ORIGINAL RESEARCH

Urinary 8-hydroxydeoxyguanosine in relation to XRCC1 rs25487 G/A (Arg399Gln) and OGG1 rs1052133 C/G (Ser326Cys) DNA repair genes polymorphisms in patients with chronic hepatitis C and related hepatocellular carcinoma

Aida A Mahmoud[1]
Mohammed H Hassan[2]
Ali A Ghweil[3]
Amany Abdelrahman[4]
Asmaa N Mohammad[5]
Hesham H Ameen[6]

[1]Medical Biochemistry Department, Faculty of Medicine, Sohag University, Sohag, Egypt; [2]Medical Biochemistry Department, Faculty of Medicine, South Valley University, Qena, Egypt; [3]Tropical Medicine and Gastroenterology Department, Faculty of Medicine, South Valley University, Qena, Egypt; [4]Medical Physiology Department, Faculty of Medicine, Sohag University, Sohag, Egypt; [5]Tropical Medicine and Gastroenterology Department, Faculty of Medicine, Sohag University, Sohag, Egypt; [6]Clinical Pathology, Faculty of Medicine, Al-Azhar University (Assiut Branch), Assiut, Egypt

Correspondence: Mohammed H Hassan
Medical Biochemistry Department, Faculty of Medicine, South Valley University, Qena, Egypt
Tel +20 109 847 3605
Email Mohammedhosnyhassaan@yahoo.com

Background and aim: DNA repair represents a protective mechanism against cell injury and cancer. 8-hydroxy-deoxyguanosine (8-OHdG) is the main ROS-induced DNA mutation. The current study aimed to evaluate urinary 8-OHdG levels in patients with chronic hepatitis C virus (HCV) and its related hepatocellular (HCC) and correlate its level to XRCC1 rs25487 G/A and OGG1 rs1052133 C/G gene polymorphisms.

Materials and methods: Urinary 8-OHdG assays were performed using HPLC technique, and XRCC1 rs25487 G/A and OGG1 rs1052133 C/G gene polymorphisms were analyzed by PCR using confronting two-pair primer method (PCR-CTPP) in 200 subjects allocated into 50 chronic HCV patients, 50 HCV-related HCC patients, and 100 controls.

Results: There were significantly increased urinary 8-OHdG levels in HCV-related HCC and chronic HCV patients when compared with the controls ($P<0.05$ for all). Urinary 8-OHdG was associated with the tumor spread. Regarding, XRCC1 (Arg399Gln), AA (Gln/Gln) genotype and A-allele were more frequent in HCC and chronic HCV patients than in the controls ($P<0.05$). ORs (95%CI) using the dominant and the recessive genetic models were; 2.1 (1.1–4.1), $P=0.032$ and 1.9 (1–3.6), $P=0.043$ respectively. For OGG1 (Ser326Cys), GG (Cys/Cys) genotype and G-allele were increased significantly in chronic HCV and HCC patients compared to the controls ($P<0.05$). ORs (95%CI) under the dominant and the recessive genetic models were; 2.1 (1.1–4.1), $P=0.032$ and 1.9 (1–3.8), $P=0.049$ respectively. Additionally, XRCC1 (AA) and OGG1 (GG) genotypes had significantly increased urinary 8-OHdG levels among patients ($P<0.05$).

Conclusions: XRCC1 (AA) and OGG1 (GG) could be considered as possible genotypic risk factors for HCV- related HCC development which were associated with significantly high urinary 8-hydroxy-deoxyguanosine levels, thus urinary 8-OHdG could be considered as non-invasive marker in follow-up chronic HCV progression into HCC.

