

Polymorphism of FOXP3 in Egyptian Patients with Hepatocellular Carcinoma (HCC)

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Abstract

Aim: This case-control study was designed to investigate the association between Forkhead box P3 (FOXP3) gene polymorphism (-3499A/G, -3279C/A and -2383C/T) and the risk of Hepatocellular carcinoma (HCC).

Subject and Methods: Genotyping polymorphism is performed using restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) in 100 Egyptian HCC patients and 100 healthy individuals.

Results: Analysis of FOXP3 (-3499 A/G) polymorphism showed a significant increase in A allele ($p < 0.05$) in healthy individuals. In contrast, frequency of G allele is significantly elevated ($p < 0.05$) in HCC patients which might be risk factor for HCC (OR = 4.5). FOXP3 -3279C/A polymorphism showed a significant increase in C allele ($p < 0.01$) in healthy individuals while the frequency of A allele is significantly elevated ($p < 0.01$) in HCC patients which might be risk factor for HCC (OR = 4.6). Genotyping of FOXP3 -2383C/T showed that the C allele was higher than T allele in controls which is increased in HCC patients. Although; no significant differences in allele frequencies between HCC patients and controls.

Conclusion: Taking into consideration that genetic polymorphisms are population specific, our data stressed the importance of FOXP3 gene polymorphism in developing HCC. Our pilot study indicates that -3279C/A and -3499A/G polymorphisms in the FOXP3 gene might play a role in HCC susceptibility in Egyptians. To the best of our knowledge, this study is the first one that examines FOXP3 polymorphism in HCC patients. Additional studies on larger population are needed to confirm our findings.

Keywords: FOXP3; HCC; HCV; Polymorphism; Egyptian

Abbreviations

α -FP: Alpha-Fetal Protein; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; CI: Confidence Interval; ELISA: Enzyme Linked Immunosorbent Assay; FOXP3: Forkhead Box P3; Hb: Hemoglobin; HBV: Hepatitis B Virus; HbsAg: Hepatitis B Surface Antigen; HCC: Hepatocellular Carcinoma; HCV: Hepatitis C Virus; OR: Odd Ratio; INR: International Normalization Ratio; Plt: Platelet Count; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; SNP: Single Nucleotide Polymorphism; Treg: Regulatory t Cell; TAE: Tris-Acetate-EDTA; WBCs: White Blood Cells

Introduction

Hepatocellular carcinoma (HCC) is considered as the fifth most common cancer and the third leading cause of cancer-related death worldwide, resulting in about 500000 deaths annually [1,2]. Despite advances in the diagnosis and treatment of HCC, a poor prognosis

with a five-year survival rate of 5% in developing countries is still unsolved problem [3]. Major risk factors for HCC development are infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), liver cirrhosis, habitual alcohol abuse, high cigarette smoking, exposure to aflatoxin and host genetic factors. The highest prevalence of HCV infection in the world was found in Egypt (~14.7%). The incidence of HCC is expected to grow largely due to HCV related cirrhosis [4]. Among the genetic factors that accelerate the progression of HCC, some single nucleotide polymorphisms (SNPs) might be considered as HCC risk factors [5-7].

Regulatory T (Treg) cells play an important role in immune regulation. Treg cells whose main functions are alleviation of inflammation and suppression of effector T cells help in keeping the balance between immunity and autotolerance. They essentially characterized by CD4+/CD25+/FOXP3+ expression [8]. Treg is believed to play a critical role in tumor immune evasion [9]. Its increase has been reported in a wide array of human malignancies, including HCC [10,11]. HCC patients had a high frequency of Tregs, and high numbers of Tregs correlated with a poor prognosis [12].

Forkhead box P3 (FOXP3) is a main regulatory factor for the development and functioning of Treg cells [13]. It acts as a main regulator in the development and function of Treg cells. Actually, the absence of a functional FOXP3 gene product has been disclosed to cause an abnormal production of Treg cells [14]. The FOXP3 polymorphism is associated with autoimmune disease such as allergic rhinitis [15], Graves' disease [16], and psoriasis [17], as well as breast cancer [18,19] and HCC [20].

