



Research article

Seven gene signature explores the impact of DAAs on the appearance of hepatocellular carcinoma in HCV infected patients



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ABSTRACT

HCV damages the hepatocytes ending with hepatocellular carcinoma (HCC). The direct-acting antivirals (DAAs) treatment has raised hopes for reducing the incidence of HCC. However, several scientific debate regarding the impact of DAAs on the occurrence of HCC in patients with cirrhosis. We aimed to study the Cirrhosis Risk Score (CRS), several clinical factors and tumor characteristics between patients who developed HCC either with or without DAAs treatment “DAA-exposed HCC patients” and “DAA-unexposed HCC patients”.

Methods: CRS was assessed via genotyping by allelic discrimination assays in HCV patients who developed de novo HCC (with DAAs (DAA-exposed HCC patients, n = 50), and without DAAs treatment (DAA-unexposed HCC patients, n = 40)). APRI, FIB-4 scores, and tumor characteristics were assessed.

Results: Around 60% and 48% of DAA-exposed HCC patients and DAA-unexposed HCC patients; respectively had high CRS scores without significant difference. DAA-exposed HCC patients showed elevated Albumin, Hemoglobin and decreased ALT, AST compared with DAA-unexposed HCC patients (P = 0.002, 0.04, <0.001 and 0.006; respectively). FIB4 and APRI didn't reach the statistical difference between the studied groups. DAA-exposed HCC patients have higher overall survival (OS) than DAA-unexposed HCC patients (median: 30 & 15 months; respectively (p = 0.019)). Moreover, no significant differences were observed between the two groups in their focal lesion characteristics.

Conclusion: All studied patients are genetically predisposed to develop HCC. Moreover, DAAs significantly improved the OS and the biochemical parameters. No differences between the two groups were detected regarding their tumor characteristics. Accordingly, the appearance of HCC after treatment is attributed to the natural course of cirrhosis.

1. Introduction

Chronic hepatitis C virus (HCV) infection is considered as the second causal agent for hepatocellular carcinoma (HCC) after hepatitis B affecting between 10%–25% of all infected patients [1]. Globally, it is estimated that HCV can infect up to 170 million individuals [1, 2].

Previously, interferon (IFN) has been selected as an ideal therapy for suppressing HCV replication and HCC development as a result of its antiviral and antitumoral activities [3]. Recently, the high rate of sustained virological response (SVR) (95–100%) has been achieved by the development of (DAAs). The later has raised hopes for reducing the incidence of HCC [4, 5]. Generally, the rate of HCC development after the achievement of the SVR was decreased to reach 0.2%–4% per year after

the complete HCV eradication. However, HCC recurrence has been reported in DAAs treated patients who performed a curative treatment for HCC, raising the scientific debate towards such a confusing issue [6]. However, the impact of DAA-based regimens on the occurrence of HCC in patients with cirrhosis is debatable [7, 8, 9, 10].

The outcome of hepatitis infection can vary dramatically, depending on both host and viral factors [11]. Several genetic variants have been identified as the predictive markers for detecting the patients at high risk to develop HCC which open the avenue towards the improvement of the treatment management based on interpersonal susceptibilities that represent a significant advance towards personalized medicine [12]. A SNP on Tolloid-like 1 gene (rs17047200), on chromosome 4, has been

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associated with the hepatocarcinogenesis in HCV infected patients treated with IFN therapy [13].

CRS is a polygenic signature firstly defined by Huang [14] and stratified the cirrhosis risk in many populations better than clinical factors [14, 15, 16, 17]. Previously we have reported that the CRS scores were higher in HCC and advanced fibrotic patients than in mild fibrotic group and approved the significance power of the CRS [18]. Application of such indexes may decrease the need for staging liver biopsy specimens among patients skipping sampling error along with intra-and interobserver variability [19].