Keywords: urinary 8-hydroxy-deoxyguanosine, high performance liquid chromatography, confronting two-pair primers, XRCC1 and OGG1 SNPs, DNA repair, hepatocellular carcinoma

Introduction

About 50–80% of patients infected by hepatitis C virus (HCV) develop chronic inflammatory disease process and hepatocellular carcinoma (HCC).[1] Chronic hepatitis C is the leading indication for liver transplantation in many parts of the world.[2,3] The number of people who are positive for HCV RNA was estimated as

1% (range: 0.8–1.14%) or approximately 71 million individuals (range: 62–79 million) worldwide.[4]

HCC commonly occurs in the setting of chronic injury and inflammation that promote DNA damage and chromosomal aberrations. DNA damage response (DDR) pathways coordinate DNA repair, cell cycle arrest, and cell death or senescence. Aberrations of DDR may affect the genes integrity, leading to hepatic injury and HCC development.[5]

Base excision repair and the nucleotide excision repair are two main DNA repair pathways. X-ray repair cross-complementing group 1 (*XRCC1*) and 8-oxoguanine DNA glycosylase (*OGG1*) are genes involved in the base excision repair pathway. Single nucleotide polymorphisms (SNPs) in the genes involved in DNA repair pathways could influence susceptibility to oxidative damage and carcinogenesis.[6]

The *XRCC1* gene is located on chromosome 19 (q13.2-13.3) and spans a genetic distance of 33 kb. It has17 exons, encoding a protein consisting of 633 amino acids and its molecular weight about 70kDa.[7] This protein interacts with three main enzymes involved in DNA single-strand break repair; poly(ADP-ribose) polymerase-1, DNA polymerase h and DNA ligase III. *XRCC1* rs25487 G/A gene polymorphism is located at the C terminus of the BRCT I domain and results in two different amino acids (Arg399Gln). The A-allele encodes for glutamine while the G-allele encodes for arginine.[8]

The *OGG1* gene is located on the short arm of chromosome 3 (3p25) and encodes 8-hydroxyguanine glycosylase that excises 8-Oxodeoxyguanosine (8-OHdG) that results from exposure to reactive oxygen species.[9] An elevated 8-OHdG content in DNA has been shown to increase the cancer risk.[10] *OGG1* gene polymorphism rs1052133 C/G (Ser326Cys) codes for two different amino acids at this site; C-allele codes for serine (Ser) and G-allele codes for cysteine (Cys). OGG1 326 Cys/Cys was reported as a low-expression genotype for 8-hydroxyguanine glycosylase and associated with human cancer susceptibility.[11] Urinary 8-OHdG is a sensitive marker of the DNA damage and a prognostic factor in HCC. A strong association was found between 8-OHdG expression and poor patient survival and clinical staging in HCC patients.[12]

As DNA repair plays a protective mechanism against cellular injury and cancer, the present study aimed to investigate the oxidative DNA damage in the form of urinary 8-OHdG levels in chronic HCV infection and HCV-related HCC, in addition to, two DNA repair enzymes genes polymorphisms rs25487 G/A (Arg399Gln) of *XRCC1* and rs1052133 C/G (Ser326Cys) of *OGG1* genes, since there

is no literature that directly points to correlation between oxidative damage levels and polymorphisms in HCC.

Materials and methods
Participants
The current cross-sectional, case-control, hospital-based study has been conducted on 200 subjects categorized into three groups: Group A included 50 chronic HCV patients without cirrhosis, group B included 50 patients with HCV-related HCC recruited from the Tropical Medicine Departments, Sohag and South Valley University Hospitals, Egypt. Group C included 100 healthy individuals who were unrelated to patients, with no history of liver disease (negative for HBsAg and HCV antibody with normal liver function tests and normal abdominal ultrasound), age and sex-matched to the patients as a control group. Selected controls should not take any type of medications through the last three months to avoid any confounding factors that may affect the results. All enrollees gave written informed consent and the ethical committee of Sohag and Qena Faculties of Medicine, Egypt approved this study, and it was conducted in accordance with the Declaration of Helsinki. We adjusted the size of the sample to achieve 80% power and 5% confidence of significance (type I error).

Diagnosis of chronic HCV was based on persistence of HCV antibodies and HCV RNA in the serum of the patients for more than 6 months. Cirrhosis was diagnosed with liver fibroscan, abdominal sonography and biochemical evidence of parenchymal damage with or without endoscopic evidence of esophageal or gastric varices.[13] The HCC diagnostic criteria were based on the guidelines proposed by European Association for the Study of the Liver (EASL).[14] Patients with associated other comorbidities eg, kidney disease, were excluded from the study.

Laboratory workup
Routine laboratory investigations including HCV antibody, HBsAg, ALT, AST, serum albumin and prothrombin concentration were done for each participant.