The FOXP3 gene is located on the Xp11.23 locus on the X chromosome. SNPs in the promoter region of FOXP3 could affect the expression and activity of FOXP3 [21,22] decreasing or eliminating functional Treg. Studies on the association between FOXP3 polymorphism and HCC are very rare [23]. Thus, this study was performed to investigate the genotype distribution of FOXP3 (NC_000023.11) promoter SNPs -3499A/G (rs3761547), -3279C/A (rs3761548) and -2383C/T (rs3761549) in Egyptian HCC patients in order to illustrate their implication on the development/prognosis of HCC.

Subjects and Methods

Study population

In this work, we carried out a hospital-based case-control study by recruiting 100 HCC patients from El Demerdash Hospital, Ain-Shams University, Egypt. The 100 cancer free healthy individuals with no history of previous liver disease, normal liver function tests, and negative HBV infection were enrolled in the study. Both HCC patients and healthy individuals have the same ethnic background. All investigations were done in accordance with the Ain-Shams University, Health and Human Ethical Clearance Committee guidelines for Clinical Researches. Local ethics committee approved the study protocol.

All HCC patients were suffering from HCV infection. They were subjected to proper full history recording, comprehensive clinical examination, routine laboratory screening including liver function tests, and abdominal ultrasound of the liver. Tumor characteristics were detected by ultrasound with or without computing tomography scan and determination of markers of viral infections and autoimmune diseases. HCV antibodies were tested by using enzyme-linked immunosorbent assay (ELISA) (Murex Biotech Ltd., Dartford, UK). Confirmation of the presence of serum viral HCV-RNA was determined by polymerase chain reaction (PCR) (Agpath-IDTM one step RT-PCR kit; Ambion, USA). Amplification conditions were; 45°C for 10 minutes (RT) followed by 45°C for 10 minutes (initial denature) and 40 cycle of 95°C for 15 seconds and 60°C for 60 seconds. Genotyping was done using innoLiPA (Innogenetics, Zwijndrecht, Belgium) according to the manufacturer's instructions. Hepatitis B surface antigen (HBsAg) was detected using a commercial kit (Sorin Biomedica, Milan, Italy). All patient groups were HCV antibody and HCV RNA positive and were negative for HBsAg.

Clinical, biochemical characteristic data as well as related risk factor including age, presence of HCV or HBV, total and direct bilirubin, albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinin, white blood cells (WBCs), hemoglobin (Hb), platelet count (Plt) and international normalization ratio (INR) and alpha-fetal protein (α -FP) were determined.

DNA Extraction

Five ml of EDTA-venous blood was collected from each subject into a sterilized vacutainer tubes. DNA was extracted by Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, USA) according to manufacturer’s protocol. The purified genomic DNA was examined using 1% agarose gel electrophoresis and ethidium bromide staining. The extracted DNA was stored at -20°C until further use.

Genotyping of FOXP3 polymorphism

Genotyping of -3499 A/G, -3279 C/A and -2383 C/T were performed by PCR-based restriction fragment length (RFLP) method [24]. Primer sequences were summarized in table 1. The reaction was performed in one tube with a final reaction volume 25 µl. PCR mixtures consisted of DreamTaq Green PCR Master Mix (2X) (Fermentas Life Science, Thermo Fisher Scientific Inc., MA, USA), 10 pmoles of each primer, and 200 ng of DNA. All PCR reactions were performed in a Biometra thermal cycler (Biometra GmbH, Germany). The conditions of amplification were: 94°C for 5 minutes and 30 cycles of denaturing at 94°C for 30s, annealing at 55°C for 30s, extension at 72°C for 30s and final extension at 72°C for 10 minutes (for -3499 A/G); 5 minutes at 94°C and 30 cycles of denaturing for 30s at 94°C, annealing for 30s at 68°C, extension for 30s at 72°C and a single final extension for 10 minutes at 72°C for (-3279C/A). For -2383C/T, initial denature at 95°C for 3 minutes followed by 30 cycles (denaturing at 94°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 1 minutes) then single final extension at 72°C for 10 minutes. PCR products were digested with proper restriction enzyme (Table 1) and visualized by agarose gel electrophoresis (4%) in 0.5X Tris-acetate-EDTA (TAE) buffer with ethidium bromide staining (10 mg/ml) under an ultraviolet illuminator and was determined proportional to the migration of a proper DNA step ladder (100 or 25bp) (Fermentas) (depending on the size of fragmented DNA).