The potential roles of hereditary determinants of HCC incidence post DAAs treatment are needed. Several studies have investigated possible molecular mechanisms triggered by DAAs that promote hepatocarcinogenesis. These mechanisms involved immune cell dysfunction, cytokine imbalance, and angiogenesis activation [16, 20, 21]. Interestingly, in cirrhotic patients previously treated for de novo HCC, DAAs showed better overall survival (OS) and lower hepatic decompensation than controls who did not receive DAAs [1, 22].

In the current study, we aim to investigate the seven gene signature in patients who developed HCC either treated or untreated with DAAs. Moreover, FIB-4, APRI scores and overall survival will be assessed in the same groups.

1.1. Patients and methods

This is a prospective case-control study. Patients with HCC were recruited consecutively and between January 2017 and March 2018 from the multidisciplinary HCC clinic, Kasr-Alainy hospital, Cairo University. Both patients groups were recruited synchronously. The study was approved by the research ethics committee of National Research Center, Cairo, Egypt, protocol number 19/071 in compliance with Helsinki Declaration 1975 revised in 2008 which contains regulatory norms and guidelines for research involving human beings. The inclusion criteria of the study were adult male and female patients, chronic hepatitis C patients confirmed by positive real time HCV-polymerase chain reaction (PCR) with or without previous DAAs therapy. Patients with HCC were diagnosed by Triphasic Computed Tomography (CT) or Dynamic Magnetic Resonance Imaging (MRI) with or without elevated AFP. Patients with HCC are naïve to anti-tumor treatment. Tumor characteristics were assessed based on radiological tumor assessment (focal lesion site, size and number, portal vein and abdominal lymph node assessment).

The exclusion criteria of the study included coexisting or other chronic liver diseases e.g., Hepatitis B virus (HBV) infection, autoimmune

hepatitis, liver cancer other than HCC, patients who refused or could not sign the informed consent. HCC management was performed different strategies (Microwave ablation, Trans-arterial chemoembolization, Combined therapy, and Surgery). Patients in the DAA-unexposed group did not receive DAA treatment prior to the diagnosis of HCC as they were either first diagnosed with HCC and were not previously known to have chronic HCV infection or refused to receive DAAs therapy or were from areas far from HCC treatment facilities. To avoid immortal time bias, all patients who were followed up less than 9 months before DAA therapy were excluded from the analysis.

Patients were subjected to full history taking and clinical assessment. Baseline laboratory tests were collected in the form of complete blood count, liver function tests, renal functions, Alpha-fetoprotein (AFP) measurement. No patient in our studied group had a history of alcoholism. HCV infection was diagnosed using a Quantitative real-time polymerase chain reaction (PCR) for HCV RNA (Artus HCV RT-PCR Quantification Kit (Qiagen GmbH) detection limit 60 IU/mL).

Liver stiffness was measured using Fibroscan and was ranked using kilo-Pascal's (or kPa) as mentioned previously by Vergniol et al (2008) as follows: F0: 0–5.4; F0 – F1: 5.5–5.9; F1: 6–6.9; F1–F2: 7–8.7; F2: 8.8–9.4; F3: 9.5–12.4; F3 – F4: 12.5–14.4; F4 \geq 14.4 kPa. In other words, scarring staged into; F0 means no scarring; F1 is mild fibrosis; F2 is moderate fibrosis; F3 is severe fibrosis; F4 is cirrhosis or advanced fibrosis. An established diagnosis of cirrhosis was obtained by abdominal ultrasonography, endoscopic evidence of portal hypertension, and transient elastography >14 kPa.

Follow-up: The initial evaluation of HCC treatment response was done after 1 month by Triphasic CT or MRI then every 3 months for 2 years and then return to routine surveillance by abdominal ultrasound and AFP every 6 months. Follow-up was performed till patients' death or till the end of the study.