Biochemical analysis of urinary 8-OHdG
Biochemical assays of of 8-OHdG in human urine samples were performed using high performance liquid chromatography (HPLC; Agilent Technologies 1200 Series, G1315D DAD; Agilent Technologies, Santa Clara, CA, USA). The urinary 8-OHdG levels were measured

according to Kawai et al,[15] with slight modifications, as done in a previously published work.[16] Frozen urine samples were defrosted at room temperature and properly mixed. A 50 μL aliquot of urine was mixed with the same volume of diluents containing the ribonucleoside marker 8-OHdG (120 μg mL^{-1}) and 4% acetonitrile (ACN) in 130 mM sodium acetate (pH 4.5) and 0.6 mM sulphuric acid. The pH of the diluted urine solution should be below 7 to obtain good separation. The urine solutions were incubated in a refrigerator at 5° C for 2–3 hours. The diluted urine samples were centrifuged at 13,000 rpm for 5 minutes using a cooling centrifuge. After centrifugation, the sample was injected automatically on an anion exchange column of HPLC (Zorbax Extend-C18 Analytical 4.6×150 mm 5-μ, Agilent Technologies, Santa Clara, CA, USA). The detector diode array detector (DAD) was at wavelength of 254 nm. The HPLC conditions for the HLC were as follows: mobile phase: 100% (NaOAC 50 mM), pH 4.6; column temperature: 35ºC; flow rate: 1.5 ml/min. The concentration of 8-OHdG was calculated from the obtained standard curves (Figure 1 and the Figure S1).

Blood collection and DNA extraction

Venous blood samples were collected in EDTA tubes, centrifuged at 3000 rpm. DNA was extracted from the puffy coat of blood samples using DNA extraction kit
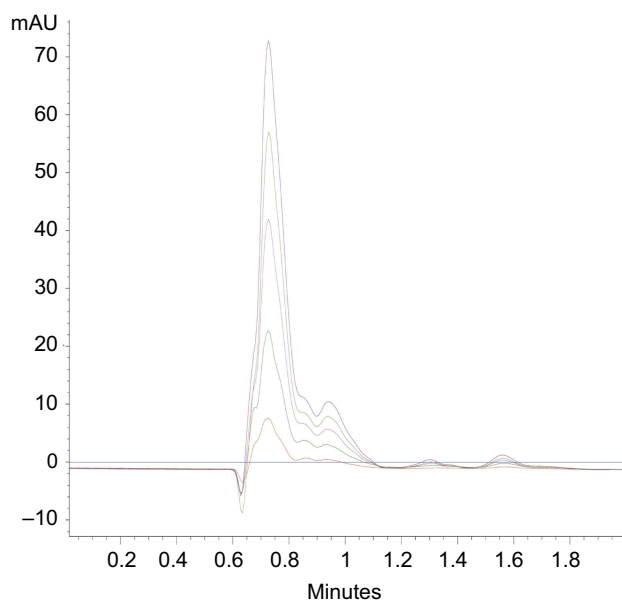

Figure 1 Calibration curves for high performance liquid chromatographic (HPLC) assay of 8-OHdG at concentrations 1, 5, 10, 15 and 20 mg/l.
Notes: Y-axis represents absorbance in mU, while X-axis represents retention time in minutes.

supplied by Jena Bioscience (Jena, Germany) as describedby the enclosed instructions.

Genotyping

DNA was amplified by the polymerase chain reaction (PCR) using the confronting two-pair primer method (PCR-CTPP).[17] This method involve the using of confronting pairs of primers (four primers in all) to determine the genotype of a SNP according to the different sizes of the generated products, without the need for sequencing or restriction enzymes, thus saving time and cost. In addition, PCR-CTPP is better than PCR-RFLP in terms of the possibility of genotyping misclassification.[17] The primer-sequences were shown in (Table 1). For the amplification, 25 μL reaction mixture containing 100–200 ng genomic DNA, 0.5 unit of Taq DNA polymerase, 2.5 of 10 x PCR buffer, 1 μL of 15 mM MgCl$_2$, 3 μL of dNTPs mix (10 mM each) and 10 pM of each primer was used.