Position	Primer sequence	PCR Product	Enzyme	Restriction product
-3499A/G (rs3761547)	Forward: 5'-CTCTGGCTCTCCATGCATGT-3' Reverse: 5'-TGCAGGGCTTCAAGTTGACAG-3'	158 bp	<i>PvuII</i>	A: 158 bp G: 136 bp and 22 bp
-3279C/A (rs3761548)	Forward: 5'-CCTCTCCGTGCTCAGTGTA-3' Reverse: 5'-CTCACCTAGCCCAGCTCTTG-3'	300 bp	<i>PstI</i>	C: 300bp A: 159bp and 141bp
-2383C/T (rs3761549)	Forward: 5'-GCCTGGCACTCTCAGAGCTT-3' Reverse: 5'-GTCTGTGGAGGCTCCGAACA-3'	942 bp	<i>BsrI</i>	C: 528bp and 377bp T: 213bp and 164bp

Table 1: Primers used in the genotyping by PCR-RFLP.

Statistical analysis

Statistical Package for Social Science (SPSS) version 19 (LEAD Technology Inc.) were used for all statistical analyses. Total and direct bilirubin, albumin, AST, ALT, creatinin, WBCs, Hb, Plt, and INR were reported as mean ± standard deviation. T-test was used to compare the distribution of these variables. The comparison of genotype and allele frequency between HCC patients and healthy individuals were analyzed by Chi-square test. Odds (OR) of genotype and allele of 95% confidence interval (95% CI) in HCC subjects versus healthy individuals were likewise calculated. The haplotype frequencies were estimated using SNPSTAT (<http://bioinfo.iconologia.net/snpstats/start.htm>). Results were considered significant at p < 0.05.

Results

Characteristics of the study population

Patient characteristics and clinical features are summarized in tables 2 and 3. One hundred healthy individuals without any history of liver disease served as normal controls. No significant difference in the albumin, creatinin and hemoglobin levels between HCC group and

healthy group. There was a significant increase in total bilirubin ($p < 0.001$), direct bilirubin ($p < 0.01$), AST ($p < 0.001$), ALT ($p < 0.001$), WBCs ($p < 0.001$), platelets count ($p < 0.001$) and INR ($p < 0.001$) in the HCC group compared with healthy group.

	Controls (No = 100)	HCC (No = 100)	P value
Tot-bilirubin (mg/dL)	0.91 ± 0.33	2.20 ± 3.35	P < 0.001
Dir-bilirubin (mg/dL)	0.13 ± 0.13	0.95 ± 2.54	P < 0.01
Albumin (g/L)	3.38 ± 1.33	3.13 ± 0.84	NS
AST (IU/L)	18.4 ± 6.86	48.1 ± 45.9	P < 0.001
ALT (IU/L)	19.0 ± 6.84	38.7 ± 35.4	P < 0.001
Creatinine (mg/dL)	0.83 ± 0.33	0.82 ± 0.44	NS
WBCs(1000/mm ³)	4.24 ± 1.13	5.58 ± 3.23	P < 0.001
Hb(mmol/L)	11.5 ± 2.09	11.4 ± 3.8	NS
PLT(1000/mm ³)	257.5 ± 94.2	144.1 ± 91.0	P < 0.001
INR%	0.98 ± 0.47	1.2 ± 0.52	P < 0.001
AFP	9.82 ± 2.68	251.27 ± 90.24	P < 0.01
P53	5.43 ± 0.29	60.82 ± 3.52	P < 0.001

Table 2: Biochemical characteristics of HCC patients and healthy controls.

