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# Association of 32-bp deletion polymorphism and promoter methylation of *PTEN* and hepatitis C virus induced hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide and the second primary aetiology of death due to cancer. Hepatitis C virus (HCV) is a major cause of chronic hepatitis which may be complicated by HCC [1]. Many oncogenes and tumour suppressor genes are associated with the development of HCC, which can be used as biomarkers in the early diagnosis and staging [2]. The gene Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), also named MMAC1 or TEP1, situated at 10q23.3, has a total length of 200 kb spanning about nine exons and eight introns, and expresses a protein of about 403 amino acids that has phosphatase action on lipid and protein [3]. PTEN acts as tumour suppressor by downregulation of phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway. Suppression of Akt pathway by PTEN resulted in tumour suppression, inhibition of insulin signalling pathway and increase in the life span. However, the role of PTEN in tumour suppression is mainly due to the inhibiting the effect of AKT pathway in proliferation, hypertrophy and anti-apoptosis. PTEN deficiency causes a decrease in its negative regulatory mechanism on the Akt pathway and hence the normal suppression effect on tumour growth and metastasis will be absent. Hepatitis C virus infections inhibition of the transcription of *PTEN* and hence reactivation of the suppressed Akt-NF-κB pathway [4]. *PTEN* has many variations affecting its expression; one of them is 32-bp deletion variant present in intron 2, but its actual mechanism in illness still obscure. Another one is the promoter methylation; an epigenetic silencing of *PTEN* and revealed to be associated with many types of cancer [5]. Therefore, our study tested the hypothesis of a link between the role of the 32-bp deletion and promoter methylation of *PTEN* in HCC patients with HCV infection.

We tested our hypothesis in a case–control study of 100 patients diagnosed as HCC with HCV, enrolled from the specialized medical hospital of Mansoura University, and 100 healthy controls. HCC patients were positive for HCV antibodies (Access BIO – RAD Co., France), confirmed by quantitative viral RNA detection using Taq Man HCV quantitative test version 2.0 (Roche Molecular Diagnostic, Branchburg, NJ, USA). Exclusion criteria were HBV and other cancers. All patients were diagnosed by clinical, laboratory and radiological investigations including abdominal ultrasound and computer tomography. Patients were further classified according to Child-Pugh grading system and Barcelona clinic liver cancer (BCLC) staging [6]. The research was approved by Mansoura University ethical committee (Code: R/19.03.470) and approval consent was obtained from each participant.

Ten mL whole blood samples were obtained in EDTA and no anticoagulation for genomic DNA and serum. Sera were subjected for routine laboratory study of complete liver functions and alpha-fetoprotein (AFP). Genomic DNA was isolated from whole blood by DNA extraction kit (Qiagen-Germany). The deletion of the PTEN 32-bp was determined by the use of two sets of primers: F; (5′-CCAGCCCTCACTAAAAACAAA-3′) and R; (5′-CAAGTGTCCAAG CAGCAAA-3). PCR program was initial denaturation at 95°C for 5 min, then 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 23 s, then at 72°C for 10 min as a final extension step. Each PCR product was electrophoresed using a 2% agarose gel. The I allele gave 241 bp fragment while D allele comprised 209 bp fragment. (Figure1). Promoter methylation was investigated by nested methylation-specific PCR (MSP) assay. DNA samples were initially treated with sodium bisulphite. Nested methylation-specific PCR (MSP) technique is composed of two stages. The first stage was done using two sets of primers F; 5′-TTTAGGGAGGGGGTTTGA-3′ and R; 5′-CCATCC TCTTAATATCTCCT-3′. PCR product gave a 529-bp fragment. The product of each PCR reaction

