

Epithelial-Mesenchymal Transition Markers in HCV-Associated Hepatocellular Carcinoma: A Multivariate Follow Up Study

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Abstract

Objective: Validated markers to predict recurrence after surgical resection of hepatocellular carcinoma (HCC) are needed. Little data is available regarding epithelial-mesenchymal transition (EMT) markers in HCC. The objective of this study was to investigate the expression of EMT markers and their correlation with clinicopathological variables and survival in hepatitis C virus (HCV)-associated HCC. **Methods:** This longitudinal study included 109 cases of HCV-associated HCC treated with surgical resection. Nine different EMT markers (vimentin, E-cadherin, N-cadherin, Stat3, Snail1, Slug, Twist1, Zeb1 and integrin $\alpha 5$) were evaluated on liver tissue from HCC cases. Twenty fresh HCC samples from the studied cases were used for gene expression of EMT markers by quantitative real time polymerase chain reaction (PCR). **Results:** EMT markers expression was 71%, 25%, 26%, 27%, 9%, 4%, 72%, 47%, 87% for vimentin, E-cadherin, N-cadherin, Stat3 snail1, slug, twist1, Zeb1 and integrin $\alpha 5$ respectively. EMT mRNA in HCC tissues correlated with protein expression by 50-70%. Vimentin was independent predictor of large tumor size ($P=0.001$), high risk of recurrence (HRR) ($P=0.006$) and shorter disease free survival ($P=0.03$) in multivariate analysis. Reduced E-cadherin was a predictor of HRR ($P=0.002$). **Conclusion:** Vimentin and E-cadherin were the most powerful prognostic EMT markers in HCV-associated HCC in prediction of recurrence.

Keywords: Epithelial-mesenchymal transition markers- hepatocellular carcinoma- prognosis- HCV

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Introduction

In spite of the rapidly developing era of direct acting antiviral regimens (DAA) in the treatment of hepatitis C virus (HCV), the incidence of hepatocellular carcinoma (HCC) is still rising (Ferlay et al., 2015; Pascut et al., 2020). Following successful virus clearance, patients continue to suffer from the sequelae of chronic HCV such as liver cirrhosis and HCC. Moreover, it has been reported recently that DAA may be claimed for the increase incidence of HCC (Musa et al., 2020). The treatment of HCC is dependent on the tumor stage at time of diagnosis. Partial or total hepatectomy with liver transplantation are the best available treatment option for patients at early stages of the disease (El-Zayadi et al., 2005). Nevertheless, HCC is associated with a high risk of postoperative recurrence and metastasis (Adam et al., 2012). The conventional clinicopathologic features of the tumor as tumor size, vascular invasion and tumor/nodal/metastases (TNM) stage are currently used for prediction of HCC

prognosis (Bruix et al., 2014). Unfortunately, HCC is a heterogeneous disease and even in patients with the same clinical and pathological characteristics, the prognosis varies (Tandon and Gracia-Tsao, 2009). Therefore, it is of great importance to find other potential molecular markers that can help in predicting HCC recurrence and metastasis (Kim et al., 2010; Pinero et al., 2020).

Epithelial – mesenchymal transition (EMT) was first identified as an essential process in cancer in 1989 (Wan et al., 2017). Later on, EMT has been found to play an important role in tumor cell invasion and metastatic potential of various cancers (Boyer et al., 1989; Soltermann et al., 2008; Sasaki et al., 2009; Iwatsuki et al., 2010; Joger et al., 2010). In case of HCC, numerous studies investigated EMT markers and their relation to the clinicopathologic features and metastasis. However most of these articles did not include survival data (Lee et al., 2006; Zhao et al., 2011; Zhang et al., 2012; Li et al., 2013; Yuan et al., 2020; Song, 2020). On the other hand, studies including survival data were analyzing

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one or two EMT markers (Yang et al., 2007; Sun et al., 2010; Cai et al., 2012; Hashiguchi et al., 2013; Yao et al., 2013; Meng et al., 2015). Investigation of multiple EMT markers with follow up data in HCC has been reported in very few studies (Yang et al., 2009; Kim et al., 2010; Nagai et al., 2016). In all these studies HCV-related HCC represented only a small fraction of the analyzed HCC cases.

In the current study, we intended to explore the value of multiple EMT markers in a cohort of 109 HCV-associated HCC patients with full follow up data. We were attempting to find out EMT marker (s) that can be used complementary to the routine prognostic factors to select cases with aggressive behavior who may need postoperative adjuvant therapy.

Materials and Methods

Specimen and data collection

One hundred and nine patients who underwent curative partial –hepatectomy or total hepatectomy with liver transplantation were enrolled in this study. The patients were selected according to HCV positivity, absence of preoperative therapy for HCC and availability of follow up data. They were obtained during the period 2012 – 2015 from National Liver Institute, Menoufia University, Ain- Shams University hospitals and Dar Al Fouad hospital. The clinical, follow up and pathologic data were retrieved from the medical and pathology records. TNM staging was done according to the American Joint Committee on Cancer (Amin et al., 2017). Hematoxylin and eosin–stained sections from the tumor tissue were re-examined to confirm the diagnosis and assess the histologic grade based on Edmondson and Steiner criteria (Edmondson and Steiner, 1954).

