Int J Clin Exp Pathol 2015;8(11):15198-15203
www.ijcep.com /ISSN:1936-2625/IJCEP0011839

Original Article

Association of *PTEN* gene polymorphisms with liver cancer risk

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Received June 23, 2015; Accepted July 27, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Objective: To find out if there are any relationship between three single nucleotide polymorphisms (SNPs) of phosphatase and tensin homolog (*PTEN*) gene (rs1234213, rs1234220, and rs2299939) and the susceptibility of liver cancer. Methods: Genotypes of the three SNPs in the *PTEN* gene were achieved utilizing polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Comparison of genotypes and alleles distribution differences between the case and the control subjects was accomplished with χ^2 test. The analysis of linkage disequilibrium (LD) and haplotypes of the three SNPs was performed using SHEsis software. We adopted odds ratios (ORs) with 95% confidence intervals (95% CIs) to show the relative risk of liver cancer. Results: TC genotype and C allele of rs1234220 polymorphism showed much more frequently in cases than in controls, reflecting that the TC genotype and the C allele may be linked to the increased risk of liver cancer (OR=2.225, 95% CI=1.178-4.204; OR=1.941, 95% CI=1.124-3.351). Rs2299939 polymorphism showed an opposite result that the GT genotype probably reduce the risk of liver cancer (OR=0.483, 95% CI=0.259-0.900). Statistical significance was not found in the distribution differences of the genotypes of rs1234213 between two groups. LD and haplotype analysis results of the three SNPs showed that the T-C-G haplotype frequency was much higher in cases than in healthy objects, which proved that the T-C-G haplotype might be a susceptibility haplotype for liver cancer (OR=3.750, 95% CI=1.396-10.077). Conclusions: *PTEN* gene polymorphisms might relate to liver cancer risk.

Keywords: *PTEN*, polymorphism, liver cancer, haplotype

Introduction

Liver cancer has the characteristics of quick development and poor prognosis. The morbidity and mortality thereof are respectively at the seventh place and the fourth place among all malignancies all over the world [1, 2]. Approximately 55% new liver cases around the world occur in China, and about 40% of the global deaths caused by liver cancer are Chinese. China has the highest liver cancer mortality rate among countries all over the world [3]. This disease is often confirmed at an advanced stage due to its obscure early symptoms. Once the disease reaches the late stage, it will develops very quickly because the rapid spreading and metastasis of cancer cells. Five-year survival rate of liver cancer is only 3%~5%. Compared with other tumors, liver cancer is characterized by late diagnosis, low excision rate and survival rate. Therefore, liver cancer is still the focus of researches on the prevention and treatment of malignant tumors in China.

Occurrence and development of liver cancer is a complex multi-stage process involving a variety of factors, pathways and genes. Mutual effects between the genetic and environmental factors can activate the protooncogene and inactivate the anti-oncogene, which will finally lead to the occurrence of tumors. Studies have successively revealed that genes associated with liver cancer susceptibility mainly comprise four kinds: protooncogene, anti-oncogene, toxicant metabolizing enzyme gene, and DNA repair gene [4]. Anti-oncogene inactivation and protooncogene activation play leading roles in the complex process of canceration. They can enable mutant cells to obtain the advantage of selective growth and achieve continuous clonal

expansion, which will cause the canceration of the mutant cells. Anti-oncogene is a kind of allogene and can inhibit cell proliferation. When this function of the anti-oncogene is lost, tumors can be induced.

Phosphatase and tensin homolog (*PTEN*) gene is the second (the *p53* gene being the first) anti-oncogene that is found to have a high mutation frequency in human tumors [5]. Also named MMAC1 gene or TEP1 gene, *PTEN* gene is located on chromosome 10q23.3, has a total length of 200 kb, consists of 9 exons and 8 introns, and encodes a kind of protein composed of 403 amino acid residues [6-8]. *PTEN* gene is the first gene that has been discovered to encode proteins which have activities of both protein phosphatase and lipid phosphatase. This gene participates in the normal physiological and pathological process of tumors, and has close relationships with the differentiation, invasion, metastasis, and prognosis of tumors [9-12].

PTEN gene mutations have shown in many different tumors, which indicate the central roles of the gene in the occurrence and development of tumors. Nevertheless, there are few reports concerning the linkage between the *PTEN* gene and liver cancer risk in our country. Therefore, we chose three single nucleotide polymorphisms (SNPs) in the *PTEN* gene (rs1234213, rs1234220, and rs2299939) to investigate the correlation of liver cancer with heredity.