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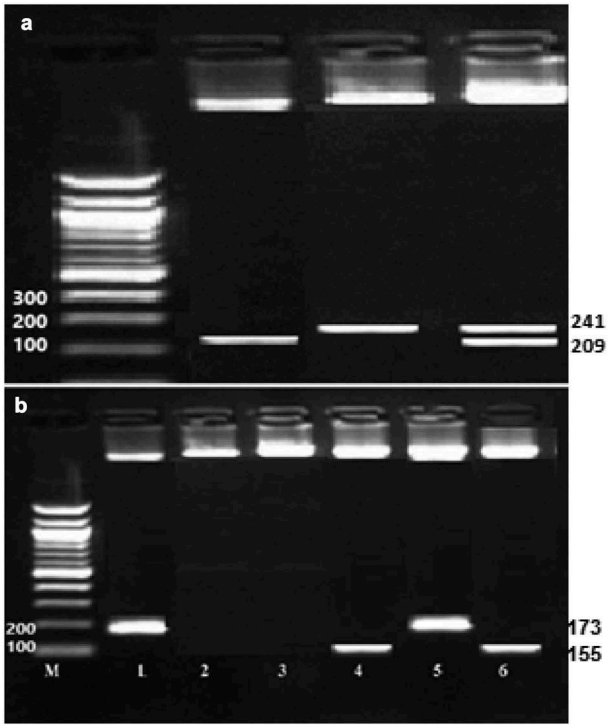


Figure 1. Agarose gel electrophoresis showing the 32-bp deletion/insertion of *PTEN* (A) using 100-bp DNA size ladder. I/D genotype (241 and 209bp bands), D/D genotype (209 bp) and I/I genotype (241bp). Promoter methylation (B), Methylated allele gives 155-bp product while unmethylated allele gives 173-bp product. Lane1, 2 (UU), lane 3, 4(MM), lane 5, 6 (UM).

Table 1. Distribution of genotypes and alleles of PTEN gene in the studied groups.

| | Patients (n = 100) | Controls (n = 100) | OR (95% CI) (p-value) |
|---|---|---|---|
| **32-pb I/D polymorphism** | | | |
| Codominant model | | | |
| II | 12 (12%) | 25 (25%) | Ref. |
| ID | 68 (68%) | 55 (55%) | 2.5 (1.18–5.6) **(0.016)** |
| DD | 20 (20%) | 20 (20%) | 0.5 (0.2–1.3) (0.12) |
| Dominant Model | | | |
| II | 12 (12%) | 25 (25%) | Ref |
| ID+DD | 88 (88%) | 75 (75%) | 2.4 (1.14–5.19) **(0.02)** |
| Recessive Model | | | |
| II+ID | 80 (80%) | 80 (80%) | Ref |
| DD | 20 (20%) | 20 (20% | 1.00 (0.5–1.9) (1.00) |
| Alleles | | | |
| I | 92 (46%) | 105 (52.5%) | Ref |
| D | 108 (54%) | 95 (47.5%) | 1.2 (0.87–1.9) (0.19) |
| **Promoter methylation** | | | |
| Codominant model | | | |
| UU | 72 (72%) | 74 (74%) | Ref. |
| UM | 20 (20%) | 20 (20%) | 1.03 (0.51–2.07) (0.94) |
| MM | 8 (8%) | 6 (6%) | 1.3 (0.45–4.14) (0.57) |
| Dominant Model | | | |
| UU | 72 (72%) | 74 (74%) | Ref |
| UM+MM | 28 (28%) | 26 (26%) | 1.1 (0.6–2.06) (0.75) |
| Recessive Model | | | |
| UM+UU | 92 (92%) | 94 (94%) | Ref |
| MM | 8 (8%) | 6 (6%) | 1.3 (0.4–4) (0.58) |
| Alleles | | | |
| U | 164 (82%) | 168 (84%) | Ref |
| M | 36 (18%) | 32 (16%) | 1.1 (0.6–1.9) (0.59) |

PTEN, Phosphatase and tensin homolog deleted on chromosome; OR (95% CI); Odds ratio 95% confidence interval; I, insertion; D, deletion; U, unmethylated; M, methylated.

was diluted and used as a template for the second PCR. The second stage was done using two pairs of primers for detection of unmethylated allele F; 5′-TGGG TTTTGGAGGTTGTTGGT-3′ and R; 5′-ACTTAACTCTAAA CCACAACCA-3′, another two sets of primers were used for methylated allele 5′-GGTTTCGGAGGTCGTCGGC-3′ and 5′-CAACCGAATAATAACT ACTAC GACG-3′. PCR program was as follows: Initial denaturation at 95°C for 5 min, then 35 cycles: 95°C for 30 s, 60°C for 30 s and 72°C for 10 s followed by 72°C for 10 min as a final extension. Unmethylated allele gave 173 bp fragment, while methylated one gave 155 bp fragment (Figure 1). Results were tested using SPSS version 22. Continuously variable data were analysed by Mann–Whitney and T-tests. Categorical data were analysed by $\chi^2$ tests. Odds ratio (95% confidence interval) was calculated to detect risk ratio. $P < 0.05$ was considered significant.