The cycling conditions of XRCC1 rs25487 genotyping were 5 min of initial denaturation at 94°C, followed by 30 cycles of 30 seconds at 94°C, 1 minute at 64°C 45 seconds at 72°C, and a final extension of 10 minutes at 72°C. For *OGG1* Ser326Cys genotyping, the cycling conditions were 10 minutes of initial denaturation at 95°C, followed by 30 cycles of 1 minute at 95°C, 1 minute at 62°C, 1 minute at 72°C and a final extension of 5 minutes at 72°C. The amplified DNA was visualized on a 2% agarose gel stained with 2 μL/100 mL ethidium bromide. The *XRCC1* Arg399Gln polymorphism was genotyped as a 447-bp band for the G (Arg) allele, a 222-bp band for the A (Gln) allele, and a 630-bp common band. The *OGG1* rs1052133 polymorphism was genotyped as a 252-bp band for the C (Ser) allele, a 194-bp band for the G (Cys) allele, and a 406-bp common band (Figures 2 and 3).

Statistical analysis

All statistical calculations were performed using the IBM SPSS version 20.0 software (IBM corporation, Armonk, NY, USA). The data were normally distributed. We represented the data as: mean ±SD or number and percent. Deviation from HWE (Hardy–Weinberg equilibrium) was tested by chi-squared analysis. The chi-squared test was also used to test the statistical significance of the association. Continuous data were compared using ANOVA with Tukey's post-hoc test. We considered a two-tailed *P*-value of less than 0.05 statistically significant. Odds ratio (95% CI) was calculated using the dominant and the recessive genetic models.

Table I Primers used for the target genomic DNA amplification

	Primers	Accession number
XRCC1 rs25487	F1: 5-TCC CTG CGC CGC TGC AGT TTC T-3 R1: 5-TGG CGT GTG AGG CCT TAC CTC C-3 F2: 5-TCG GCG GCTGCC CTC CCA-3 R2: 5-AGC CCT CTG TGA CCT CCC AGG C-3	NC_000019.10
OGG1 rs1052133	F1: 5-CAG CCC AGA CCC AGT GGA CTC-3 R1: 5-TGG CTC CTG AGC ATG GCG GG −3 F2: 5-CAG TGC CGA CCT GCG CCA ATG −3 R2: 5-GGT AGT CAC AGG GAG GCC CC −3	NC_000003.12
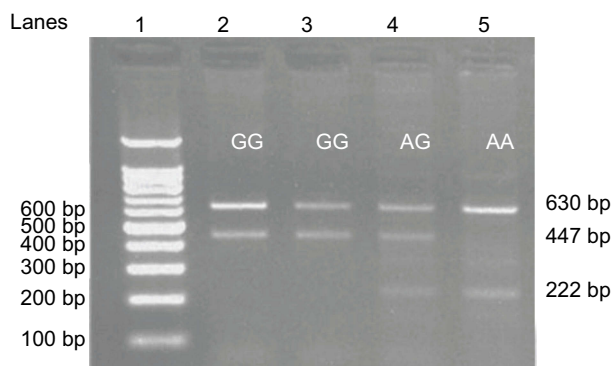
Figure 2 Detection of *XRCC1* rs25487 A/G (Arg399Gln) single nucleotide polymorphism using the confronting two-pair primer method (PCR-CTPP).
Notes: Lane 1: 100 bp DNA ladder. Lane 2,3 are wild type homozygote (GG), non-mutant (447-bp and 630-bp). Lane 4 is mutant heterozygote (AG) (222-bp, 447-bp and 630-bp).Lane 5 is mutant homozygote (AA) (222-bp, and 630-bp).
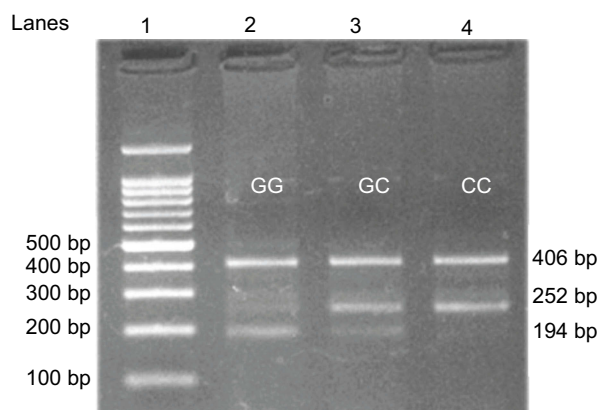
Figure 3 Detection of *OGG1* rs1052133 C/G (Ser326Cys) single nucleotide polymorphism using the confronting two-pairprimer method (PCR-CTPP).
Notes: Lane 1: 100 bp DNA ladder. Lane 2 is mutant homozygote (GG) (194-bp and 406-bp). Lane 3 is mutant heterozygote (GC) (194-bp, 252-bp and 406-bp). Lane 4 is wild type homozygote (CC), non-mutant (252-bp and 406-bp).

