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# The association between the survivin gene -31G/C polymorphism and risk of hepatocellular carcinoma in Egyptian population

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**Abstract**

**Background**: Survivin is one of the most crucial apoptosis inhibitory genes, playing a basic role in hepatocellular carcinoma (HCC) development and progression. The current study aims at investigating the relationship of survivin polymorphism with the risk of HCC in Hepatitis C virus-infected Egyptian population.
**Methods**: the study was conducted on 120 Egyptian individuals divided into: healthy controls, fibrotic patients, cirrhotic patients and HCC patients (30 subjects/group). History and risk factors were collected and recorded for all cases. Polymorphism of the survivin gene, including one locus (rs9904341) was chosen for genotyping using (PCR-RFLP) technique.
**Results**: No statistically significant difference was detected in the genotype or allele distribution of HCC subjects in respect to the controls (P>0.05). On the other hand, a significant difference could be detected in genotype or allele distribution in fibrosis and cirrhosis HCV patient groups compared to healthy controls (P<0.05). A significant difference in genotype or allele distribution was detected in HCC group compared to cirrhosis group (P<0.05). No statistically significant difference was found in genotype or allele distribution of fibrosis cases relative to the controls.
**Conclusion:** No relationship between survivin gene (-31G/C) polymorphism rs9904341 and the risk of HCC could be found in HCV-infected Egyptian population. However, a positive correlation could be detected between survivin gene polymorphism and the risk of cirrhosis in the same population. The study also identified a relationship between survivin gene polymorphism in cirrhosis relative to fibrosis as well as between survivin gene (–31G/C) polymorphism rs9904341 in HCC relative to cirrhosis.

**Keywords**
survivin, -31G/C polymorphism, hepatocellular carcinoma, fibrosis

## Introduction

Hepatocellular carcinoma (HCC) is the 6th most common tumor on the planet and the third most regular reason for disease related demise, which speaks to a noteworthy and continually rising health care cost all through the world. It is to be noted that almost 55% of the newly reported cases of HCC are found in China **[1, 2].** Worldwide, approximately >50 million people are chronically infected by hepatitis C virus (HCV) and are at risk of developing chronic inflammation of the liver, cirrhosis and HCC, with 1.5 million new infection each year **[3]**. One of the main causes of neoplasm pathogenesis is the disturbance in the intricate balance between cell survival and apoptosis signals **[4]**. It's broadly acknowledged that apoptosis is a key player in cell/tissue homeostasis, where its dysregulation induces various disorders including malignancies **[5-7].**

Survivin is a member of the inhibitors of apoptosis (IAPs) protein group [8]. It is associated with cell cycle control, hindrance of the apoptosis pathways and microtubule steadiness **[9]**, assuming a basic part in carcinogenesis and cancer progression. Survivin manipulates mitochondrial apoptosis pathways, as it binds and represses caspase-3, caspase-7, and caspase-9 [**10]**. In addition, it interferes with the apoptotic pathways initiated by other apoptotic mediators including IL-3, Fas, and Bax **[11, 12]**. Survivin is expressed by activated hepatic stellate cells (HSCc), which represent a main player in hepatic fiborgenesis. Within these cells, survivin induces their proliferation and stimulates them to deposit extracellular matrix (ECM) leading to fibrotic scarring. This fibrogenic effect of surviving was shown to be mediated via TGF-β and Wnt/β-catenin pathways **[13,14]**.

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Some reports have linked survivin to angiogenesis as well, which is a fundamental step for carcinogenesis **[12]**. Survivin shows developmentally regulated expression levels, where it is widely expressed in the embryonic tissues but this expression becomes more restricted in differentiated tissues **[8]**. It is noteworthy to mention that surviving expression is upregulated in most human malignancies including HCC, where it is considered as an indicator for chemotherapy resistance and poor prognosis as well **[9, 16-19]**. Interestingly, animal models for inhibited survivin expression showed ameliorated fibrogenesis and improved hepatic functions, suggesting survivin as promising therapeutic target for managing hepatic fibrosis. **[20].**