This work was approved by the Research Ethical Committee (IRB) of the Faculty of Medicine, Ain–Shams University (FWA00006444) according to the declaration of Helsinki for medical research involving human subjects. Patients that had samples from fresh frozen liver tissue had informed consent. All data and specimens were anonymized.

Immunohistochemistry

Formalin–fixed, paraffin embedded tissue sections were obtained from all 109 resected HCC specimens. Ten normal liver tissue samples were taken from healthy donors (after informed consent) to be used as control. All tissue sections were dewaxed and hydrated. After antigen retrieval in citrate buffer and blocking the endogenous peroxidase activity, the tissue sections were incubated with the primary antibodies. The following antibodies with their catalog number were: Vimentin, MS–129–R7, E-cadherin, MS–9470–R7, N-cadherin, 030076, Stat 3, S 7971 – 20C, Snai1, 031251, Slug, 213491, Twist1, 214438, Zeb1, 214442 and Integrin 5, C 2405 - 02S. Vimentin and E-Cadherin antibodies were purchased from Thermo Fisher Scientific (UK) and were ready to use. The remaining antibodies were purchased from US Biological (MA, USA) and used at a concentration of 1:100. The incubation period for vimentin and E-Cadherin was one

hour at room temperature. Incubation with the rest of antibodies was overnight at 4°C. As a negative control, the primary antibody was replaced by nonimmunized mouse serum. Appropriate positive control tissue sections were used as recommended by the manufacturer. The staining system was the mouse/ rabbit specific HRP-DAB detection kit (catalog no. TP-060-HL, Thermo Fisher Scientific, UK). This system was applied according to the manufacturer’s instructions.

Interpretation of immunostaining

For vimentin, the cases were classified as negative or positive (>5% of tumor cells showed cytoplasm staining). For E-cadherin and N-cadherin, the results were categorized as either reduced expression or preserved expression (>90% of tumor cells showed membranous staining) (Hashiguchi et al., 2013). Stat3 expression was evaluated according to Xiang et al.; 2002 as negative (0-15% of tumor cells showed nuclear staining) or positive (> 15% positive tumor cells). For Snai1, Slug and Twist1, positivity was considered when $\geq 50\%$ of the tumor cells revealed nuclear staining (Losic et al., 2020). Zeb1 expression was regarded as positive if at least 1% of the tumor cells has positively stained nuclei (Sun et al., 2010). Integrin-5 immunostaining was categorized according to Yao et al.; 1997 as follows: 1 (no staining), 2 (fewer than 15% positive membranous staining of the tumor cells), 3 (15% - 50% positive cells), 4 (more than 50% positive cells). For statistical analysis, 1 and 2 were grouped as negative and 3 and 4 as positive.

Real time PCR analysis

Twenty frozen tissue specimens from the 109 HCC patients and ten normal liver tissues were used to analyze the expression of vimentin, E-cadherin, Snai1, Twist1 and Zeb1 mRNA as selected EMT markers. β -Actin as house keeping gene was used as a reference gene. Total RNA was isolated from frozen tissue using an RNeasy Plus Minikit (Qiagen, Valencia, CA, USA). RNA quality was assessed by using NanoDrop2000 (Thermo Fisher Scientific, UK). The ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA, a ratio of 1.8- 2.1 is an indicator of purified RNA.

For cDNA synthesis, we used one microgram from total RNA and random primers were used using the RevertAid H Minus Reverse Transcriptase provided by (ThermoFisher Scientific Inc., Canada). In a total volume of 20 μ l real time PCR reactions were performed using SYBR Green (Life Technologies, CA) and was conducted on the Applied Biosystems Step One™. PCR reactions were as following 15 minutes incubation at 95°C followed by 40 cycles for 15 seconds at 94°C, and annealing temperature of 55 °C for 30 seconds and 70°C for extension for another 30 seconds . After PCR amplification, the Δ Ct is calculated by subtraction of the B-Actin Ct from each sample Ct. The following equation $2^{-\Delta\Delta Ct}$ was used for data analysis.

The primer sets used were as the following.

Vimentin F: 5'-GTTGACAATGCGTCTCTGGC-3'

R: 5'-CGATTAGGACAGCTGTTCCCT-3'

E-cadherin F: 5'-GGAACCTCTGTGATGGAGGTCA-3'

R: 5'-AAGCGATGGCGGCATTGTA-3'
 Snail F: 5'-GTTTACCTTCCAGCAGCCCTA-3'
 R: 5'-CAGGACAGAGTCCCAGATGAG-3'
 Twist1 F: 5'-ACGAGCTGGACTCCAAGATG-3'
 R: 5'-CACGCCCTGTTTCTTTGAAT-3'
 Zeb1 F: 5'-TGCAGAAAATGAGCAAACCAT-3'
 R: 5'-GTGAAAATGCACTGGTGTTC-3'

Statistical analysis

The relationships between EMT markers and clinicopathologic factors were investigated by Chi square test. Overall survival (OS) and disease free survival (DFS) were analyzed by Kaplan-Meier survival curve and their relationships to the expression of EMT markers were assessed using long-rank test. The correlations between the levels of expression of various EMT markers were evaluated by Spearman's rank correlation test. Finally, multivariate analysis was performed by applying Cox proportional hazard model to identify the EMT markers

with independent prognostic value. A P value less than 0.05 was considered statistically significant.