Results

The current study has been carried out on 200 subjects who were allocated into three groups; group A included 50 patients with HCC: 32 patients (64%) were males and 18 (36%) were females; group B included 50 patients with

chronic HCV: 72% were males and 28% were females. They were comparable with group C which included 100 healthy controls: 68 (68%) subjects were males and 32 (32%) subjects were females. The mean ages of the three groups were 55.7 years ±11.9, 43 years ±11.8 and 50.7 years ±9 respectively with non-significant differences regardingage and sex, indicating age and gender matching (Table 2). Their serum levels of liver enzymes, serum albumin and prothrombin concentration were presented in (Table 2)

There were significantly higher mean ±SD urinary levels of 8-OHdG among group A (203.4 mg/L ±25.2) and group B (135.2 mg/L ±30.9) when compared with group C (28.4±7.7) ($P<0.05$ for both) with significantly higher levels among group A vs group B (<0. 05), (Table 2).

Genotypes and alleles distribution study revealed no deviation from HWE for XRCC1 *rs25487* and *OGG1* rs*1052133* gene polymorphisms. XRCC1 *rs25487* genotypes distribution showed a significantly higher frequency of AA and significantly lower GG genotypic frequency in chronic HCV and HCC patients vs controls (x^2=7.4, P=0.024), with non-significant differences between the three groups regarding the AG genotype (P>0.05). Additionally there were non-significant differences in genotypes distribution between HCC and chronic HCV patients (x^2=0.24, P=0.89). Frequency of G-allele decreased significantly with significant increase in A-allele frequency in patients' groups when compared to the controls (x^2=7.3, P=0.007). Regarding OGG1 rs1052133 genotypes distribution, the GG genotype and G-allele were more prevalent, with significant lower CC genotype and C allele, in patients than in the controls (P<0.05). Additionally there were nonsignificant differences in genotypes or allelic distribution between HCC and chronic HCV patients. ORs (95%CI) in all patients versus the controls using the dominant and the recessive genetic models were shown (Table 3). There were nonsignificant differences as regards the genotypic or allelic frequencies of both SNPs

Table 2 Demographic, clinical and laboratory data of the studied groups

Variables	Group A HCC (N=50)	Group B chronic HCV (N=50)	Group C controls (N=100)
Age (mean ± SD, years)	55.7±11.9	43±11.8	50.7±9
Gender, N (%) Males Females	32 (64%) 18 (36%)	36 (72%) 14 (28%)	68 (68%) 32 (32%)
ALT (mean ±SD, U/l)	68.5±21.6	61.7±12.4*	28.5±10.5*
AST (mean ±SD, U/l)	40.6±15	37.99±10.49*	25.42±6.79*
Albumin (mean ±SD, g/dl)	2.72±0.92	3.9±0.94*	3.8±0.8*
Prothrombin concentration (mean ±SD, %)	55.69±5.7	84.37±9.2*	94.68±5.5*
Cirrhosis, N (%)	50 (100)	0 (0)	–
Lobe affected, N (%) Single Both	36 (72%) 14 (28%)	– 	–
Number of lesions, N (%) Single Multiple	19 (38%) 31 (62%)	– 	–
Urinary 8-OHdG (mg/L)	203.4±25.2	135.2±30.9*	28.4±7.7*

Note: *$P<0.05$.
Abbreviations: HCC, hepatocellular carcinoma; 8-OHdG, 8-hydroxydeoxyguanosine; ALT, alanine transaminase; AST, aspartate transaminase.

in relation to the gender or liver function tests (ALT, AST, albumin and prothrombin concentration) or tumor spread.