1.2. Cirrhosis risk score (CRS) assessment

The 7 SNPs identified previously by Huang et al. [14] were genotyped using a real-time PCR protocol based on the pre-validated TaqMan MGB™ probe for allelic discrimination assay (Applied Biosystems) as described previously in our manuscript [18]. The seven SNPs are shown in Table 1. Also, we consciously used the classification launched in the original publication by Huang et al. [14] (2007a) CRS >0.7 signifies patients with high risk of advanced liver fibrosis, CRS <0.5 signifies a low risk of fibrosis, a CRS of 0.5–0.7 signifies an intermediate risk, and upon the score the patient was assigned to appropriate risk category.

Table 1. Demographic laboratory features of DAA-exposed HCC patients and DAA-unexposed HCC patients.

		Group 1 (DAA-exposed HCC patients) N = 50		Group 2 (DAA-unexposed HCC patients) N = 40		P-value
Age (years)		59.00 (54.0–63.0)		60.0 (55.00–66.50)		0.289
Sex	Males	N	%	N	%	0.062
	Females	38	76.0%	23	57.5%	
		12	24.0%	17	42.5%	
Diabetes mellitus		11	22.0%	7	17.5%	0.596
Hypertension		14	28.0%	9	22.5%	0.552
Hemoglobin (13–17 gm/dl)		12.00 (11.00–13.00)		12.8 (11.4–13.97)		0.041
Platelets (150–450 x 10) mm3		101.00 (72.50–126.25)		110.00 (81.25–160.0)		0.344
Serum albumin gm/dl		3.55 (3.20–4.00)		3.20 (2.90–3.57)		0.002
International Normalization Ratio		1.20 (1.20–1.30)		1.20 (1.10–1.30)		0.190
*Alanine transaminase U/L		40.03 \pm 15.78		50.19 \pm 18.35		< 0.001
*Aspartate transaminase U/L		50.43 \pm 19.92		57.98 \pm 18.81		0.006
*Bilirubin(T) mg/dl		1.3902 \pm 0.91683		1.2127 \pm 0.55911		0.543
Serum creatinine		1.00 (0.90–1.20)		1.00 (0.90–1.07)		0.678
Alfa-fetoprotein		56.00 (7.00–822.0)		200.00 (14.42–468.75)		0.132
Cirrhosis Risk Score		0.73 (0.59–0.86)		0.62 (0.59–0.77)		0.446

All Data are expressed as median. *Data are expressed as mean \pm sd. The P values in bold type refer to statistically significant values.

1.3. FIB-4 and APRI scores estimation in DAA-exposed and DAA-unexposed HCC patients

The FIB-4 and APRI scores were detected in DAA-exposed HCC patients (n = 50) vs. DAA-unexposed HCC patients (n = 40).

The FIB-4 and APRI formula fibrosis scoring system (FIB4) AST to platelet ratio index (APRI) and were calculated using the following formulas, as originally reported by [19, 24] respectively, and as described in our manuscript [18].

1.4. Overall survival

The overall survival of HCC patients was calculated from the date of the patient's first visit to the multidisciplinary HCC clinic until the date of death or the end of the study. Survival and recurrence curves were plotted by the Kaplan-Meier method and compared using the log-rank test according to Chan et al (2004).

1.5. Statistical analysis

SPSS 16.0 was used for data analysis. The information was displayed as mean standard deviation. The effect of differences was established by computing the odds ratio with the 95 percent confidence interval for categorical variables and comparing them with the χ^2 or Fisher exact tests, as appropriate (95%CI). For comparisons across many groups, 1-way ANOVA or the nonparametric Kruskal-Wallis test were applied, depending on the distribution of the variable. $P \leq 0.05$ was considered significant. The log-rank test was used to compare the survival curves that were plotted using the Kaplan-Meier method. The link between various etiologies and the development of HCC after DAAs treatment was clarified using logistic regression analysis.

2. Results

2.1. Patients' demographic and clinical data

This case control study included 90 patients with HCC. There was no significant difference on comparing between curative and non-curative treatments between the two groups. According to the previous intake

of Direct-Acting Antiviral therapy patients were divided into 2 groups: Group 1 (50 patients): who had HCC after receiving DAAs treatment (DAA-exposed HCC patients), Group 2 (40 patients): who had HCC without receiving DAAs treatment (DAA-unexposed HCC patients). DAAs treatment encompassed Sofosbuvir, daclatasvir \pm ribavirin for 12 weeks of therapy. All patients included in the study achieved SVR prior to HCC development.