Results

Clinicopathologic features

The ages of the 109 patients included in the study ranged from 44 to 75 years with mean age 57.7 ± 9.1 . They were 90 males (82.6%) and 19 females (17.4%). The clinicopathologic data are given in Table 1. The overall survival (OS) ranged from 12 to 26 months (median 24 months). The disease free survival ranged from 6 to 24 months (median 12 months).

Immunoexpression of EMT markers

Normal liver tissue samples showed positive immunostaining for E-cadherin, N-cadherin and integrin $\alpha 5$. Whereas, none of the remaining studied EMT markers revealed immunoexpression in normal liver tissues. The

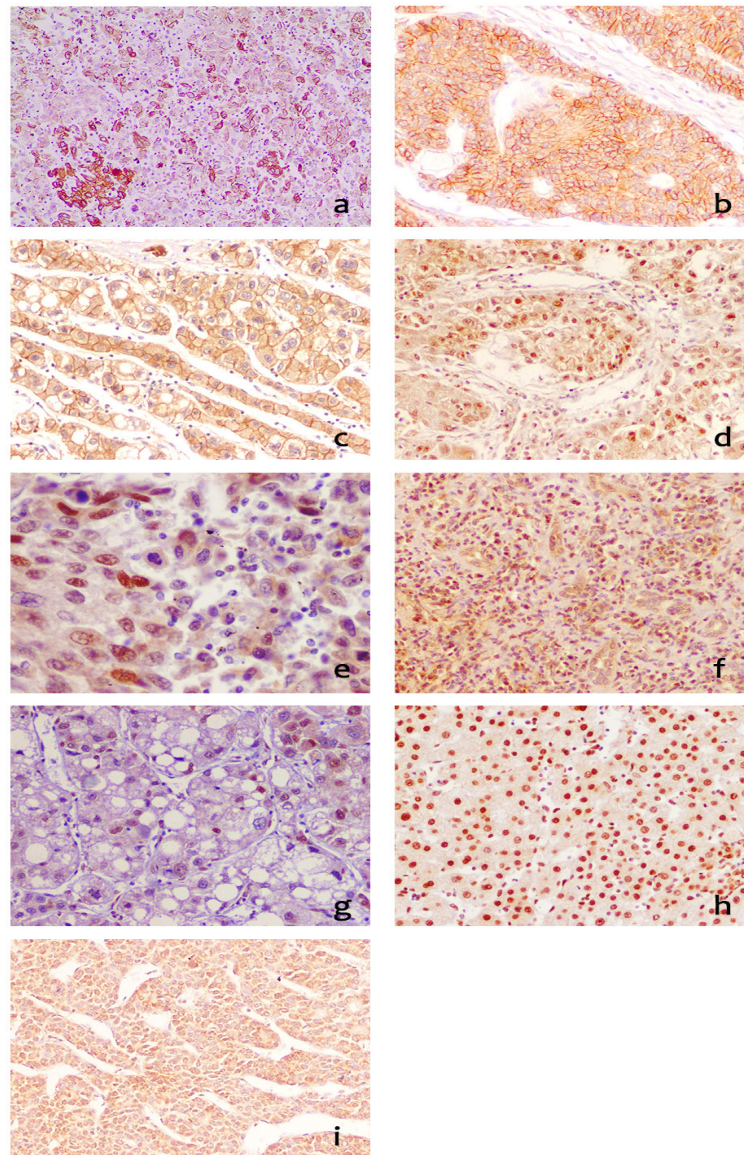


Figure 1. Cases of Hepatocellular Carcinoma Showing. (a), cytoplasmic staining of vimentin; (b), preserved membranous expression of E-cadherin; (c), preserved membranous expression of N-cadherin; (d), nuclear staining for Stat3; (e), nuclear staining of Snail; (f), nuclear staining of slug; (g), nuclear staining of Twist1; (h), nuclear staining of Zeb1; (i), cytoplasmic staining for integrin $\alpha 5$. Original mag. X200