Regarding patients with HCC who exhibited AA genotype for *XRCC1 rs25487*, they showed significant higher mean ±SD of urinary levels of 8-OHdG than those with AG or GG genotypes (229 mg/L ±28, 192 mg/L ±8 and 191 mg/L ±13 respectively) with similar findings among chronic HCV (149 mg/L ±30, 107 mg/L ±5, 102 mg/L ±7 respectively), $P<0.05$ for all. Regarding mean ±SD of urinary levels of 8-OHdG in relation to *OGG1* rs1052133 genotypes distribution, GG genotype revealed significant higher urinary excretion of 8-OHdG among HCC patients (239 mg/L ±12) and

Table 3 Genotypic and allelic frequencies of *XRCC1* rs25487 and *OGG1* rs1052133 among the studied groups

Genotypes and alleles	Group A HCC (N=50) N (%)	Group B HCV (N=50) N (%)	X^2 (P-value)	Group C Controls (N=100) N (%)	X^2 (P-value)	Dominant model OR (95%CI), P-value	Recessive model OR (95%CI), P-value
XRCC1 rs25487 AA AG GG	15 (30) 25 (50) 10 (20)	14 (28) 24 (48) 12 (24)	0.24 (0.89)	15 (15) 50 (50) 35 (35)	7.4 (0.024)	1.9 (1–3.6), 0.043	2.3 (1.15–4.65), 0.019
G-allele A-allele	45 (45) 55 (55)	48 (48) 52 (52)	0.18 (0.67)	120 (60) 80 (40)	7.3 (0.007)		
OGG1 rs1052133 CC CG GG	9 (18) 27 (54) 14 (28)	8 (16) 26 (52) 16 (32)	0.2 (0.9)	30 (30) 52 (52) 18 (18)	6.6 (0.037)	2.1 (1.1–4.1), 0.032	1.9 (1–3.8), 0.049
C-allele G-allele	45 (45) 55 (55)	42 (42) 58 (58)	0.18 (0.67)	112 (56) 88 (44)	6.2 (0.012)		

Table 4 Mean ±SD of urinary 8-OHdG levels (mg/L) according to genotypes among the studied groups

XRCC1 rs25487	AA	AG	GG	P-value
Group A (HCC)	229±28	192±8	191±13	<0.05
Group B (HCV)	149±30	107±5	102±7	<0.05
Controls	28±8	27±7	26±6	>0.05
OGG1 rs1052133	GG	CG	CC	
Group A (HCC)	239±12	204±18	201±27	<0.05
Group B (HCV)	171±9	138±26	134±31	<0.05
Controls	29±7	28±8	27±9	>0.05

patients with chronic HCV (171 mg/L ±9) when compared with CG (204 mg/l ±18 and 138 mg/l ±26 respectively) and CC genotypes (201 mg/l ±27 and 134 mg/L ±31 respectively), $P>0.05$ for all with nonsignificant differences among the controls (Table 4).

In addition, mean ±SD of urinary levels of 8-OHdG was significantly higher among HCC patients with both lobes affected (232.2±27) than one lobe (191.7±12), and in those with multiple malignant hepatic lesions (211±28) than those with single lesion (190±11), $P<0.05$ for both, (Table 5).

Discussion

Egypt is considered among the countries with highly prevalent HCV infection with 5.3 million subjects with HCV antibodies positive, of whom 3.7 million were positive for HCV-RNA.[18] Furthermore, HCC account for 11.75% of digestive tract malignancies and 1.68% of cancer in general among Egyptians.[19] HCC has an increasing incidence in Egypt due to the high prevalence of HBV and HCV as primary risk factors.[20]

8-hydroxy-deoxyguanosine is a key marker for measuring the effect of endogenous oxidative damage to DNA and plays a role in initiation and promotion of cancer.[21] Although oxidative stress occurs frequently in chronic hepatitis C patients, but its role in further progression into HCC is not well known.[22] The results of the current study showed an increase in urinary 8-OHdG level in patients with HCC and HCV patients than the controls with higher levels in HCC than in HCV group.

