ORIGINAL ARTICLE

Association of Polymorphisms of X-ray Repair Cross-complementing 1 (XRCC1) Protein and Aflatoxin B1 (AFB1) in Egyptian Patients with Hepatocellular Carcinoma

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ABSTRACT

Background: *Hepatocellular carcinoma (HCC) is the most prevalent malignancy affecting developing populations Aflatoxin B1 (AFB1), an established carcinogen produced by certain strains of fungi, particularly Aspergillus parasiticus and Aspergillus flavus. The carcinogenic effects of AFB1 in hepatocellular carcinoma (HCC) are ascribed to AFB1-DNA adduct formation with p53 gene mutation.* ***Objective:*** *The present study aimed was to investigate the prevalence of single nucleotide polymorphism (SNP) in rs 25487 of X-ray cross complementing group 1 protein (XRCC1) in association with AFB1 in patients with hepatocellular carcinoma (HCC).* ***Methodology:*** *The study included 100 patients with HCC and 100 healthy control subjects. Blood samples were withdrawn from each subject and SNP in rs 25487 XRCC1 was estimated by real time polymerase chain reaction (PCR) and determination of blood levels of AFB1.* ***Results:*** *There was a dramatic increase in the level of aflatoxin B1 in patients (1.02±0.7ng/gm) compared to control subjects (0.8±0.6ng/gm), P=0.01. A significant increase in mutant genotypes CT and TT was found in HCC patients (43% and 7%, respectively) compared to control subjects (27% and 1%, respectively), with a notable increase in the dominant genotype CC in healthy controls (72%) compared to patients with HCC (50%), P=0.002. There was also a statistically significant increase in C allele (85.5%) in the control subjects compared to patients with HCC (71.5%) with a significant increase in T allele in patients (28.5%) compared to control subjects (14.5%), P=0.001. Furthermore, a significant difference in level of aflatoxin B1 was detected in patients with different genotypes of rs25487 XRCC1, with high levels of aflatoxin B1 found in patients with CT genotype (1.2±0.5), P=0.002.* ***Conclusion:*** *This study revealed the relationship of SNP of XRCC1 rs25487 with hepatocellular carcinoma, showing a significant association between this SNP and the increased level of aflatoxin B1 in patients with HCC.*

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent malignancy affecting developing populations with mortality associated with complications of HCC making it the third common cause of malignancy-related death [1,2]. Although HCC in Egypt is primarily caused by hepatitis C virus (HCV) infection, several non-viral factors such as tobacco and aflatoxin (AFB1) have been implicated in development of this aggressive cancer [3].

Aflatoxin B1 (AFB1), an established carcinogen produced by certain strains of fungi, particularly *Aspergillus parasiticus* and *Aspergillus flavus*, are known to contaminate inadequately stored human food products such as peanuts, corn, rice, and wheat[4]. Ingestion of these carcinogenic substances results in formation of AFB1 adducts associated with breakage of DNA strands, damage to DNA bases, as well as oxidative damage. The carcinogenic effects of AFB1 in hepatocellular carcinoma (HCC) are ascribed to AFB1-DNA adduct formation with p53 gene mutation[5]. While those adducts can be inhibited by the DNA repair system involved in base excision repair, strand break repair, and nucleotide excision repair[4], the carcinogenic effect of AFB1 is enhanced with increased amount of

DNA damage[6,7]. Consequently, this inter communication between AFB1 and DNA repair gene mechanism may be associated with development of HCC[3].

The DNA repair protein known as X-ray repair cross-complementing group 1 protein (XRCC1) promotes protection of cells against ionizing radiation and chemical agents by interacting with several repair enzymes, including DNA repair enzyme, DNA ligase III, DNA polymerase b and poly (ADP-ribose) polymerase (PARP), with resultant activation of the single-strand break repair (SSBR) pathway[8]. Therefore, it is possible that polymorphisms of XRCC1 genes may affect the efficacy of DNA repair. To date, three polymorphisms of XRCC1 at conserved sequences have been known[9].

Limited studies are currently available regarding the association of polymorphisms of XRCC1 encoding gene and AFB1 as a potential risk factor in development of HCC. As a result, this study group aimed to investigate the prevalence of a single nucleotide polymorphism (SNP) in rs 25487 of XRCC1 gene in association with AFB1 exposure in patients with HCC.

METHODOLOGY

This case-control study was conducted on 100 patients with hepatocellular carcinoma (HCC) recruited from Mansoura University Hospital in Mansoura, Egypt, during the period from January 2018 to January 2019. An additional 100 healthy subjects were recruited as a control group during the same study period. Criteria for patients included in this study were age above 18 years, negative virology for hepatitis B virus (HBV) and HIV, and diagnosis of HCC by clinical and radiological investigations such as magnetic resonance imaging (MRI) and computerized tomography (CT)[10]. Written consent was obtained from each study participant following approval by the ethical committee of faculty of medicine, Mansoura University.