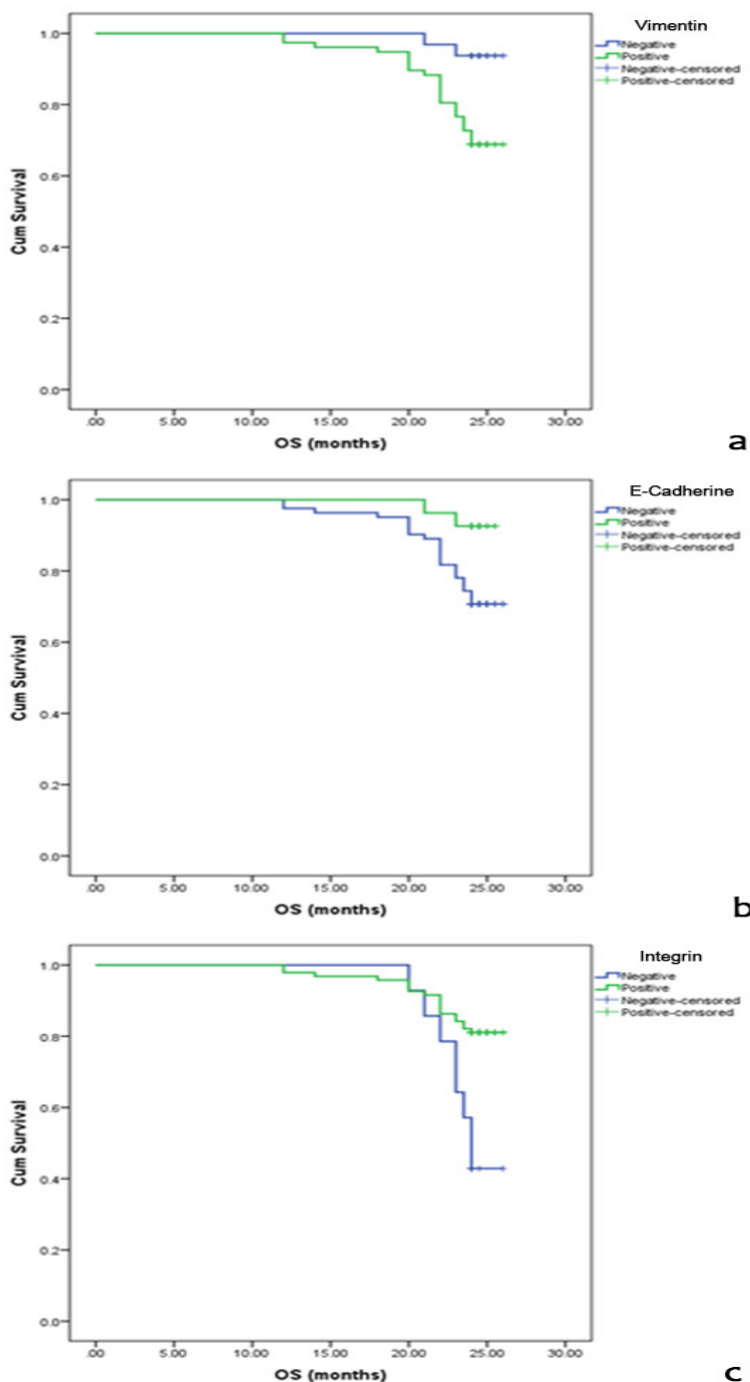


Figure 2. Kaplan-Meier Survival Curve Showing the Relationship between Overall Survival and the expression of (a) Vimentin (P=0.007), (b) E-cadherin (P=0.03), and (c) integrin α 5 (P=0.003)

immunohistochemical results of EMT markers in liver tissue from HCC cases are shown in Table 2 and Figure 1.

PCR data

The results showed that HCC cases had higher expression regarding vimentin, E-cadherin, Snai1, Twist1 and Zeb1 when compared with normal liver tissues.

Relationship between EMT markers and clinicopathologic factors

Table 3, shows the relationships between the 9 investigated EMT markers and the clinicopathologic

variables in the HCC cases as estimated by Chi square test.

Survival analysis

Long rank test revealed that the rate of 2-year overall survival (OS) was significantly less for patients whose tumors showed vimentin positivity ($X^2 = 7.3$, $P = 0.007$, Figure 2A), reduced E-cadherin expression ($X^2 = 5.0$, $P = 0.03$, Figure 2B) and integrin positivity ($X^2 = 9.1$, $P = 0.003$, Figure 2C). The rest of the EMT markers had no value in predicating the OS. Regarding the rate of the 2-year disease free survival (DFS), it was less for patients with vimentin positivity ($X^2 = 13.7$, $P = 0.000$, Figure 3A),

Table 1. Clinicopathologic Data of 109 Hepatocellular Carcinoma Cases Included in the Study

Feature	Number (%)
Age	
≤ 50 years	18 (16.5)
> 50 years	91 (83.5)
Alpha fetoprotein (ng/ML)	
≤ 400	60 (82.2)
> 400	13 (17.8)
Tumor size (cm)	
≤ 5	73 (67.0)
> 5	36 (33.0)
Histologic grade	
I	41 (37.6)
II	53 (48.6)
III & IV	15 (13.8)
Vascular invasion	
Absent	75 (68.8)
Present	34 (31.2)
TNM stage	
I	39 (35.8)
II	51 (46.8)
III	19 (17.4)
Recurrence	
Absent	39 (35.8)
present	70 (64.2)

reduced E-cadherin ($\chi^2 = 7.7$, $P = 0.006$, Figure 3B) and Zeb1 negativity ($\chi^2 = 7.0$, $P = 0.008$, Figure 3C).

Independent prognostic EMT markers

Multivariate Cox regression analysis (Table 4) showed that vimentin was an independent predictor of larger tumor size ($P=0.001$), vascular invasion ($P=0.004$), increased risk of recurrence ($P=0.006$) and shorter DFS ($P=0.03$). Reduced E-cadherin expression was an independent predictor of poor tumor histologic grade ($P=0.002$) and high risk of recurrence ($P=0.002$). Snail expression predicts larger tumor size ($P= 0.02$) as well as poor histologic grade ($P=0.01$). Zeb1 predicts earlier TNM stage ($P=0.02$) and integrin α 5 expression independently predicts shorter OS ($P=0.02$).