Survivin expression is cell cycle-regulated mainly on the transcriptional level, peaking during the G2/M phase, and promptly declining during the G1 stage **[21-22]**. A few single nucleotide polymorphisms (SNPs) have been distinguished in survivin gene, including -31G/C, -625G/C and -644C/T. Polymorphism at the location -31, where G is substituted for C (rs9904341) is a well-known polymorphism at the promotor region (CDE/CHR binding site), that is common in tumor cell lines. It promotes overexpression of survivin both on the mRNA and protein level **[23,24].** A few studies linked survivin gene polymorphisms to human tumors including HCC **[25-27].**

Up to our knowledge, no studies investigated the association of HCC with survivin - 31G/C polymorphism in the Egyptian population. In view of the crucial role of survivin in cancer and the relationship of its gene polymorphisms with different tumors, we postulated that polymorphism in survivin gene may be linked to HCC pathogenesis. To test this theory, we investigated the association of survivin polymorphism with HCC in Egyptian population infected with HCV.

## 2-Materials and methods

### 2.1. Study population

The current study is a progressing epidemiological study of HCC directed in Faculty of Medicine, Minia University, Minia, Egypt. The current study was conducted in accordance with the Code of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The study protocol was approved by Faculty of Pharmacy, Minia University research ethics committee. All subjects were hereditary inconsequential Egyptian from Minia governorate. A written informed consent to enroll in the current study was obtained from all patients and controls included in the study.

Subjects were personally interviewed face-to-face by trained interviewers to collect demographic data as well as related risk factors such as tobacco smoking, alcohol drinking, and medical history of viral hepatitis type B and C.

Approximately 5 ml venous blood was drawn from each subject in a blind fashion, and samples were preserved at -20°C or -80°C. Blood samples were collected from 30 patients with HCC, 30 HCV patients who developed cirrhosis and 30 HCV patients who developed fibrosis in the division of gastroenterology between January 2016 and December 2016. During the same time, 30 healthy subjects with no proof of hepatocellular or other malignancy who visited the clinic for well-being registration were enlisted as the control group. The 30 healthy control subjects did not have a history of any liver diseases and serologically negative for hepatitis B or C infection.

Each individual included in the study was subjected to biochemical evaluation of ALT, AST, total bilirubin, direct bilirubin and serum creatinine using commercially available kits according to manufacturer's instructions (Biodiagnostics®, UK). HCV patients were positive for HCV antibody and HCV-RNA. Liver fibrosis and cirrhosis were diagnosed based on typical morphologic findings from CT, ultrasonography and Fibroscan®, pathological examination, and the laboratory features. The diagnosis of HCV-related HCC was based on histological findings in addition to elevated serum AFP levels above 400 ng/mL with positive liver images on CT, MRI, or ultrasonography. Controls were negative for both HCV antibody and HCV-RNA.

The HCC symptomatic criteria depended on the rule proposed by European Association for the Study of the Liver (EASL) **[28]**. Patients were recruited to the HCC group when the patient had at least one risk factors: HCV-based fibrosis, or cirrhosis in addition to one of the following: AFP levels >400 ng/ml and at least one positive computed tomography (CT) scan [28], MRI, or hepatic angiography; or AFP levels <400 ng/ml and at least two positive CT, MRI, or hepatic angiography.

Histopathological investigations were performed for cases that did not satisfy the greater part of the clinical non-invasive analytic criteria of HCC. Cirrhosis patients are diagnosed via liver biopsy, stomach sonography, and esophageal or gastric varices **[29]**. Patients with cirrhosis were classified into three grades based on their clinical status **[30]**. Serum HBsAg and Anti-HCV were surveyed utilizing an immunoassay (Abbott Laboratories, Abbott Park, IL, USA). Serum AFP focuses was measured by miniaturized scale molecule protein immunoassay (Abbott Laboratories, AXSYM, USA).

### 2.2. DNA extraction

A 5 mlvenous blood sample was collected from each subject into a test tube containing EDTA as anticoagulant. Genomic DNA was isolated from the whole blood samples using High Pure PCR Template Preparation Kit® (Roche Diagnostics. GmbH, Mannheim, Germany) according to the manufacturer's protocol.

### 2.3. Polymerase chain reaction-restriction fragment length polymorphism analysis

To identify the genotype of the surviving polymorphism, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed as described previously **[23]**. The 341 base pair (bp) section incorporating the G to C polymorphic site in survivin promoter region was amplified using a specific pair of primers;

Forward: 5`GTT CTT TGA AAG CAG TCG AG 3`

backward: 5`GCC AGT TCT TGA ATG TAG AG 3`.