2.2. Tumor characteristics data

The DAA-exposed HCC patients and DAA-unexposed HCC patients were analogous in terms of gender distribution, age, diabetes, and hypertension. For both patients group similar number of hepatic focal lesions was recorded (31 single, 8 double, 11 multiple lesions for DAA-exposed HCC patients and 26 single, 5 double, 9 multiple lesions for DAA-unexposed HCC patients) ($p = 0.9$). Site of focal lesion in DAA-exposed HCC patients was 32/5/13 in right/left and both lobes while in DAA-unexposed HCC patients was 30/4/6 in right/left and both lobes ($p = 0.43$). Around 3 cm mean size of their largest focal lesion (median 3; 2.5–3.9) for DAA-exposed HCC patients and (median 3.08; 1.7–3.7) for DAA-unexposed HCC patients ($p = 0.34$); see Figure 1.

All demographic and laboratory features of patients are shown in Table 1. DAA-exposed HCC patients showed significant lower hemoglobin ($p = 0.04$), ALT ($p < 0.001$), AST ($p = 0.006$) and higher serum albumin levels ($p = 0.002$) compared to DAA-unexposed HCC patients, while the decline in platelets, AFP and increment in bilirubin was not significant for the same comparison ($p > 0.05$ for all parameters).

2.3. Estimation of CRS score in DAA-exposed HCC patients and DAA-unexposed HCC patients

Upon CRS Values, patients were grouped into high risk (CRS > 0.7), intermediate risk (CRS 0.5–0.7), or at low risk of cirrhosis (CRS < 0.5).

No difference was noticed in the CRS score among DAA-exposed HCC patients compared to DAA-unexposed HCC patients. CRS value was distributed as follow: in DAA-exposed HCC patients ((7)14% low risk (13) 26% intermediate risk, and (30) 60% high risk) than in DAA-unexposed HCC patients ((6)15% low risk (15) 37.5% intermediate risk, and (19) 47.5% high risk) ($p = 0.5$).

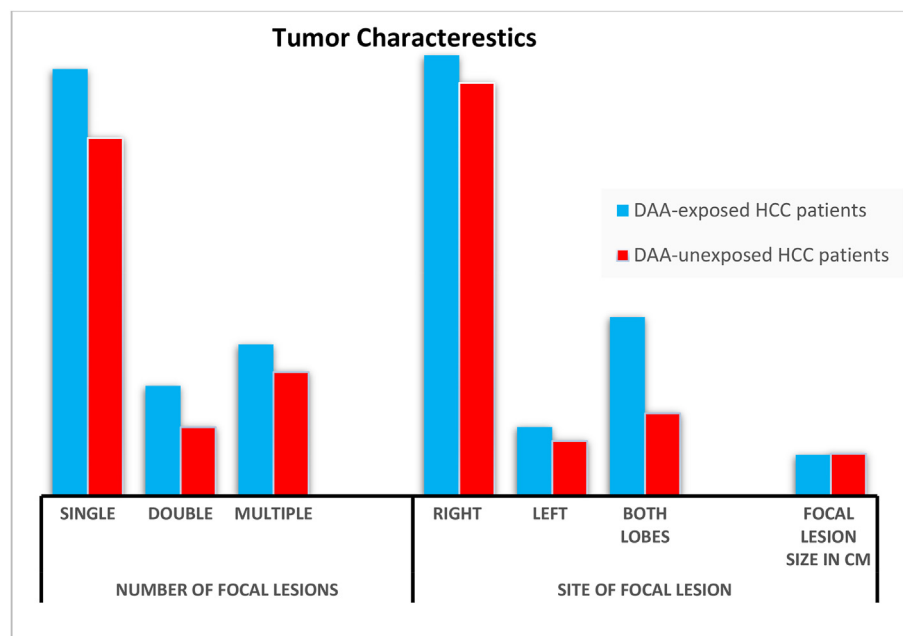


Figure 1. Tumor characteristics in DAA-exposed HCC patients & HCC DAAs free patients. Number, site, and size of hepatic focal lesions in 50 HCC patients treated with DAAs (DAA-exposed HCC) and 40 HCC patients who did not receive DAAs (DAA-unexposed HCC) were compared ($p > 0.05$ for all).