Correlation between EMT markers

Figure 4, showed the correlation between the expression levels of the EMT markers. This figure demonstrated that most of the EMT markers are correlated with each other. The main correlations can be categorized into two groups. First, significant positive correlation between each two of the following: vimentin expression, E-cadherin reduced expression, Twist expression and Zeb1 expression. The only exception was the relationship between Zeb1 expression and either vimentin expression or reduced E-cadherin expression which was an inverse correlation. The second group included Stat3, Twist1

Table 2. Immunohistochemistry of EMT Markers in 109 Hepatocellular Carcinoma Cases

Marker	Negative (%)	Positive (%)
Vimentin	32 (29.4)	77 (70.6)
E-cadherin	82 (75.2)	27 (24.8)
N-cadherin	81 (47.3)	28 (25.7)
Stat 3	80 (73.4)	29 (26.6)
Snail	99 (90.8)	10 (9.2)
Slug	105 (96.3)	4 (3.7)
Twist1	31 (28.4)	78 (71.6)
Zeb1	58 (53.2)	51 (46.8)
Integrin α 5	14 (12.8)	95 (87.2)

and Zeb1 which showed positive correlation between the expression levels of any two markers.

Discussion

Hepatocellular carcinoma (HCC) is an aggressive disease with high rate of recurrence and metastasis (Forner et al., 2012). The value of the traditional clinicopathologic parameters in predicting the patient outcome after curative surgery is limited (Bruix et al., 2014). This indicates the need for other potential markers that can be of prognostic value in HCC (Kim et al., 2010). Several molecular techniques have proposed a gene signature for predicting prognosis of HCC patients (Kim et al., 2010; Nagai et al., 2016; Villanueva et al., 2007; Wang et al., 2007). Nevertheless, these methods cannot be applied for routine practice due to their tedious technology (Dupuy and Simon, 2007). Immunohistochemistry is now considered a reliable and simple method which is available in routine pathology laboratories (Chen, 2000). The process of epithelial–mesenchymal transition (EMT) has been reported to be a key factor in cancer development and progression including metastasis and recurrence (Ye and Weinberg, 2015; Smith and Bhowmick, 2016). Numerous studies investigated the prognostic value of EMT in HCC. However, most of these studies, if not all, are from East Asia, mainly China (Iwatsuki et al., 2010; Nagai et al., 2016). Up to our knowledge, no reports are available from the Middle East especially Egypt, where HCC is a very common malignancy (Pascut et al., 2020).

In this study we assessed the value of nine EMT markers in a cohort of 109 HCC patients by correlating them to the conventional clinicopathologic factors as well as recurrence, overall survival (OS) and disease free survival (DFS). It was astonishing to find that the level of reduced E-cadherin expression (75%) and the positive expression of vimentin (71%), Twist1 (72%) and Zeb1 (47%) were much higher than that reported in many immunohistochemical studies (Bruix et al., 2014; Zhang et al., 2012; Yao et al., 2013; Yang et al., 2007; Sun et al., 2010). This difference may be due to variation in methodology, types and dilution of antibodies or interpretation of results. Alternatively, we speculate that the higher level of EMT markers in our patients as compared to other studies is due to the fact that all cases

Table 3. Relationship between Immunohistochemical Expression of EMT Markers and Clinicopathologic Variables

Variables	Vimentin			Reduced Ecadherin		Reduced Ncadherin		Stat 3		Snail		Slug		Twist1		Zeb1		Integrin α 5	
variable	N	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P	N	P
Age (years)																			
≤ 50	18	16	NS	13	NS	11	NS	4	NS	0	NS	0	NS	15	NS	9	NS	14	NS
> 50	91	61		69		70		25		10		4		63		42		81	
Gender																			
Male	87	65	NS	70	NS	66	NS	24	NS	9	NS	3	NS	67	NS	44	NS	78	NS
Female	19	12		12		15		5		1		1		11		7		17	
Grade																			
I	41	28		25		30		8		1		1		25		14		35	
II	53	37	NS	43	0.02	43	NS	17	NS	5	0.02	3	NS	40	NS	29	NS	48	NS
III & IV	15	12		14		8		4		4		0		13		8		12	
AFP (ng/ML)																			
≤ 400	60	47	NS	43	NS	43	NS	11	NS	0	0.002	2	NS	38	0.009	24	NS	50	NS
> 400	13	11		10		8		3		2		1		13		3		13	
Tumor size																			
≤ 5cm	73	58	0.004	57	NS	51	NS	18	NS	3	0.009	2	NS	51	NS	33	NS	60	0.03
> 5cm	36	19		25		30		11		7		2		27		18		35	
Stage																			
I	39	23		29	NS	31	NS	12	NS	4	NS	1	NS	29	NS	24	0.01	33	NS
II	51	41	NS	39		34		9		2		2		36		16		44	
III	19	13		14		16		8		4		1		13		11		18	
V-invasion																			
Absent	75	46	0.002	51	0.009	57	NS	22	NS	5	NS	2	NS	51	NS	37	NS	65	NS
Present	34	31		31		24		7		5		2		27		14		30	
Recurrence																			
Absent	39	19	0	23	0.003	32	NS	10	NS	5	NS	1	NS	27	NS	25	0.007	35	NS
Present	70	58		59		49		19		5		3		51		26		60	

