164*, recognizing the sequence 5′-CTGCA$^{\downarrow}$G-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 A at the polymorphic site were digested into two fragments, 276 bp and 55 bp, while those with G were not because of the absence of a PstI restriction site. Samples yielding 276 bp and 55 bp fragments were scored as AA, those with single 331 bp fragments as GG, and 331 bp, 276 bp and 55 bp as GA. This assay was illustrated in Fig. 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%.

2.4. 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 χ^2 test (for categorical variables). The 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 χ^2 test with degree of freedom equal to 1 among cases and controls, respectively. Pearson's χ^2 test was used to determine whether there was any significant difference in allele and genotype frequencies between patients and controls. The associations between PD-1.3 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 G allele of PD-1.3 was used as the reference in calculating ORs and 95% CIs. Probability levels less than 0.05 were used as a criterion of significance.