Materials and methods

Objects of study

This case-control study obtained the informed consent of all the included subjects as well as the permission of the Ethnics Committee of Shandong Provincial Hospital affiliated to Shandong University. The process of sample collecting accorded with the ethnical guidelines of National Human Genome Research Institute.

105 cases were confirmed with liver cancer in Shandong Provincial Hospital affiliated to Shandong University during January of 2013 to December of 2014 included 56 males and 49 females aged 25-72, and had a mean age of 45.8±7.2. Cases were all newly diagnosed, and related antitumor therapies such as radiotherapy, chemotherapy, and biotherapy had not been performed before they entered the hospital. After entering the hospital, they were diagnosed with liver cancer, using routine examinations, histopathological examinations, and(or) imaging examinations like B-ultrasonic wave, CT or nuclear magnetic resonance. Patients who ever experienced immunosuppressive therapies or have the following diseases were not accepted into our study: general acute or chronic inflammation, long-term use of nonsteroidal antiinflammatory drugs, malignant tumors in other parts of the body, extensively destroyed lungs; hepatitis A, C, D, and E, toxic hepatitis, autoimmune hepatitis, alcoholic hepatitis, drug hepatitis, primary and secondary biliary cirrhosis, recurrent liver cancer, metastatic liver cancer, Budd-Chiari syndrome (BCS), Wilson disease or infections of pathogens like HIV, HCV and syphilis, hepatic parasitosis, and liver diseases like hapatitis and liver cirrhosis induced by diabetes, fatty liver, metabolic disorder and vascular disease.

111 healthy individuals (60 men and 51 women, mean age 46.2±7.9 ranged from 23 to 75) were recruited from the physical examination populations of the above hospital during the same period. Case and control groups had the same nation, age and gender composition, similar smoking and drinking status, and no blood relationship was found among people in both groups. Physical examination reports of people in the control group showed that they had normal liver functions and no liver diseases. We ruled out people having the following medical histories or diseases: hepatitis virus infections, chronic inflammatory disease, tumors of all kinds, family history of diseases, medical histories of tumors and autoimmune diseases, and alcoholism history.

Clinical data of the research subjects and collection of DNA samples

Clinical characteristics like age, gender, and smoking and drinking histories of all subjects were recorded. According to the standards of WHO, those who smoke more than one cigarette every day for more than a year are considered to have a smoking history.

We collected 5 ml morning fasting peripheral venous blood from each person, then antico-

Table 1. Primer sequences

Gene	SNP	Primer sequence		Length
PTEN	rs1234213	Forward	5'-CTACATCTCTGATTCCTTAT-3'	291 bp
		Reverse	5'-TAAGGAATCAGAGATGTAGG-3'	
	rs1234220	Forward	5'-TTTGGAAATAGAAAAACCTT-3'	229 bp
		Reverse	5'-TAAGGTTTTTCTATTTCCAA-3'	
	rs2299939	Forward	5'-CTCAAACTCCTGACCTCGGG-3'	277 bp
		Reverse	5'-TCACCCGAGGTCAGGAGTTT-3'	

Table 2. Basic characteristics of the cases and the controls

Characteristics	Gender		Age ($\bar{x}$±s)	Smoking	
	Male	Female		-	+
Case n (%)	56 (53.33)	49 (46.67)	45.8±7.2	73 (69.52)	32 (30.48)
Control n (%)	60 (54.05)	51 (45.95)	46.2±7.9	78 (70.27)	33 (29.73)
P	>0.05		>0.05	>0.05	

agulated the blood using ethylene diamine tetraacetic acid (EDTA), and finally stored the blood samples in a refrigerator at -80°C. DNA extraction was performed with a genomic DNA extraction kit (Beijing Tiangen Biotech Co., Ltd), and the DNA samples were preserved at -20°C for future detection.

Genotyping with PCR-RFLP technique

Three pairs of primers of *PTEN* rs1234213, rs1234220, and rs2299939 polymorphisms were designed with Primer 5.0 software. Shanghai Sangon Biotech Co., Ltd accomplished the primer synthesizing. The forward and reverse primer sequences were described in **Table 1**.