All data are presented as mean ± SD. Total bilirubin (Tot-bil), Direct bilirubin (Dir-bil), Albumin (Alb), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Creatinine (Cr), White blood cells (WBCs), Hemoglobin (Hb), Platelet (PLT), International normalization ratio (INR), Alpha-fetoprotein (AFP).

Parameter	HCC patients (N = 100)
Number of Focal Lesion	
1	61%
2	11%
3	6%
Multiple	22%
Size of focal lesions (Mean ± SD)	4.84 ± 2.33
Portal vein thrombosis	
No	86.9%
Main LT	4.0%
Main RT	3.0%
Segmental LT	3.0%
Segmental RT	1.1%
Main PV	2.0%
Ascites	
No	84.8%
Mild	7.2%
Moderate	4.0%
Sever	4.0%
Child classification	
A	55.8%
B	29.9%
C	14.3%

Table 3: Clinical characteristics of HCC patients.

Association between FOXP3 polymorphisms and HCC

Analysis of FOXP3 (-3499 A/G) polymorphism (Table 4) showed a significant increase in A allele ($p < 0.05$) in healthy individuals. In contrast, frequency of G allele is significantly elevated ($p < 0.05$) in HCC patients which might be risk factor for HCC (OR = 4.5). FOXP3

-3279C/A polymorphism showed a significant increase in C allele ($p < 0.01$) in healthy individuals. In contrast, frequency of A allele is significantly elevated ($p < 0.01$) in HCC patients which might be risk factor for HCC (OR = 4.6). Genotyping of FOXP3 -2383C/T showed that the C allele was higher than T allele in controls which is increased in HCC patients. Although; no significant differences in allele frequencies between HCC patients and controls.

Position	Controls	HCC	OR (95% CI)	P
-3499A/G (rs3761547)				
Allele Frequency (N,%)				
A	162 (97.5%)	97 (82.2%)	0.266 (0.079 - 0.88)	P < 0.05
G	4 (2.5%)	9 (7.8%)	4.5 (1.36 - 15.13)	P < 0.05
-3279C/A (rs3761548)				
Allele Frequency (N,%)				
C	147 (88.5%)	74 (62.7%)	0.217 (0.118 - 0.398)	P < 0.001
A	19 (11.5%)	44 (37.3%)	4.600 (2.500 - 0.430)	P < 0.001
-2383C/T (rs3761549)				
Allele Frequency (N,%)				
C	137 (82.5%)	92 (77.9%)	0.749 (0.414 - 1.353)	NS
T	29 (17.5%)	26 (22.1%)	1.33 (0.738 - 2.41)	NS

Table 4: Genotype and allelic frequency of FOXP3 SNPs (-3499A/G,-3279C/A and -2383C/T) in controls and HCC patients.

P: p value (significant); NS: Not Significant.

Haplotypes emerged (ACC, ACT, AAC, AAT, GCT and GCC) from the estimates of haplotype frequencies is presented in table 5. Both ACC is the most frequent haplotypes in both groups, and GCC was the least frequent. The frequency of the ACC haplotype showed a significant increase ($p < 0.01$) healthy individuals over the HCC patients. The ACC haplotype might be a protective factor for HCC (OR = 0.265). The AAT haplotype showed a significant increase ($p < 0.001$) in HCC patients over the healthy ones. The AAT haplotype might be a risk factor for HCC (OR = 16.3).

Haplotype	Controls	HCC	OR (95% CI)	P
ACC	70.1%	40.2%	0.264 (0.146 - 0.481)	< 0.001
ACT	12.9%	8.1%	0.725 (0.278 - 1.838)	NS
AAC	9.7%	8.6%	0.979 (0.324 - 2.379)	NS
AAT	2.6%	24.4%	16.3 (3.750 - 71.133)	< 0.001
GCT	3.0%	2.7%	1 (0.196 - 5.077)	NS
GCC	0.3%	1.2%	5.1 (0.241 - 107.620)	NS

Table 5: Haplotype frequency of the FOXP3 SNPs (-2383C/T,-3279C/A and -3499A/G) in controls and HCC patients.