The PCR reaction mixture consisted of 1 µl genomic DNA (~ 250 ng), 2 µl of each primer (10 pmol primer), 12.5 µl Master Mix® and 7.5 µl nuclease free water.

The PCR amplification program used was as follows: initial denaturation for 5 min at 94 $^0$C, followed by 35 cycles of: 30 s at 94 $^0$C, 90 s at 57 $^0$C and 90 s at 72 $^0$C, followed by a final extension step at 72 $^0$C for 5 min.

After confirming a successful PCR amplification by 1.5% agarose gel electrophoresis, each PCR product was individually digested by EcoO109I (37$^0$C, 16 hrs) (New England Bio labs Inc., Beverly, MA) and separated on 3% agarose gel against a standard DNA ladder. Test samples yielding 236 bp and 105 bp fragments were scored as GG, those with single 341 bp fragment as CC, and those with 341 bp, 236 bp and 105 bp as GC.

### 2.4. Statistical analysis

Collected data was analyzed using Graph Pad prism version 7software. The data was presented as mean ± standard deviation (SD) or standard error of means (SE) for quantitative variables, and number and percentage for qualitative values. Statistical differences between groups were tested using Chi Square test for qualitative variables or independent T-test and ANOVA (analysis of variance) with post Hoc Bonferroni test for quantitative normally distributed variables. Nonparametric Mann Whitney test and kruskal-Wallis test were used for quantitative variables which aren't normally distributed. Logistic regression analysis was done to test for significant predictors of outcome variable. P-values less than or equal to 0.05 were considered as statistically significant.

## 3. Results

### 3.1. General characteristic of the subjects

The clinical data of all the studied groups are illustrated in table 2.

This table shows the mean values ± standard error of the mean for age in control group (29.9 ± 1.751), fibrosis group (37.9 ± 2.208), cirrhotic group (58.53 ± 2.148) and HCC group (58.17 ± 1.474). All groups were significantly older compared to healthy control group ($p<0.05$). No significant differences in age were found between HCC and cirrhotic group (p value >0.05).

Biochemical data including ALT, AST, Albumin, serum creatinine, hemoglobin, direct bilirubin, total bilirubin are presented in table 1.

Only HCC group, but not cirrhotic or fibrotic group, showed a significant increase in ALT levels compared to healthy control **[31].** However, both HCC and cirrhotic group showed significant increase in their AST, total bilirubin, direct bilirubin, and serum creatinine levels compared to healthy control group. It is to be noted that both groups had significantly lower hemoglobin levels compared to healthy control ($p<0.05$) **[32, 33].**

Fibrosis group showed a significant increase in total and direct bilirubin and a significant reduction hemoglobin level compared to healthy control ($p<0.05$), whereas no significant differences could be detected regarding the other tested parameters ($P > 0.05$) **[34, 35].**

### Genotype percentage distribution of survivin (- 31G/C) polymorphism

The genotype distribution of survivin gene polymorphism was in Hardy-Weinberg equilibrium. In addition, the minor allele frequency (MAF) was found to be 0.26. The percentage distributions of the diverse genotypes for survivin -31G/C polymorphism can be seen in Table 2. The allelic frequencies of HCC cases (G: 0.45, C: 0.55) are not significantly different compared to those of the control cases (G: 0.35, C: 0.65) (p=0.2636). The allelic percentage of cirrhosis cases (G: 0.74, C: 0.26) were significantly different compared to those of the control cases (G: 0.35, C: 0.65) (p=>0.0001) and the allelic percentage of fibrosis cases (G: 0.57, C: 0.43) were significantly different compared to control cases (G, 0.35; C, 0.65) (p= 0.0172).