Table 4. Multivariate Analysis of Prognostic Value of EMT Markers According to Real Time PCR Results

Marker	Tumor size	Histologic grade	Vascular invasion	TNM stage	Recurrence	Overall Survival	Disease free Survival
Vimentin							
Odds ratio	0.15	—	8.7	—	3.99	3.71	2.1
95% CI	0.05 - 0.46		1.99-37.93		1.48 - 10.80	0.83 - 16.67	1.06-4.17
P	0.001		0.004		0.006	NS	0.03
E cadherin							
Odds ratio	—	0.21	0.24	—	0.16	0.39	0.74
95% CI		0.08 - 0.56	0.05 – 1.14		0.05 – 0.53	0.09 – 1.76	0.36 – 1.51
P		0.002	NS		0.002	NS	NS
Snail							
Odds ratio	5.63	3.72	—	—	—	—	—
95% CI	1.36 – 23.30	1.32 – 10.47					
P	0.02	0.01					
Zeb1							
Odds ratio	—	—	—	2.39	1.04	—	0.67
95% CI				1.19 – 4.82	0.41 – 2.66		0.41 – 1.10
P				0.02	NS		NS
Integrin α 5							
Odds ratio	7.58	—	—	—	—	0.35	—
95% CI	0.95 – 60.47					0.15 – 0.81	
P	NS					0.02	

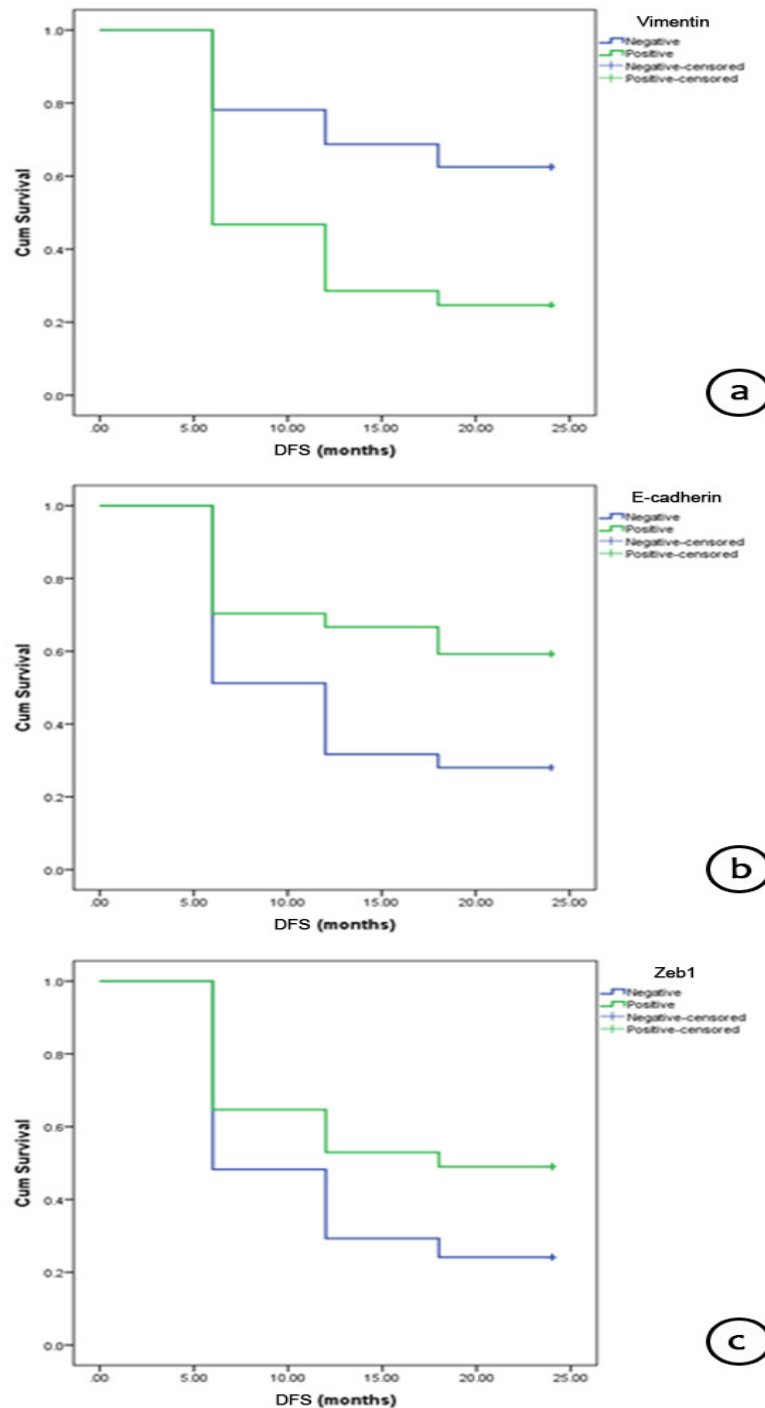


Figure 3. Kaplan-Meier Survival Curve Showing the Relationship between Disease Free Survival and the Expression of (a) Vimentin (P=0.000), (b) E-cadherin (P=0.006), and (c) Zeb1 (P=0.008)