The patients consisted of 76 males and 24 females, the controls 55 males and 45 females ($p = 0.002$). Mean [SD] of the age of the patients was 50.1 [5.5] years versus 49.5 [8.6] in the controls ($p = 0.5$). The median (IQR) of AFP was higher in HCC group as compared to healthy group 200 (66–300) ng/ml and 6 (4–8) ng/ml, respectively)($p < 0.001$). Genotype frequency of 32-pb I/D polymorphism showed that the ID genotype was more frequent in patients as compared with the controls (Table 1). The risk of HCC in the dominant model was higher in patients with ID or DD genotype. PCR determination of promoter methylation of *PTEN* revealed no distinction in the frequency of different genotypes and alleles between patients and controls. Of the 68 patients with ID genotype, 42 (62%) were BCLC grade C and D, whilst 58 patients (85%) were Child-Pugh grade B and C. Regarding the relationship between 32-bp I/D gene variants and AFP in the patients, the median (IQR) were 155 (30–400), 230 (100–300) and 90 (25–250) in II, ID, DD genotypes, respectively ($p = 0.09$), whilst in the promoter methylation genotypes they were 205(75–300), 166 (37–271), 184(30–313) in UU, UM, MM genotypes, respectively ($p = 0.5$).

We hypothesised a link between the 32-bp deletion and the epigenetic promoter methylation of *PTEN* with the incidence of HCC in HCV infection. We found that the incidence of HCC was increased with ID or DD genotypes of 32-pb I/D. Our results were in contrast with the finding of Ding et al. [7] who showed no relationship between this polymorphism and HCC incidence in a Chinese population. *PTEN* encodes a phosphatase enzyme which can regulate the cell

cycle, stimulate cell apoptosis, inhibit the tumor angiogenesis, invasion and metastasis of tumours through a network of signal transduction pathways. Polymorphisms in *PTEN* can decrease the phosphatase activity of its product, so increasing liver cancer cells malignant transformation and metastasis [8]. The diminished production of PTEN protein in human HCC is associated with more aggressive tumour behaviour, larger tumour size and decreased life span [9]. There was no association between PTEN gene and the level of AFP, a finding in contrast to the result of Zhou and Li [10] who found that HCC patients with decreased or absent PTEN protein expression are often associated with elevated AFP and increased rate of cancer cell spread. However, at $p = 0.09$ we acknowledge the possibility of a false negative due to small numbers. The differences in our results with other previous studies may be attributed to that the incidence of *PTEN* genotypes differ between ethnic groups, different sample size, limited studies of this polymorphism in HCC, and other genetic variations associated with HCV infection which may predispose to HCC

Hepatitis C virus is the major cause of HCC, and HCV core protein is a significant determinant in the pathological events of HCV. The core protein of HCV resulted in reactivation of the downstream PI3K/AKT signalling pathway; hence, the normal negative regulatory mechanism of PTEN gene for cell division and metastasis will be lost, and malignant transformation of tumours will be accelerated [11]. Previous studies showed that *PTEN* is involved in HCV-induced hepatic dysfunction [12], and that low PTEN protein activity is associated with the incidence of several hepatic-related disorders as liver cancer and viral infection cirrhosis [8].

The epigenetic silencing of *PTEN* through hypermethylation of CpG sites in its promoter region has been considered as a possible mechanism leading to *PTEN* inactivation [13]. Our study revealed that there were no differences in the frequency of different genotypes and alleles of promoter methylation of *PTEN* between patients as compared with the controls. In accordance with Yu et al. [14] who showed that the *PTEN* promoter in all liver tumors was unmethylated. In contrast, Wang et al. [15] found that the decrease in the expression of *PTEN* in HCC might be due to promoter methylation which plays a fundamental role in HCC development and progression. To explore and confirm the functions of *PTEN*, studies on larger number of HCC patients should be done.

This work represents an advance in biomedical science because it shows that the 32-bp deletion polymorphism of *PTEN* is linked to HCC in individuals with HCV, but *PTEN* promoter hypermethylation is unrelated to this disease

## Disclosure statement

No potential conflict of interest was reported by the authors.

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