Table 5 Mean ±SD of urinary 8-OHdG levels (mg/L) in terms of tumor extension

Lobe affected		Number of lesions	
Single	Both	Single	Multiple
191.7±12	232.2±27*	190±11	211±28*

Note: *P<0.05.

In the current study, AA genotype and A-allele of *XRCC1* rs25487 G/A increased significantly in the patients with HCC when compared to the controls. The *XRCC1* gene encodes one of the major repair factors involved in base excision repair, as it fixes base damage and DNA single strand breaks caused by alkylating agents and ionizing radiation and *XRCC1* gene polymorphisms were found to be associated with the risk of several cancers. Many studies reported the association between *XRCC1* rs25487 G/A gene polymorphism and different types of cancer. *XRCC1* rs25487 G/A was reported to be associated with nonmelanoma skin cancer,[23] childhood lymphoblastic leukemia in Asians,[24] Hodgkin's disease,[25] and HCC.[26] The amino acid changes from Arg to Gln at codon 399 (exon 10) of *XRCC1* protein may alter the protein function. It has been shown that these changes are associated with significant alterations in DNA repair capacity and increase the susceptibility to several diseases.[27] In contrary, Mattar et al,[28] reported *XRCC1*Arg194Trp but not *XRCC1* Arg399Gln as associated risk factor for HCV related HCC development in Egypt that could be explained by the ethnic, geographic differences and the complex heterogeneity of HCC.

The findings of the current study also revealed risk association between *OGG1* rs1052133 C/G (Ser326Cys) gene polymorphism and chronic HCV related HCC. G/G (Cys/Cys) genotype and G- allele increased significantly in HCC patients when compared to the controls. *OGG1* rs1052133 C/G (Ser326Cys) gene polymorphism was found involved in base excision repair and was found associated with the risk of bladder,[29] gallbladder,[30] lung,[31] and breast[32] cancers. *OGG1* Ser326Cys polymorphism may alter *OGG1* mRNA levels leading to delayed repair of oxidative DNA damage. In silicocomputational biology tools reported that *OGG1* Ser326Cys polymorphism may change the functional and structural characteristics of the *OGG1* protein, so the 326Cys variant is smaller and more hydrophobic than the wild type. That change in the shape of *OGG1* protein leads to deleterious effects on its function.[33]

In addition,our results revealed that AA genotype and A-allele of *XRCC1* rs25487 G/A and G/G (Cys/Cys) genotype and G- allele of *OGG1* rs1052133 C/G were increased significantly in the patients with chronic HCV which was in line with Almeida et al.[34] This could be explained by the possible effects of HCV infection in preventing many DNA repair processes to enhance chromosomal instability in both hepatocytes and monocytes, which could explain the immunological perturbation and oncogenicity of HCV infection.[35] Chronic inflammation induces reactive oxygen species production that cause damage of the cellular molecules including

DNA, which if not repaired will resultin malignant transformation.

Moreover, patients having AA genotype of *XRCC1* rs25487 G/A, and G/G (Cys/Cys) genotype of *OGG1* rs1052133 C/G, exhibited significant higher urinary 8-OHdG levels.This could be explained by the impaired DNA repair ability of the proteins coded by these two genes as a result of the mutation with subsequent accumulation of the oxidation induced damaged DNA molecules (8-OHdG). *OGG1* can removes 8-OHG by cleaving the N-glycosyl bonds between the oxidized guanine and the deoxyribose backbone, leaving an apurinic/apyrimidinic site and the presence of G allele cause downregulation of *OGG1* with subsequent increase in the 8-OHdG levels.[36,37] 8-OHdG was found associated with increased risk for HCC and hepatic 8-OHdG levels are useful as markers to identify the high-risk individuals.[22] Furthermore, urinary levels of 8-OHdG were significantly higher among HCC patients with both lobes affected rather than one lobe and in those with multiple malignant hepatic lesions than those with single lesion, indicating its association with the tumor spread and with a need for further evaluation.

Conclusion

XRCC1 rs25487 G/A, and *OGG1* rs1052133 C/G polymorphisms are more prevalent in HCV and HCC patients than in controls, indicating impaired base excision repair induced HCV infection which may play a role in HCV related HCC development via impaired DNA repair protein activities as proved by the raised urinary 8-OHdG levels. Urinary 8-OHdG might be a noninvasive marker for the prediction of HCC in chronic HCV patients.