included in the present work were HCV-associated in contrast to those studies where HCV infection was not present or limited to few or some of the investigated HCC cases (Yang et al., 2009; Sun et al., 2010; Zhao et al., 2011; Zhang et al., 2012; Yao et al., 2013; Hashiguchi et al., 2013; He et al., 2014; Luo et al., 2016; Yuan et al., 2020). The role of HCV in inducing the process of EMT was pointed out by several studies (Battaglia et al., 2009; Bose et al., 2012; Akkari et al., 2012; Iqbal et al., 2014; Kwon et al., 2015). Battaglia et al., (2009) found that in HCC, the expression of HCV-derived core protein switched the cellular response to transforming growth factor β from

inhibition of growth to induction of EMT. A more recent study reported that HCV – infected hepatocytes secrete osteopontin which binds with cell surface receptors and triggers signaling cascade that promotes EMT (Iqbal et al., 2014). Last but not least, Kwon et al., (2015) highlighted that HCV induces signaling molecules that trigger EMT generation.

Multivariate analysis demonstrated that the most important one among all EMT markers included in the current study was vimentin which proved to be an independent indicator of the tumor size (P = 0.001), vascular invasion (P = 0.004) and more importantly

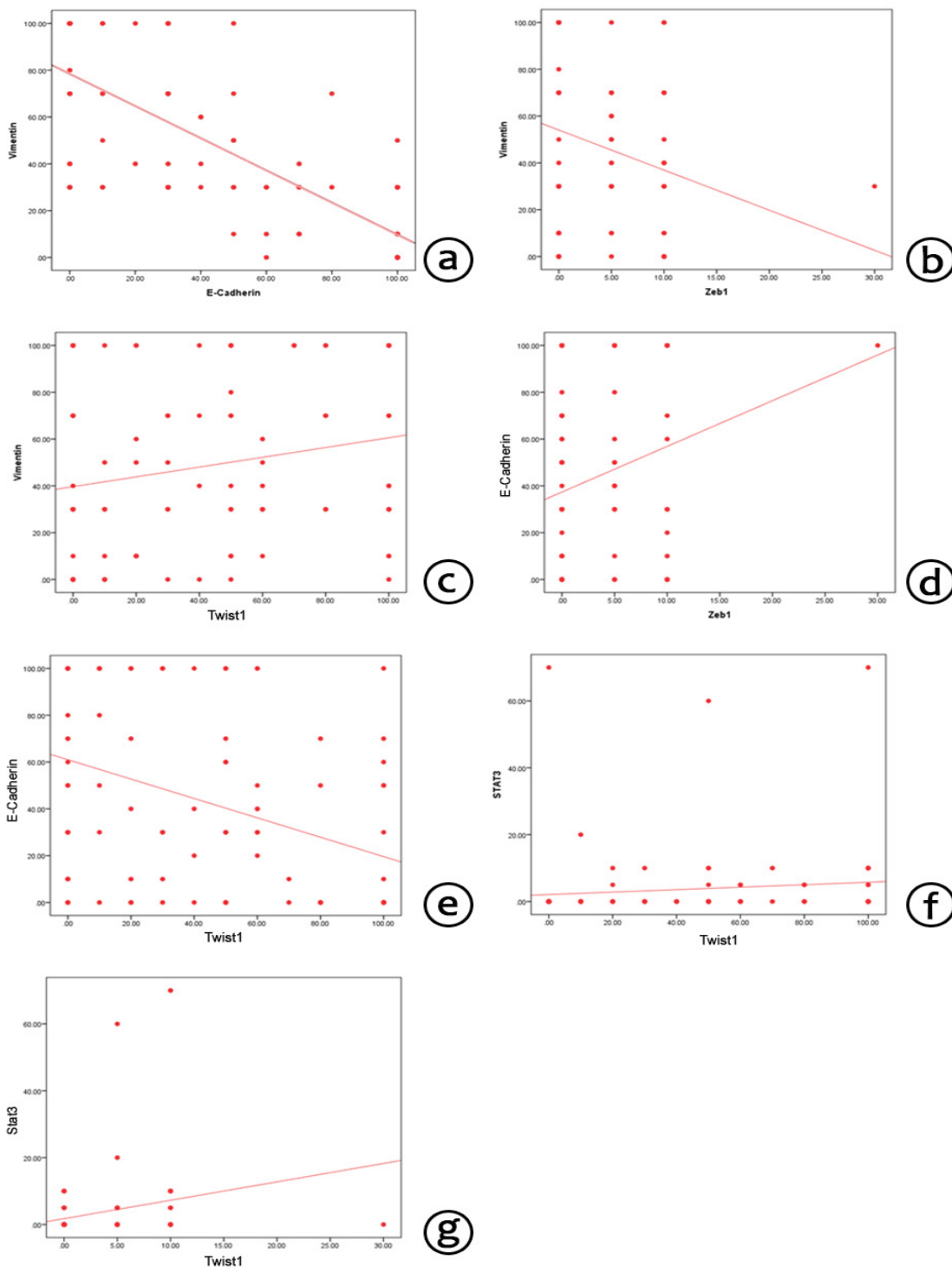


Figure 4. Spearman Rank Correlation between: (a) Vimentin and E-cadherin ($r=-0.764$, $P=0.000$), (b) Vimentin and Zeb1($r=-0.220$, $P=0.028$), (c) Vimentin and Twist1 ($r=0.215$, $P=0.03$), (d) E-cadherin and Zeb1 ($r=0.216$, $P=0.03$), (e) E-cadherin and Twist1 ($r=-0.386$, $P=0.000$), (f) Stat3 and Twist1($r=0.327$, $P=0.001$), (g) Stat3 and Zeb1 ($r=0.290$, $P=0.003$) and Zeb1 and Twist1 ($r=0.203$, $P=0.04$)