Polymerase chain reaction-restriction length polymorphism (PCR-RFLP) technique was adopted to analyze the *PTEN* gene polymorphisms. In each 20 μl PCR reaction system, there were 2.0 μl 10× PCR Buffer liquid, 1.5 μl $MgCl_2$, 1.0 μl dNTP, 0.5 μl forward primer, 0.5 μl reverse primer, 0.5 μl Taq DNA polymerase, 2.0 μl template DNA, and the rest was filled with sterile water. PCR reaction conditions were presented as follows: predenaturation at 95°C for 6 min; denaturation at 94°C for 50 s, annealing at 56°C for 50 s, and extension at 72°C for 50 s (35 cycles); and extension at 70°C for 5 min. Nco I enzyme was applied to digest the PCR products. Agarose gel electrophoresis (AGE) (2-3%) was carried out at 90 V for 30 min, with the running buffer being 1× TAE. The results of AGE were observed by E-Gel imager so as to determine the genotypes of each SNP.

Statistical analysis

The recording and analyzing of the genotype and allele data were performed with PASW Statistics 18 software. Genotype and allele distributions were checked by Hardy-Weinberg equilibrium (HWE), and set the statistical significance level at *P*>0.05. We achieved analysis of the linkage disequilibrium (LD) and haplotypes of the three SNPs with SHEsis software. Chi-square test was used to detect whether there were any differences in distribution conditions of genotypes and alleles of the three SNPs between the case and control groups. Differences were regarded to be statistically significant when the *P* value was smaller than 0.05. Association strength between the three SNPs and liver cancer risk were evaluated by odds ratios (ORs) with 95% confidence intervals (95% CIs).

Results

Basic characteristics of the research subjects

As could be known from **Table 2**, the age, gender, and smoking conditions of the case group were not significantly different from those of the control group (*P*>0.05).

Relevance analysis of the PTEN gene polymorphisms and liver cancer susceptibility

Distribution conditions of genotypes and alleles of *PTEN* gene rs1234213, rs1234220, and rs2299939 polymorphisms in the case group and the control group are detailed in **Table 3**. HWE test of the three SNPs in the healthy controls was passed, which indicated the high representativeness of the control subjects.

TC genotype and C allele of *PTEN* rs1234220 may increase the risk of liver cancer (OR=2.225, 95% CI=1.178-4.204; OR=1.941, 95% CI=1.124-3.351). However, the GT genotype of *PTEN* rs2299939 may lead to reduced risk of liver cancer (OR=0.483, 95% CI=0.259-0.900). Meanwhile, we discovered no statistical signifi-

Table 3. Genotype and allele distributions of *PTEN* polymorphisms

SNP	Genotype/allele	Case n=105	Control n=111	χ^2 (*P*)	OR (95% CI)
rs1234213	CC	26 (24.76)	28 (25.23)	-	1
	CT	50 (47.62)	57 (51.35)	0.029 (0.865)	0.945 (0.491-1.819)
	TT	29 (27.62)	26 (23.42)	0.229 (0.633)	1.201 (0.566-2.547)
	C	102 (48.57)	113 (50.90)	-	1
	T	108 (51.43)	109 (49.10)	0.234 (0.628)	1.098 (0.753-1.601)
rs1234220	TT	68 (64.76)	89 (80.18)	-	1
	TC	34 (32.38)	20 (18.02)	6.213 (0.013)	2.225 (1.178-4.204)
	CC	3 (2.86)	2 (1.80)	0.548 (0.459)	1.963 (0.319-12.078)
	T	170 (80.95)	198 (89.19)	-	1
	C	40 (19.05)	24 (10.81)	5.802 (0.016)	1.941 (1.124-3.351)
rs2299939	GG	79 (75.24)	69 (62.16)	-	1
	CT	21 (20.00)	38 (34.24)	5.343 (0.021)	0.483 (0.259-0.900)
	TT	5 (4.76)	4 (3.60)	0.016 (0.899)	1.092 (0.282-4.228)
	G	179 (85.24)	176 (79.28)	-	1
	T	31 (14.76)	46 (20.72)	2.616 (0.106)	0.633 (0.402-1.093)

Table 4. Linkage disequilibrium and haplotype analysis of the three SNPs

SNP1-SNP2-SNP3	C-T-G	C-T-T	T-T-G	T-C-G
Case 2n=210	56	40	78	18
Control 2n=222	70	44	90	6
χ^2 (*P*)	-	0.205 (0.651)	0.114 (0.735)	7.530 (0.006)
OR (95% CI)	1	1.136 (0.653-1.977)	1.083 (0.681-1.723)	3.750 (1.396-10.077)

cance between rs1234213 polymorphism and liver cancer risk.