Laboratory Investigation

Ten milliliters of blood were withdrawn from each participant and divided in two aliquots. One aliquot was used for determination of hepatitis C IgG by ELISA system (Roche-diagnostic, Basal, Switzerland), alpha fetoprotein (AFP) measurement by enzyme-linked immunosorbent assay (ELISA-DRG International Inc., USA.) and complete liver functions tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and albumin by spin react kits (tra. Sta. Coloma, 7. 17176 St. Esteve de Bas. GIRONA-Spain) and AFB1 by immunoassay (Ridascreen-R-Biopharm AG, Demstadt, Germany). The second aliquot was blood with EDTA for molecular study of XRCC1 SNP by real time PCR.

AFB1 measurement by ELISA

Aflatoxin B1 (AFB1) level in serum samples was estimated by (Ridascreen-R-Biopharm AG, Demstadt, Germany) after extraction by EASI-EXTRACTH R-Biopharm- AG An der Neuen Bergstraße 17 Darmstadt, Germany)

Aflatoxin immunoaffinity column (Scotland). The kit was a ready-to-use kit with microplate coated with antibodies specific for aflatoxin B1 to which serum was added. Wash was then performed and a second antibody bound to AFB1 and enzyme conjugate was added. Bound aflatoxin antibodies were competed by free aflatoxin and aflatoxin enzyme conjugate. Any unbound enzyme conjugate was washed and chromogen substrate was added to the wells and transformed into a blue product by bound enzyme conjugate. The yellow color that developed after adding the stop solution was measured by ELISA plate reader at 450 nm. The absorbance was inversely proportional to sample aflatoxin concentration. Results were expressed as ng/gm.

Real-time PCR for Arg-399Gln (rs 25487) SNP of XRCC1 DNA Extraction

DNA was extracted by the use of Qiagen min blood DNA extraction kit (Qiagen-Germany) 1500 District Avenue, Suite 2097, Burlington, MA 01803, USAa and DNA was stored at -20°C until amplification procedures.

Real Time PCR

The amplification and detection were performed by the use of real-time PCR master mix (Intron Biotechnology, Korea). The used concentration of extracted DNA was 20ng/µl in the quantitative PCR reaction mix. DNA concentration was determined by measuring the absorption at 260 nm using a Nanodrop spectrophotometer.(Thermofisher-Waltham, Massachusetts, USA)

The PCR mix per well consisted of 10µL of 2x master mix solution, 7µL nuclease-free water, 2µL PCR primers, TaqMan probe, and 1µL template DNA. The real-time PCR instrument (Applied Biosystems, SDS v 2.1 software, Step One System, RQ Manager 1.2) was programmed for denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 92°C for 15 seconds and annealing/extension at 60°C for 60 sec[11].

Statistical Analysis

Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 24. Quantitative data was presented as mean, standard deviations and ranges Descriptive statistical data was expressed as percentage. Comparison between percentages was done using Chi-Square test. P value was considered significant at $P<0.05$.

RESULTS

In this study, 100 hepatocellular carcinoma (HCC) patients with mean age ±SD 51.1±7.8 years and 100 healthy control with mean age ±SD 49.7±8.7 years were included. HCV IgG antibodies were positive in 79% of patients, while significantly elevated levels of ALT, AST and total bilirubin were found in patients when compared to control subjects (P=0.0001). In addition, patients demonstrated aflatoxin B1 (AFB1) levels 1.02±0.7 while controls exhibited reduced values of 0.8±0.6, a difference that was significant (P=0.01) (table 1).

Table 1: Comparison of demographic and laboratory data between patients with HCC and healthy control

Parameter	HCC group (n=100)	Control group (n=100)	P
Age (Mean±SD) years	51.1±7.8	49.7±8.7	P=0.2
Sex (No., %) Male Female	 67 (67%) 33 (33%)	 69 (69%) 31 (31%)	P=0.4
HCV-IgG (No., %)	79%	0 (0%)	
Albumin (Mean±SD) gm/dl	3.7±0.6	4.0±0.5	P=0.0001
ALT (Mean±SD) IU/l Median Quartile	 42.5 27-65	28.9±4.7	P=0.0001
AST (Mean±SD) IU/l Median Quartile	 55 35-84	27.9±3.9	P=0.0001
Bilirubin (Mean±SD) mg/dl	2.4±1.9	0.9±0.1	P=0.0001
Total leucocytes (Mean±SD) x10^3cmm/ml	7.2±4.5	7.5± 2.1	P=0.4
Aflatoxin B1 (Mean±SD) ng/gm	1.02±0.7	0.8±0.6	P=0.01
AFP (Mean±SD) ng/ml Median Minimum Maximum	 6.00 70 2000	3.8±2.5	P=0.0001

An considerable increase in the mutant genotypes CT and TT were found in patients (43% and 7%, respectively) compared to control subjects (27% and 1%, respectively), with a great increase in the dominant genotype CC in 72% of healthy controls compared to 50% of patients with HCC (P=0.002). Furthermore, a statistically increase in C allele (85.5%) was detected in control subjects compared HCC patients (71.5%) while the T allele was increased in patients (28.5%) compared to control subjects (14.5%) (P=0.001) (Table 2).