recurrence ($P = 0.006$) and DFS (0.03). This agrees with previous reports (Satelli et al., 2011; Li et al., 2013; Mima et al., 2013). The prognostic value of vimentin in HCC can be explained by various mechanisms. First, vimentin as an EMT marker promotes tumor angiogenesis and thus stimulates invasion and metastasis (Zhang et al., 2013). Second, EMT markers including vimentin are suggested to have an anti-apoptotic action which leads to tumor growth

(Shang et al., 2013; Sui et al., 2014). Third, vimentin may induce cancer stem cell generation in cancer patients and accordingly enhance tumor growth and recurrence (Li and Zhou, 2017).

The next important EMT marker in our study was E-cadherin which was found to be an independent predictor of histologic grade ($P = 0.002$) and recurrence ($P = 0.002$). This result was previously reported by others

(Sun et al., 2010; Zhang et al., 2012; Cai et al., 2012; Hashiguchi et al., 2013; Liu et al., 2017). The important role of vimentin and E-cadherin was further supported by the significant positive correlation between the levels of vimentin expression and E-cadherin reduced expression. In fact, these findings were expected. Vimentin and E-cadherin are the main pillars in the process of EMT, since EMT is manifested by changing the epithelial morphology of the cells through losing the epithelial markers mainly E-cadherin and acquiring the spindle cell morphology via expression of the mesenchymal markers as vimentin (Cai et al., 2012; Zheng and Kang, 2014).

Other EMT markers that showed independent prognostic value included Snail, Zeb1 and integrin α 5. The rest of markers included in our study (N-cadherin, Stat3, Slug and Twist1) had no independent prognostic value. These data agree with some studies (Yang et al., 2009; Kim et al., 2010; Sun et al., 2010; Nagai et al., 2016; Song et al., 2020). Other studies found that some of these EMT markers, especially N-cadherin and Twist1 had prognostic value in HCC. Yet, most of these studies did not apply multivariate analysis to assess the independent prognostic value of these markers (Iwatsuki et al., 2010; Zhao et al., 2012; Li et al., 2013; Yao et al., 2013; Hashiguchi et al., 2013, Liu et al., 2017).

One of the important points investigated in our study was the relationship between the various EMT markers. Vimentin, E-cadherin, Twist1 and zeb1 were significantly correlated with each other. On the other hand, Stat3, Twist1 and Zeb1 were also significantly correlated with each other. These results suggest that EMT markers act interdependently. In other words, there is a cross-talk or signal axis relating these markers to each other to act cooperatively rather than individually to promote EMT in HCC. The relationship between various EMT markers in HCC patients was the subject of controversy. Li et al., 2013 reported that the level of E-cadherin did not correlate with snail, slug or Zeb1. Yang et al., (2009) found that E-cadherin correlated with Snail and Twist, but not with Slug. Other studies demonstrated that E-cadherin correlates negatively with Twist, while Zhang et al., (2012) could not achieve such a correlation.

The most important limitations in this study were the relative small number of cases and short follow up period. Conversely, it has several points of strength.

First, up to our knowledge, this is the initial study from the Middle East investigating EMT in HCC. Second, we analyzed a relatively good number of EMT markers with follow up data and multivariate analysis. Last, but not least, All HCC cases included in this work were HCV-associated which allowed us to disclose the effect of HCV on EMT expression in HCC. However, this needs further investigations with HCV-negative control cases.

In conclusion, our results demonstrated that: 1) The level of EMT markers in Egyptian HCC patients is higher than that reported in the literature. Although HCV infection seems to be repressible of this high EMT level, further studies are recommended to confirm this speculation. 2) EMT markers play an impotent role in HCC prognosis and they act cooperatively as shown by the significant correlation between most of them. 3) More importantly,

vimentin and E-cadherin proved to be strong independent predictors of patient prognosis, especially recurrence. These two markers are available in any routine pathology laboratory with easy and simple immunohistochemical technique. Therefore, we recommend these two markers in HCC patients with curative resection for prediction of the potential risk of recurrence.

Author Contribution Statement

Helal, T: PI of STDF grant, study concept and design, writing the manuscript. Aref, A: performed the gene expression analysis for EMT, literature search. Gomaa, A: managing all clinical & survival data for HCC cases from NLI. Nada, O: interpretation of immunohistochemical results. Abdelghaffar, M: managing clinical & survival data for HCC cases from Ain Shams. Farouk, K: managing clinical & survival data for HCC cases from Dar Al-Fouad Hospital. Ehsan, N: interpretation of immunohistochemical results, drafting the manuscript.

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This study was approved by the Ethical Committee (IRB) of Faculty of Medicine, Ain Shams University, Cairo, Egypt following the Declaration of Helsinki for medical research involving human subjects.

Conflict of interest

All authors declare no conflict of interest.

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