Discussion

Treg cells are acquaint as T cells in charge of suppressing potentially deleterious activities of Th cells and are mainly characterized by the expression of FOXP3+ transcriptional factor. FOXP3+ acts as a main regulator in the development and function of Tregs [25,26]. The absence of a functional FOXP3 gene product revealed to cause regulatory T cells abnormal production [14]. It has been displayed that

FOXP3 is expressed not only in Treg but also in tumor cells of cancer patients; its expression level and function may symbolize a new mechanism of cancers immune evasion [27]. FOXP3 gene might be a tumor suppressor and its inactivation might contribute to the development of cancer. In other previously reported study, FOXP3 has a similar immunosuppressive effect in liver cancer [23]. To date a few publications are available regarding HCC and FOXP3 polymorphisms. Thus, our study pursued to evaluate FOXP3 gene polymorphisms and its association with the development of HCC.

Our results showed that the FOXP3 -3499 A/G (rs3761547) and -3279 C/A (rs3761548) polymorphism was significantly associated with HCC while FOXP3 -2383 C/T (rs3761549) polymorphism was not associated with HCC. The -3279 C/A (rs3761548), situated in the promoter region of the gene, might theoretically affect gene expression, resulting in FOXP3 mRNA instability [23].

Our data showed that the frequency of the FOXP3 -3499 (rs3761547) G allele is significantly elevated in HCC patients and might be risk factor for HCC. No previous results have been documented in cancer patients. Several studies have been performed on patients undergoing renal transplantation with contradictory results. Zhuo., *et al.* [28] studied genetic polymorphisms of FOXP3 SNP (rs3761547) in 114 Chinese renal transplant patients who were on tacrolimus (TAC)-based maintenance immunosuppression. They demonstrated that the TAC-induced acute nephrotoxicity was not associated with FOXP3 rs3761547 polymorphism. No significant difference in the genotype frequencies of rs3761547 variants between patients with and without rejection history in allograft rejection in renal transplantation [29]. The same results were reported by several other investigators on other diseases such as Hosseini., *et al.* [30] in Behcet's disease (BD) patients in Iranian population. Song., *et al.* [31] stressed on the importance of rs3761547 A allele is one of the major alleles associated with an increased risk of Psoriasis vulgaris (PV) in Chinese Han population. The study of Inoue., *et al.* [24] in autoimmune Thyroid patients found no change in genotype and allele frequencies of FOXP3 -3499AG A/G polymorphism among studied groups.

FOXP3 -3279C/A (rs3761548) A allele frequency could be considered risk factors for HCC. The FOXP3 gene rs3761548 (C/A) polymorphisms, located on the promoter region of the FOXP3 gene, is one of the most common SNPs [22]. The A allele correlates with a decrease in FOXP3 expression [24]. Therefore, HCC patients with the -3279AA genotype might have Treg cells with weak suppressor activity, as FOXP3 controls Treg function [32]; it might be severe for such patients to achieve a proper anti-cancer response. In accordance with the presented results, several studies have confirmed the association AA genotype and A allele on increasing cancer risk. The study of Jiang., *et al.* [33] which investigated the association between FOXP3 gene polymorphism and susceptibility to differentiated thyroid cancers (DTC) stressed on the association of A allele with high risk of DTC. They found an increase in AA/AC genotypes in cancer patients assuming that these genotypes might be a risk factor DTC in Chinese Han Population. AL-Hajaj and AL-Battat [34] study evaluated FOXP3 gene polymorphism in breast cancer patients from Iraq and they found a positive association for (AA) homozygous genotype in relation to breast cancer development. The same results were obtained on Triple Negative Breast Cancer (TNBC) by Lopes., *et al.* [19]. In the Chinese patients suffering from colorectal cancer (CRC), Chen., *et al.* [20] revealed that the AA, AC, and the combined A variant genotype (AA/AC) conferred a significantly greater risk of CRC. The data obtained by He., *et al.* [35] showed that the A allele of rs3761548 significantly increase the prohibition of developing non-small cell lung cancer (NSCLC) in Chinese Han population. On the other hand, the present study results are contradictory to Jahan., *et al.* [18] results which demonstrated a lack of association of FOXP3 -3279C/A polymorphisms with breast cancer formation.