**Table 1.** The clinical data of all studied groups (Mean ± SEM)

| Parameters | Group 1 Control (n=30) | Group 2 Fibrosis (n=30) | Group 3 Cirrhosis (n=30) | Group 4 HCC (n=30) |
|---|---|---|---|---|
| | Mean±SEM | Mean±SEM | Mean±SEM | Mean±SEM |
| **Age in years** | 29.9 ± 1.751 | 37.9 ± 2.208 ** | 58.53 ± 2.148 **** | 58.17 ± 1.474 **** |
| **ALT(U/L)** | 51 ± 0 | 53.45 ± 7.141 | 50.73 ± 4.142 | 154 ± 31.29 ** |
| **AST (U/L)** | 40 ± 0 | 53.24 ± 6.671 | 72.86 ± 8.466 *** | 193.2 ± 53.93 ** |
| **T. Bilirubin (mg/dL)** | 1.2 ± 0 | 0.7583 ± 0.07251 **** | 4.559 ± 1.55 * | 3.644 ± 0.4735 **** |
| **D. Bilirubin (mg/dL)** | 0.4 ± 0 | 0.2457 ± 0.04316 *** | 2.739 ± 1.113 * | 2.16 ± 0.3734 **** |
| **S.Creatinine ( mg/dL)** | 0.8947 ± 0.04171 | 0.874 ± 0.04698 | 1.553 ± 0.1785 ** | 1.588 ± 0.1626 *** |
| **HB (g/dL)** | 13.86 ± 0.3826 | 12.85 ± 0.268 * | 9.073 ± 0.4539 **** | 9.92 ± 0.404 **** |
| **Albumin (g/dL)** | 4 ± 0 | 4.153 ± 0.08384 | 2.441 ± 0.1498 **** | 2.278 ± 0.09158 **** |

Abbreviations: * indicate a statistically significant difference compared to control group. *P*-Value <0 .05 means a statistical significant difference.

**Table 2.** allele percentage distribution of Survivin (- 31G/C) polymorphism among cirrhosis, fibrosis, HCC and control group and the relation between this and hepatocellular cancer danger.

| | G | C | Fischer's exact p value in comparison to control group | Odds ratio with 95% CI (confidence interval) | Fischer's exact p value in comparison to control group |
|---|---|---|---|---|---|
| **Control** | 21 | 39 | ------- | Control vs fibrosis 2.43 (1.16, 5.06) | ---------- |
| **Fibrosis** | 34 | 26 | 0.0275* | Fibrosis vs cirrhosis 2.01 (0.977,4.53) | Fibrosis vs cirrhosis P=0.0844$^{ns}$ |
| **Cirrhosis** | 44 | 16 | 0.0001*** | Cirrhosis vs HCC 3.35 (1.56,5.06) | Cirrhosis vs HCC p<0.0028*** |
| **HCC** | 27 | 33 | 0.2732 | Fibrosis vs HCC 1.6 (0.25, 1.19) | Fibrosis vs HCC P=0.2732 $^{ns}$ |

Abbreviations: * indicate a statistical significant difference with control group *P*- Value <0.05 means a statistical significant difference (Comparison between these alleles frequencies by chi square test).

### 3.2. Genotype frequency distribution of survivin -31G/C polymorphism

The distribution frequency of the diverse genotypes for survivin -31G/C polymorphism are presented in Table 3. The allelic frequencies of HCC subjects are not significantly different from the control subjects (p = 0.2636). In contrast, the allelic frequencies of both the cirrhosis and the fibrotic subjects were significantly different from the control subjects (p <0.0001, and p = 0.0172, respectively).

**Table 3.** Allele frequency distribution of Survivin (-31G/C) polymorphism among cirrhosis, fibrosis, HCC and controls and the relation between this and hepatocellular cancer risk.

| | G | C | *p*-value |
|---|---|---|---|
| **Cirrhosis** | 44 (74%) | 16 (26%) | >0.0001 (****) |
| **Fibrosis** | 34 (56%) | 26 (43%) | 0.0172 (*) |
| **HCC** | 27 (45%) | 33 (55%) | 0.2636 ( n.s ) |
| **Control** | 21 (35%) | 39(65%) | ----- |

Abbreviations: * indicate a statistical significant difference with control group P- Value <0 .05 means a statistical significant difference. (Comparison between these alleles frequencies by chi square test).

### 3.3. Survivin -31G/C polymorphism and risk of hepatocellular carcinoma

This association was examined among patients with cirrhosis, fibrosis and hepatocellular carcinoma and compared with control healthy patients .We analyzed the genotype of survivin gene on (-31 G/C) using PCR and RFLP. The restriction analysis product of each PCR sample was visualized on a 2% agarose gel and classified according to the position on DNA fragments into GG, CC and GC genotypes (figure 1). Lanes 1, 3 and 4 are CC genotypes, lanes 2 and 5 are GC genotypes.