2.4. FIB-4 and APRI scores estimation in DAA-exposed and DAA-unexposed HCC patients

The median of, FIB4 median varied between DAA-exposed HCC patients (0.81; 0.54–1.24) and DAA-unexposed HCC patients (0.63; 0.46–0.94) ($p = 0.08$) without reaching statistical significance. Similarly, APRI of DAA-exposed HCC patients (0.85; 0.58–1.27) differed from median of APRI in DAA-unexposed HCC patients (0.61; 0.48–0.91) ($p = 0.08$) (Table 2).

2.5. Survival analysis

The mean survival duration in DAA-exposed HCC patients was 24.33 months with median 30 (95%CI for median 26.1–33.9) months this was significantly higher than the mean duration of survival of patients in DAA-unexposed HCC patients which was 17.48 months with median 15 months (95%CI for median; 11.9–18.1); p -value 0.019 (Table 3, Figure 2). Regarding the survival analysis, no difference has been observed in the high-risk group (based on their CRS values) between the DAA-exposed HCC patients and DAA-unexposed HCC patients.

2.6. Regression analysis for factors associated with the occurrence of HCC

Logistic regression analysis for prediction of HCC was calculated by comparing the current study group DAA-exposed HCC patients ($n = 50$) with DAA-unexposed HCC patients ($n = 40$). Coefficients describe mathematical relationship between variables. P -value between coefficients indicates whether these relationships are significant or not. Inverse correlations were found in CRS, sex, Albumin and INR between the studied group ($p = 0.007, 0.01, 0.001$ and 0.004 respectively; Table 4).

3. Discussion

The HCV infection has an inflammatory effect and can promote the carcinogenesis [2]. Knowing that patients with CHC who were treated with DAAs and achieved SVR-12 are still at risk of the development of HCC particularly those with advanced fibrosis and cirrhosis [9, 26]. Several studies have investigated possible molecular mechanisms triggered by DAAs that promote hepato-carcinogenesis [10]. A supportive hypothesis postulated that the DAAs disrupt the immune system balance due to the sudden eradication of the HCV viral titer which can induce cancer [9]. Contradictory, Cheung et al., screened cirrhotic HCV infected patients treated with DAAs, followed up several complications as liver cancer, and concluded in the beneficial role of DAAs even in advanced liver disease stages [27].

In our study no statistical difference was found between the DAA-exposed HCC patients and DAA-unexposed HCC patients in terms of demographic data (age, gender, diabetes, and hypertension), number, site, and size of hepatic focal lesions (around 3 cm mean size of their largest focal lesion). In an analogous study no differences were observed

Table 2. Assessment of FIB4, APRI and CRS values among HCC post DAAs and DAA-unexposed HCC patients.

	Group 1 (DAA-exposed HCC patients)		Group 2 (DAA-unexposed HCC patients)		p value
APRI	0.85	(0.58–1.27)	0.61	(0.48–0.91)	0.06
Fib-4	0.81	(0.54–1.24)	0.63	(0.46–0.94)	0.08
CRS low risk (<0.5)	7	14.0%	6	15.00%	0.45
CRS Intermediate risk (0.5–0.7)	13	26.0%	15	37.50%	
CRS high risk (>0.7)	30	60.0%	19	47.50%	

Data represented as median.

Table 3. Median survival time in DAA-exposed HCC patients and DAA-unexposed HCC patients.