Disclosure

The authors report no conflicts of interest in this work.

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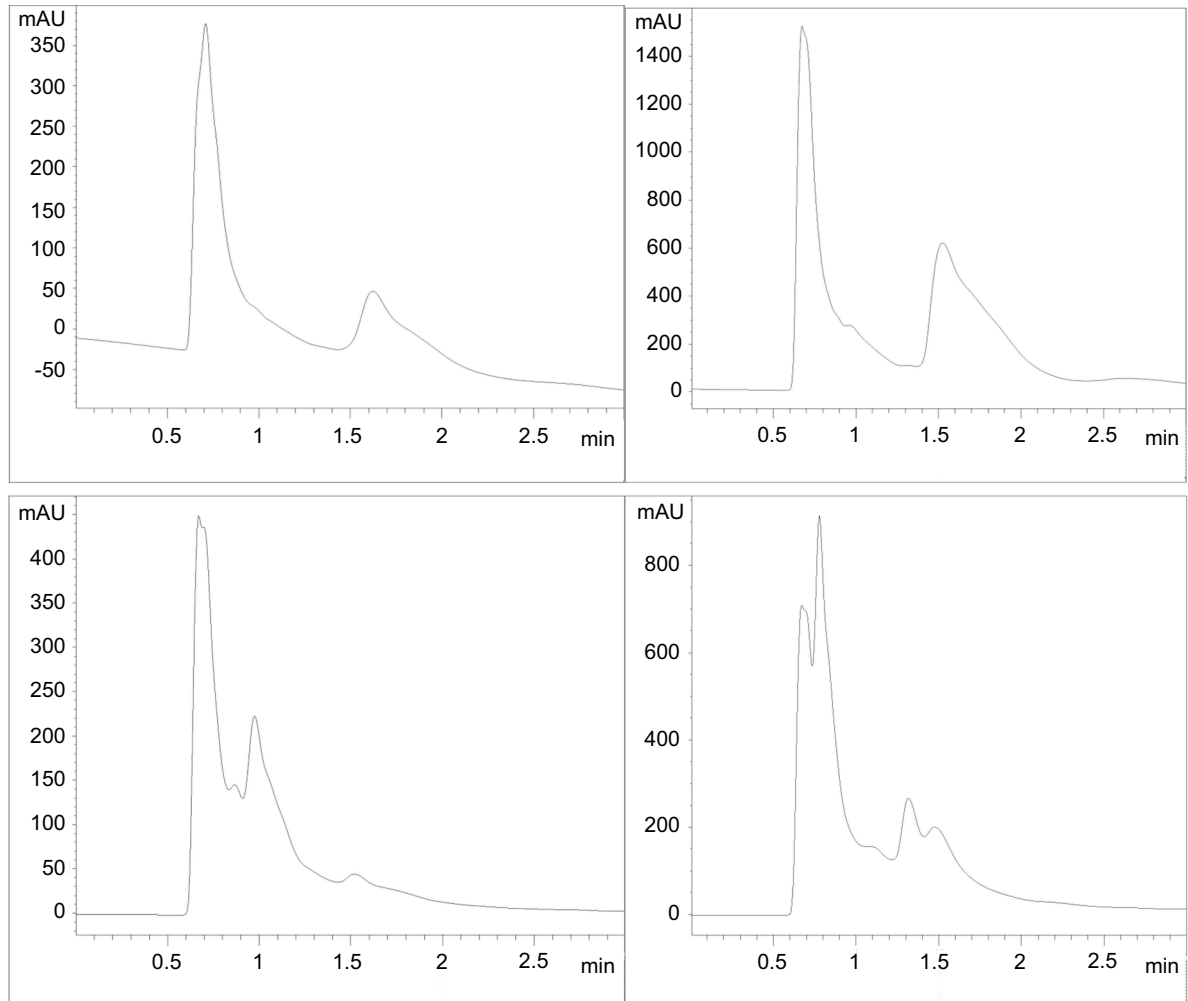
Supplementary materials

Figure S1 High performance liquid chromatography analysis curves of 8-hydroxy-deoxyguanosine for some included subjects.