Relationship of the haplotypes formed by the three SNPs and liver cancer

We constituted the haplotypes of the three SNPs (rs1234213-rs1234220-rs2299939) according to the genotyping data thereof in both groups. 4 haplotypes which frequencies were higher than 0.05 were selected to analyze the association with liver cancer (**Table 4**). As it shown in **Table 4**, the T-C-G haplotype had much higher frequencies in the cases than in the controls (*P*=0.006), suggesting that individuals carrying the T-C-G haplotype can possibly have higher risk of liver cancer (OR=3.750, 95% CI=1.396-10.077).

Discussion

Liver cancer is one of the most common malignant tumors that pose great threat to the health and life of human beings. There are an average of more than 500,000 new liver cancer cases and about 1000,000 deaths caused by liver cancer each year worldwide. The incidence of liver cancer shows an upward tendency ever since the 1990s. The occurrence of liver cancer is due to interactions of genetic factors and environmental factors [13, 14]. Major risk factors of liver cancer include infection of chronic hepatitis viruses (hepatitis B virus (HBV) and hepatitis C virus (HCV)), hereditary liver disease, cirrhosis, non-alcoholic fatty liver disease (NAFLD), metabolic disease, diabetes, the intaking of aflatoxin B1 from food, long-term excessive drinking, smoking, long-term oral contraceptive taking among females, lack of detoxicating vitamins and selenium, chronic and inorganic lead poisoning, and iron-overload [15-17]. Liver cancer occurrence is the result of long-time exposure to risk factors and the accumulation of toxins. Liver cancer risk of an individual is depended, to a great degree determined by hereditary susceptibility. As the third generation of genetic markers, SNP is one of the most common genetic mutations of human beings. Genetic differences between individuals have become the major focus in researches, including the genetic susceptibility to liver cancer [18].

Genetic factors associated with the occurrence and development of tumors are very complicated, and may include the inactivation of the anti-oncogene, the activation of the protooncogene, epigenetic alteration, genomic instability, chromosome gain and deletion [19, 20]. Current studies have shown that the mutation, deletion or low expression of the anti-oncogene *PTEN* exist in the formation and development process of most tumors because the tumor suppression function thereof is affected [21-23]. It has been proved that the *PTEN* gene play an important role in the occurrence and development of liver cancer [24-26]. Park et al. carried out a study on the genetic changes of the *PTEN* gene in liver cancer occurrence, and demonstrated that the gene inactivation caused by mutation and allele drop-out of the *PTEN* gene was very significant in the pathogenetic mechanism of liver cancer [27].

PTEN gene encodes bispecific phospholipase, and can adjust and control the cell cycle, promote the cell apoptosis, inhibit the tumor angiogenesis and the invasion and metastasis of tumors through complex signal transduction network. *PTEN* gene is the only anti-oncogene which can achieve the dephosphorylation of the lipid, and is widely expressed in normal histocytes of the human body. *PTEN* polymorphisms can sharply reduce the phosphatase activity, enhance malignant proliferation and invasion abilities of liver cancer cells, promote cell motility and inhibit cell apoptosis, and thus aggregate the vicious transformation of cells. Genetic and epigenetic changes (including loss or abnormalities of the expression) of the *PTEN* gene in liver cancer are linked to the loss of heterozygosity and homozygosity, and hypermethylation or mutation of the promotor. Among the common polymorphisms in the *PTEN* gene, there are 12 in the intron region, 2 in the intron-exon boundary, 1 in the exon region, and the other 2 in the 3' UTR region [28].

In this study, we analyzed the correlation between *PTEN* rs1234213, rs1234220, and rs2299939 polymorphisms and liver cancer, and finally ascertained the existence of the correlation. The results of our study showed that there might present a linkage between the TC genotype and C allele of the *PTEN* rs1234220 and the raised risk of liver cancer. Meanwhile, GT genotype of rs2299939 polymorphism possibly protect against the occurrence and development of liver cancer. Besides, rs1234213 polymorphism might have no relevance with liver cancer occurrence. All these results reflected that the SNPs in the *PTEN* gene might be involved in the occurrence and development process of liver cancer. Furthermore, as to the linkage of the haplotypes of *PTEN* polymorphisms with the susceptibility of liver cancer, we found that the T-C-G haplotype can promote the liver cancer occurrence.

Through exploring the susceptibility genes of liver cancer, more knowledge about the pathogenesis of liver cancer can be obtained. Theoretical basis can also be provided for the prevention and treatment measures of liver cancer. However, the occurrence of liver cancer is not depended by a single gene, it is caused by interactions of mutations in several different genes. Further studies should pay more attention to gene-gene interactions and gene-environment interactions, so that the understanding of liver cancer can be deepened and better prevention methods can be obtained.

Disclosure of conflict of interest

None.

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