Data analysis revealed a differential distribution of survivin (-31G/C) genotypes among the test populations. The frequencies of the GC, CC and GG genotypes among control healthy persons were 30%, 50%, and 20% respectively, while that among cirrhosis patients were 26.6%, 13.4%, and 60%, respectively and among fibrosis patients the frequencies were 60%, 13.4%, and 26.6% respectively.

On the other hand, the frequencies of these genotypes among HCC patients are 56.7%, 26.6%, and 16.7%, respectively (table 4). There was a significantly different distribution of the survivin (-31G/C) genotypes among the study subject (*p=0.0041).*

**Table 4**. The genotype frequencies of survivin (-31G/C) in different groups**.**

| Genotype | Control N=30(%) | Cirrhosis N=30(%) | Fibrosis N=30(%) | HCC N=30(%) | *p*-value |
|---|---|---|---|---|---|
| **GC** | 9(30%) | 8(26.6%) | 18(60%) | 17(56.7%) | |
| **CC** | 15(50%) | 4(13.4%) | 4(13.4%) | 8(26.6%) | 0.0041 ** |
| **GG** | 6(20%) | 18(60%) | 8(26.6%) | 5(16.7%) | |

Abbreviations: * indicate a statistical significant difference with control group P- Value <0 .05 means a statistical significant difference.

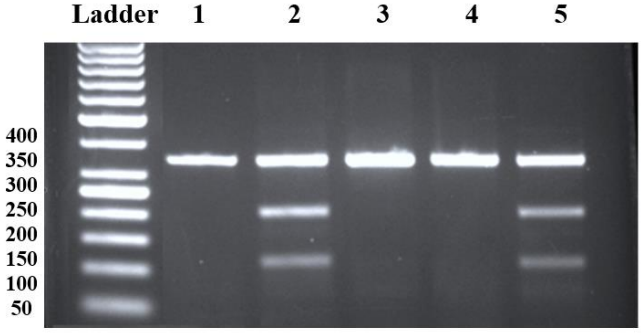


**Figure 1.** Agarose gel analysis for the digest of survivin (-31G/C) PCR products. Samples were separated on 2% agarose gel. lanes 1, 3 and 4 are CC genotypes, lanes 2 and 5 are GC genotypes.

### 4. Discussion

Apoptosis and apoptotic signal has been shown to play a major role in the pathogenesis of malignancies by a huge number of studies. Apoptosis is a fundamental mechanism that allows the biological system to get rid of abnormal cells, and hence prevents carcinogenesis. Disabling apoptosis allows the accumulation of genetic errors by prolonging the cell cycle, turning the cells insusceptible to cytotoxicity, such insusceptibility plays a crucial role in carcinogenesis **[4, 16]**. Since survivin works as an

apoptosis inhibitor, it is questionable if subjects with survivin -31G/C polymorphism may have improved ability to wipe out cells with DNA damage via apoptosis.

The current study explored whether the survivin -31G/C polymorphism could modulate vulnerability to HCC. Survivin -31G/C polymorphism was chosen based on its location within the CDE/CHR binding site, which allows this polymorphism to influence the binding of molecules that control cell cycle-dependent transcription of survivin gene, thereby taking part in the deregulation of survivin in HCC. In contrast to our expectations, the study revealed no significant difference in distribution of survivin -31G/C genotype between HCC cases in the Egyptian population. No significant association was detected between -31G/C polymorphism and risk of HCC. Interestingly, genotype variation decreased the HCC chance, in spite of the fact that the effect was not statistically significant. Our presented data are in accordance with that of previous research groups demonstrating no association between the survivin -31G/C polymorphism and risk for different malignancies including cervical tumor, gastric malignancy, esophageal squamous cell carcinoma, pancreatic cancer, and acute myeloid leukemia **[37-41]**. However, it is to be noticed that a few studies identified positive association between -31G/C polymorphism and some malignancies including lung malignancy, sporadic colorectal disease, and urothelial tumor **[42, 43, 19]**. A rational explanation for this variation could be regarded to the different signaling pathways which become altered in each kind of tumor, resulting in a positive or a negative association between survivin polymorphism and risk of tumors. In addition, the contribution of hereditary polymorphisms to the risk for tumor might be reliant on the ethnic background of studied population, and environmental components that impact that population. Demographic or ethnic contrasts have been accounted for with respect to the genotype frequency of several polymorphisms.