Group	Median Survival Time (Months)			
	Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
DAA-exposed HCC patients	30.000	1.994	26.091	33.909
DAA-unexposed HCC patients	15.000	1.587	11.890	18.110
Overall	18.000	2.476	13.147	22.853

between de novo and recurrent HCC patients in their tumoral characteristics [6].

There is much focus on genetic determinants and the development of HCC after DAAs therapy in recent years such as human leukocyte antigen, IL28B, IL-15 and OAS variants [10, 12, 28, 29, 30, 31].

We evaluated the CRS, APRI and FIB4 methods, as noninvasive tools to discriminate between DAA-exposed HCC patients and DAA-unexposed HCC patients. APRI and FIB4 medians varied slightly between DAA-exposed HCC patients and DAA-unexposed HCC patients. CRS values in the studied patients' groups were not statistically different from each other and around 60% of DAA-exposed HCC patients and 48% of DAA-unexposed HCC patients had high CRS score without reaching significant difference. Additionally, in the end stage liver disease patients who are eligible for liver transplantation, the majority had high CRS score [32]. This is illustrated by the fact that HCC originated on top of liver cirrhosis, all patients who developed HCC have elevated CRS values. Those finding suggest that individuals who develop HCC already have their own genetic background affecting the severity of the disease even before the initiation of DAAs treatment and could not be considered due to DAAs usage.

Of note in our earlier work, CRS, APRI and FIB4 parameters discriminated between HCC and no HCC in HCV infected patients [18]. The role of other clinical factors on the prediction of liver cancer has been studied [33]. In this regard, study concluded a novel score (HMC-CU) for the diagnosis of HCC in a complete absence of genetic determinants; utilizing AFP, age, gender, haemoglobin, s-albumin, INR levels [33].

An effective DAAs treatment may not only eradicate HCV, but concurrently repair parameters of liver fibrosis induced by HCV infection [22]. In DAA-exposed HCC patients, serum albumin was elevated, while ALT & AST were dropped than in DAA-unexposed HCC patients. The latter notion is a surrogate marker of DAA-related improvement of liver function. Hypoalbuminemia increases the risk of HCC development and in HBV and HCV-related HCC [34]. Congruent study showed that albumin levels improved in patients only achieved SVR post DAAs treatment [34].

Patients with DAA-exposed HCC patients exhibited better OS than patients with DAA-unexposed HCC patients. Our data were similar to that of the previous studies in other populations [35], this may be explained via long-term maintenance of liver function and accordingly hepatic decompensation reduction. Recent retrospective study highlighted that DAAs treatment prolonged progression-free and OS rates compared not only to the control group but also to IFN treated patients [36].

Current study has some limitations. Firstly, these results were taken from a somewhat small number of patients. Therefore, larger sample size is warranted to confirm our findings. Secondly, assessment of the functionality of these SNPs associated with the clinical outcome of HCC is crucial in future studies. Thirdly, longitudinal study is needed to approve our notion.

Finally, we concluded that all the recruited patients are genetically at high risk for the development of HCC. Moreover, no differences were observed between the two groups in their focal lesion's characteristics. Also, the biochemical parameters such as (albumin, ALT, AST) and OS have been improved considerably in patients who received DAAs compared with those who didn't receive DAAs treatment, so the DAAs therapy may improve the care of HCV for these very sick patients. In the