Table 2: Comparison of rs25487 XRCC1 genotypes between control and patients with HCC

rs25487 XRCC1	Control (n=100)		HCC (n=100)		OD-95%CI	P
	No.	%	No.	%		
CC	72	72%	50	50%	Reference 0.5-0.8	P=0.002
CT	27	27%	43	43%		
TT	1	1%	7	7%		
CT+TT	---	---	---	---		
C allele	71	85.5%	43	71.5%		P=0.001
T allele	29	14.5%	57	28.5%		

Regarding aflatoxin B1 (AFB1) levels in patients with different genotypes of rs25487 XRCC1, those with CT genotype demonstrated the highest level of AFB1 at 1.2±0.5 ng/gm (P=0.002) (Table 3). However, there was no characteristic difference between AFB1 levels in patients with regard to HCV-IgG presence or absence (P=0.3) (Table 4), nor was there any notable association between the presence of HCV IgG and different genotypes of rs25487 XRCC1 (P=0.8).

Table 3: Aflatoxin B1 level in relation to different rs25487 XRCC1 genotypes

rs25487 XRCC1	**Aflatoxin B1** (Mean±SD) ng/gm	**P**
CC (n=50)	0.8±0.7	
CT (n=43)	1.2±0.5	P=0.002
TT (n=7)	0.9±0.4	

Table 4: Aflatoxin B1 level in patients in relation to HCV-IgG

	Aflatoxin B1 (Mean±SD) ng/gm	**P**
HCV status		
Positive	1.0±0.7	P=0.3
Negative	1.1 ±0.6	

DISCUSSION

The hepatocarcinogenic efficacy of aflatoxin B1 (AFB1) occurs as an outcome of AFB1-DNA adduct formation associated with mutation of tumor suppressor gene p53, ultimately resulting in DNA damage, an effect countered by the defense mechanisms of the DNA repair gene 3. Based on this data, the current study group aimed to estimate the prevalence of rs25487 XRCC1 polymorphism in patients with HCC and its association with aflatoxin B1 (AFB1) level.

A significant increase in the mutant genotypes CT and TT were observed in patients with HCC when compared to control subjects in the current study, a finding that agree with an earlier report of increased mutant genotype TT and decreased wild genotype CC in patients with hepatocellular carcinoma[3]. Several previous studies had reported that about fifty single nucleotide polymorphisms (SNPs) in the coding region of XRCC1 gene were associated with substitutions of amino acids. Of these, the polymorphism in the rs25487 region of XRCC1 was linked to low DNA repair function [12-15], with reports of an association between this polymorphism and increased environmental risk for cancer development [13-15].

Moreover, it has been noted that the reduced ability for DNA repair consequent to gene polymorphism actually increases the carcinogenic effects associated with exposure to aflatoxin B1 (AFB1)[3], suggesting a possible AFB1 exposure-induced environment-gene interaction included in the carcinogenic process of HCC. The current study showed a significant difference for the level of AFB1 in patients with different genotypes of rs25487 XRCC1, with particularly incressing levels of AFB1 seen in patients with CT genotype. This finding perhaps demonstrated the increased AFB1 carcinogenic effects related to reduced DNA repair due to genetic polymorphism, a conclusion supported by several meta-analyses examining different exposure levels of AFB1 [14-16].

In Egypt, Hepatocellular carcinoma (HCC) is primarily attributed to HCV infection, a major health problem affecting this population. The current study demonstrated 79% positivity of HCV IgG antibodies in studied patients, a finding that concurs with the established fact that approximately 80% of HCC cases occur in the patients with cirrhosis or advanced fibrosis subsequent to either hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [17]. It has been noted that HCV-induced induction of hepatocarcinogenesis likely occurs due to occurence of a microenvironment of cirrhosis or cirrhotic tissue that provides the setting for the direct carcinogenic effect of HCV protein[18]. This hypothesis supports findings from our study of an insignificant difference in the occurence of different genotypes of XRCC1 rs25487 in patients with positive HCV IgG, noted that genetic factors related with DNA repair might not play a role in HCV pathogenesis. Confirmation of this finding will require further longitudinal studies.

Otherwise, the present study demonstrated insignificant differences in AFB1 levels between patients whatever presence or absence of HCV IgG, possibly indicating that HCV and AFB1 constitute separate extrinsic risk factors for induction of HCC. Further comprehension of HCC etiology and risk factor pathogenesis may support appropriate selection of investigative and therapeutic interventions [19].

CONCLUSION

This study examined the association of SNP of XRCC1 rs25487 with hepatocellular carcinoma, showing a notably association between this SNP and increased level of aflatoxin B1 in patients with HCC. further studies with a larger number of patients are required for further evaluation.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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