In the current study, a significant positive association connected between the risk of cirrhosis and survivin-31G/C polymorphism in all statistical analyses when compare with healthy control subjects, where HCV patients with GG genotype were more susceptible to cirrhosis than CC and GC genotypes.

When comparing the frequency of genotypes between cirrhosis patients and fibrosis patients (both with a history of HCV infection), a significant correlation was detected, where fibrotic patients with GG genotype were the most susceptible to cirrhosis compared to GC and CC genotypes in the test population.

Also a significant correlation was detected between the frequency of genotypes of survivin -31G/C polymorphism between cirrhosis patients and HCC patients, where cirrhotic patients with GC genotype were the most susceptible to HCC.

It is to mention that no significant correlation could be detected between control healthy persons and fibrosis patients when comparing the frequency of survivin -31G/C polymorphism.

In agreement with our findings and as mentioned before that survivin - 31G/C promoter polymorphism was shown by some groups not to be involved in different carcinogenesis **[37, 39-43]**, so it is possible that other molecular and cellular mechanisms are engaged with survivin over-expression in hepatocellular carcinogenesis. For example, survivin expression can be deregulated in malignancy by a several mechanisms, including enhancement of the survivin locus on chromosome 17q25, demethylation of survivin exons, expanded promoter activity, expanded upstream signaling in the phosphatidylinositol 3 kinase and epigenetics changes by promoter methylations and demethylation of survivin exons **[44-46]**.

The mutual role of survivin in modulating apoptosis and cell cycle, as well as its differential expression in tumors versus normal tissues causes this member of IAPs family to be a potential promising target for various therapeutic choices **[10, 15]**.

There are some lines of evidence suggesting the involvement of survivin in hepatocarcinogenesis **[10, 40, 47]**. In HCC cells, over-expression of survivin speeds up the G1 checkpoint by releasing p21WAF1/Cip1 from the p21/cyclin-subordinate kinase 4 (CDK4) complexes and enabling the interaction between caspase-3 and p21, which suppresses cell death signaling **[10]**. Recently published studies reported that HCC cells have more elevated amounts of survivin mRNA however less survivin protein compared with paired non-tumor and cirrhotic liver tissues **[48, 49]**. Over-expression of survivin was found in 70% of HCC patients, where it was linked to poor prognosis (high nuclear and histologic grade, micro-vascular invasion, and increased proliferation), local recurrence, and shorter disease free survival **[40, 47]**. However, there is a likewise dispute in the literature regarding the prognostic significance of survivin in HCC, where some studies showed that lower survivin protein levels in HCC cells were associated with more advanced disease stage and with poorer prognosis **[48]**. Further studies are necessary to demonstrate these variations.

Despite the data presented in the study, it still has some limitations. First, the present study was hospital-based case–control study, so patients were chosen at a single institution in one governorate. Accordingly, the test population may have been unrepresentative of hepatocellular carcinoma patients in the general Egyptian community. In addition, control subjects chosen for the study were also enrolled at a similar hospital.

Second, this study is constrained by the generally modest number of cases and controls. A nationwide study including a bigger number of subjects is expected to clear up the debate between our findings and literature.

Third, the study was limited to Egyptian population because of the variety in allele recurrence between various ethnic backgrounds has been observed for survivin - 31G/C polymorphism.

Finally, due to the lack of information on survivin expression as indicated by survivin -31G/C genotype in HCC, future work should be done so as to investigate the relationship between levels of survivin in healthy and HCC liver tissues with regard to various genotypes of survivin polymorphism.

## Conclusion

Our findings show on one hand that survivin -31G/C polymorphism doesn't have a significant role in genetic vulnerability to HCC among the Egyptian population. On the other hand, there is a significant association between survivin-31G/C polymorphism and risk of cirrhosis. A significant association between fibrosis and cirrhosis could be found, where fibrotic patients with GG genotype are more susceptible to cirrhosis than GC and CC genotypes in Egyptian population.

In addition, a significant association between cirrhosis and HCC could also be detected, where cirrhotic patients with GC genotype are more susceptible to HCC than GG and CC genotypes in Egyptian population.

Since this is a primary report investigating survivin -31G/C polymorphism and its association with the risk of HCC, further investigations are required to verify our data on a bigger scale, including patients of various ethnic backgrounds to better comprehend survivin - 31G/C polymorphism and susceptibility to HCC.

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