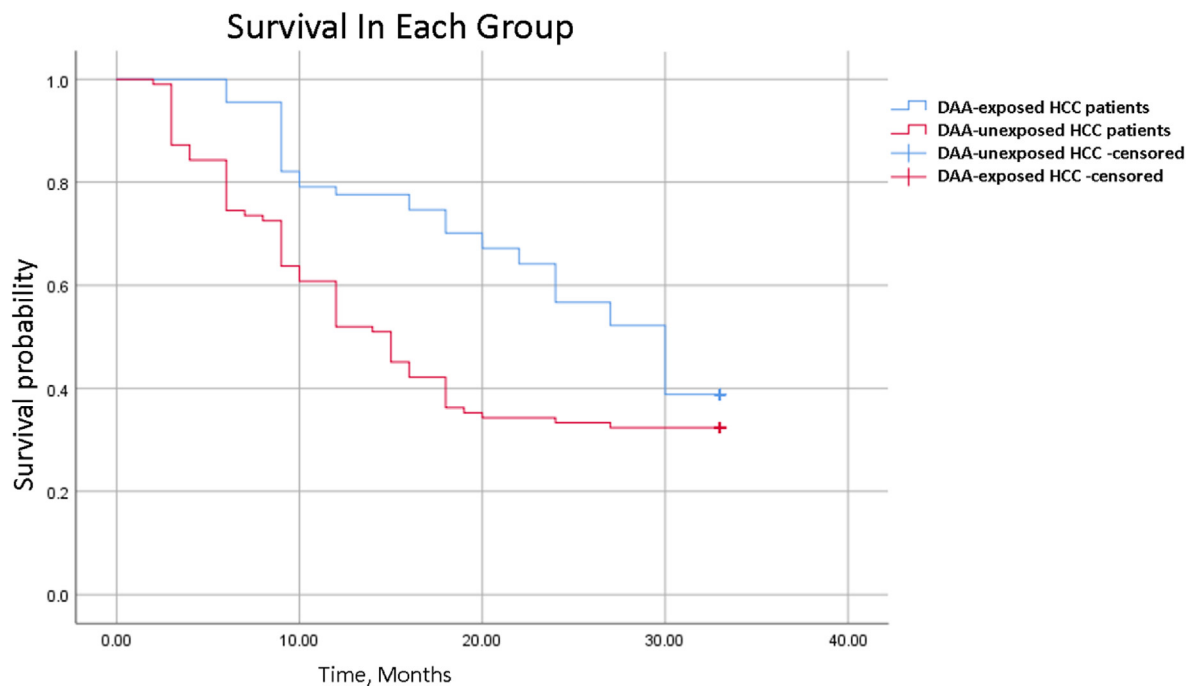


Figure 2. Kaplan-Meier curve for OS in DAA-exposed HCC patients & DAA-unexposed HCC patients. Overall survival (OS) in 50 HCC patients treated with DAAs (DAA-exposed HCC) and 40 HCC patients who did not receive DAAs (DAA-unexposed HCC). the log-rank test was generated ($p = 0.019$).

Table 4. Regression analysis for factors associated with the occurrence of DAA-exposed HCC patients.

	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		
(Constant)	6.598	2.642		2.498	0.015
CRS	-2.257-	0.819	-0.339-	-2.754-	0.007
age	0.009	0.026	0.059	.346	0.731
sex	-.612-	0.245	-0.288-	-2.503-	0.015
DM	0.175	0.306	0.071	.573	0.568
HT	0.139	0.271	0.061	.513	0.609
Hb	0.099	0.058	0.187	1.702	0.093
WBc	0.010	0.074	0.016	0.133	0.895
creatinine	0.196	0.637	0.037	0.307	0.760
Alb	-0.768-	0.207	-0.456-	-3.710-	<.001
AST	0.008	0.011	0.182	0.731	0.467
ALT	-0.003-	0.017	-0.072-	-0.195-	0.846
INR	-1.874-	0.623	-0.340-	-3.008-	0.004
PLT	0.001	0.002	0.076	0.455	0.650
APRI	0.007	0.010	0.246	0.732	0.467
FBI	-0.286-	0.591	-0.143-	-0.484-	0.630

P value < 0.05 is significant (-) refers to an inverse correlation.

current study, the appearance of HCC after DAAs therapy is attributed to the natural course of cirrhosis that can induce the development of HCC.

Declarations

Author contribution statement

Reham Dawood: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mai Abd El-Meguid: Performed the experiments; Analyzed and interpreted the data.

Ghada Maher Salum: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Hend Ibrahim Shousha; Ahmed Elsayed; Mohamed Mahmoud Nabeel: Analyzed and interpreted the data.

Ashraf Abdelaziz; Ayman Yosry: Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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