Inoue., *et al.* [24] study on autoimmune thyroid patients suggests that -3279C/A FOXP3 polymorphism, a reduction in FOXP3 expression with the A allele correlates with indicating that the -3279AA genotype might contribute to the severity of the immune response. On the other side, Wu., *et al.* [36] study in Unexplained Recurrent Spontaneous Abortion (URSA) in the Chinese Han population which genotyped four common polymorphisms of FOXP3 gene in 146 unrelated URSA patients who had histories of at least two successive miscarriages with unexplained etiology to determine if functional polymorphisms at the FOXP3 loci are associated with URSA. They found that -3279C/A genotype was significantly different between the URSA group and control group. Hassannia., *et al.* [37] performed their study on Iranian patients suffering from Allergic Rhinitis (AR) and they demonstrated that FOXP3 -3279AA genotype and A allele in the patient group were increased significantly in comparison with the controls.

The frequency of the FOXP3 -2383C/T in our study shows insignificant change in the distribution of all genotypes between healthy subjects and patients. Our results are in accordance with, Jahan., *et al.* [18] in breast cancer Indian patients who found no association of FOXP3 -2383C/T polymorphisms with breast cancer. Sakaki., *et al.* [38] in HCV infection study showed that FOXP3 did not differ between patients with chronic HCV infection and healthy individuals. Our results are contradictory to the study of Chen., *et al.* [23] in hepatitis B-related HCC Chinese patients who suggested that FOXP3 -2383C/T in the FOXP3 gene might be associated with HCC. Their study pointed to a concept that FOXP3 polymorphism may create a susceptibility to chronic hepatitis B (CHB) and cirrhosis, with HCC just a result of this susceptibility. Also, in the study of Mojtahedi., *et al.* [39] in Colorectal Cancer patients, a significant association of metastatic colorectal cancer was observed with T allele in men and C/T (T/T) genotype in women and a significant association between metastasis and FOXP3 polymorphism. In PV patients, Song., *et al.* [31] suggested that the homozygote for major alleles of CC had high susceptibility to PV and high frequency homozygote increase the risk of PV. Also Inoue., *et al.* [24] study in autoimmune thyroid patients suggest that in the -2383C/T polymorphism, the distribution of alleles and genotypes did not differ between normal subjects and patients with autoimmune disease.

The role of Treg cells in HCV has been assessed, particularly in the chronic forms of the infection. There is strong evidence that viral persistence is associated with different populations of Tregs that mediate the suppression of virus-specific T cells in HCV infections [39]. Elevated levels of intra-hepatic and circulating FOXP3+ Tregs have been described in chronic hepatitis C [40-44]. Speletas., *et al.* [45] observed a significant increase of FOXP3 in both HCV and HBV patients compared to healthy individuals, which was positively correlated with the intensity of inflammation. Higher mRNA expression of FOXP3 was observed in the HCV patients, with maximum increase in cirrhotic patients. The increased FOXP3 mRNA expression was positively correlated with increased AST and ALT levels in HCV patients [46]. Compared to patients with active disease and no prior treatment, Germanidis., *et al.* [47] reported down-regulated liver mRNA expression of FOXP3 in patients maintained on-treatment following 5 years of remission.

Conclusions

We can conclude that our pilot study indicates to the importance of the genetic background in HCC Egyptian patients. Polymorphisms at -3499A/G (rs3761547) and -3279C/A (rs3761548) in the FOXP3 gene might be associated with HCC. Although, the sample size of the present association study is likely insufficient for detecting all the associations between these SNPs and HCC, A precise genetic association study involving multiple centers and regions may provide a sound foundation for further research into the involvement of the FOXP3 gene with liver cancer. Further research is also required to clarify the molecular mechanization by which FOXP3 affects the HCC development.

Conflict of Interest

The authors declare that they have no conflicts of interest concerning this article.

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