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Expression of Podoplanin in Hepatocellular Carcinoma in a Sample of Egyptian Population – Immunohistopathological Study

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Abstract

BACKGROUND: Hepatocellular carcinoma (HCC) is a highly incident malignancy with a dreadful prognosis. It evolves through a multistep process, with a contribution from different stromal cells like cancer associated fibroblasts. Podoplanin is a glycoprotein that influences epithelial mesenchymal interplay facilitating the tumor invasion.

AIM: The aim of the study was to evaluate the immunohistochemical expression of Podoplanin in HCC in cancer associated fibroblasts (CAFs) and malignant hepatocytes as well as assessing the lymphovascular density, and correlating them with the clinicopathological parameters.

METHODS: Sixty formalin-fixed paraffin-embedded HCC tissue blocks were retrieved from the pathology Department of the National Hepatology and Tropical Medicine Research Institute and Kasr Al-aini Hospital during the period of January 2012 till December 2019. The specimens were obtained through partial or total hepatectomy inclusion criteria included HCC cases obtained through resection type biopsy and those having no history of pre-operative cancer therapy, while cases with insufficient data, core biopsy, and marked necrosis were excluded from the study. Tumor tissue blocks were immunostained for Podoplanin and its expression was interpreted in lymphatic vessels, CAFs, and malignant hepatocytes.

RESULTS: Podoplanin expression in CAFs and malignant hepatocytes was detected in the majority of HCC cases (81.7%) and (88.3%), respectively. The malignant hepatocytes showed increased expression of Grade 1 immunostaining (36.7%). High lymphovascular density was detected over the majority of the cases (73.3%). Podoplanin expression was significantly correlated with higher mean age, male gender, presence of viral infection, cirrhosis, and higher tumor grade. Unifocal tumor mass, tumor size <5 cm, and presence of invasion showed a significant correlation with Podoplanin in malignant hepatocytes and CAFs for the formers and the later, respectively.

CONCLUSION: Podoplanin is highly expressed in HCC, which could be used as a prognostic marker for lymphangiogenesis. Furthermore, within the malignant hepatocytes and CAFs suggesting a role in hepatocellular tumorigenesis. Podoplanin targeted therapy can be investigated to slow down the tumor progression and metastasis.

Introduction

Hepatocellular carcinoma (HCC) is a universal health trouble that ranks 6th and 3rd in the incidence and the mortality of malignant tumors. In Egypt, it is highly recorded due to the increased incidence of the hepatocarcinogenic hepatitis C virus, combined with the limited therapy options and the refractoriness to therapy [1], [2]. The malignant tumorigenesis involves a composite interplay between the neoplastic and the stromal cells through epithelial-mesenchymal crosstalk [3]. The stromal cells include different cell types such as cancer-associated fibroblasts (CAFs) and myofibroblasts. They originate locally from the progenitors of the resident fibroblasts or myofibroblasts, the vasculogenic cells through endothelial mesenchymal, and epithelial-mesenchymal transitionor bone marrow precursor cells [4], [5]. CAFs differentiation is mediated by growth factors (e.g., transforming growth factor β, platelet-derived growth factor, and fibroblast growth factor 2). They are excreted from neoplastic and non-neoplastic cell types such as immune cells [6], [7], [8]. CAFs contribute to tumor initiation and progression through the secretion of growth factors, cytokines, and chemokines [9], [10], [11]. It also potentiates the invasion and the dissemination through the matrix metalloproteinase that facilitates basement membrane invasion [12]. Equivalent effects are exerted at the metastatic sites; promoting the disseminated cells proliferation, protective cancer niches formation, angiogenesis, and modulation of ECM. A plethora of action mediated by metastasis-associated fibroblasts, which could represent a variant of CAFs [13], [14]. HCC microenvironment showed abundance of CAFs [15], they are allocated in the HCC fibrous septae, capsule, and blood sinusoids [16], [17]. Furthermore, liver metastasis is associated with stellate cells activation which may contribute to create a cancer niche and angiogenesis [18]. The potential use of CAFs in tumor target therapy is investigated to suppress their proliferation, and their cytokines

secretion to inhibit HCC progression [19]. Podoplanin is a normally detected transmembrane sialoglycoprotein within the lymphatics, endothelial cells, lung, heart, and mesothelial cells [20], [21]. Over-or neo-expression was detected in lymphangiomas, seminomas, mesothelioma, granulosa cell tumors [22], [23], and squamous cell carcinoma [24]. Podoplanin induces remodeling of the actin component of the tumor cells cytoskeleton facilitating tumor cell motility and thereby tumor invasion [25]. In addition, it induces dendritic cell (DC) migration through a complex interaction between DC receptor C-type lectin-like receptor-2 (CLEC-2) and the Podoplanin that is expressed on fibroblastic reticular cells and lymphatic endothelial cells. This causes an increase in the migratory potential of the DCs and may also contribute to the migration of cancer cells along with the lymphatic networks [26]. Podoplanin exerts platelets aggregating potentials; mediated in part by its extracellular domain that consists of the EDxxVTPG segment platelet aggregating domain (PLAG) [27], and in another part by the Podoplanin receptor (CLEC-2), which was detected on human platelets [28], [29]. This platelet aggregating capacity helps malignant cells to escape from the host immune system, and enhance their metastatic potential [30]. A tumor-targeted therapy can inhibit Podoplanin platelet aggregating function by blocking antibodies that direct against CLEC-2 [31] or inhibit the interaction of the PLAG domain and CLEC-2 with a less resultant propensity for tumor metastasis [29], [32]. The Podoplanin expression of the lymphatic vessels can evaluate lymphovascular density (LVD) and lymphovascular invasion, which can detect nodal occult metastasis [33], [34], and it may be used for risk-based stratification of the patients within the same TNM stage, for further consideration of adjuvant chemotherapy [34], [35]. It is also expressed in the malignant hepatocytes, and high expression was significantly correlated with a higher histological grade. Podoplanin expression in HCC thus can be used as a prognostic marker or for the future trials in new therapeutic strategies [36]. The objective of this study was to evaluate Podoplanin expression in the epithelial and the mesenchymal cells in HCC by assessing its presence in the malignant hepatocytes, the (CAFs) and also evaluating LVD in HCC and correlation with clinic-pathological parameters.

Materials and Methods

Study population and tumor criteria

A retrospective study was conducted on 60 paraffin blocks of HCC from the pathology department of the National Hepatology and Tropical Medicine Research Institute and Kasr Al-aini Hospital during the period from January 2012 to December 2019. The samples were obtained through partial or total hepatectomy procedure. The study attained approval by the Ethical Committee for the use of the patient samples in medical research. The relevant patients' personal records were retrieved from the files.

Inclusion criteria

Resection type specimen and absence of pre-operative cancer therapy were included in the study.

Exclusion criteria

Insufficient clinical data, inadequately fixed specimens, and tumor with marked necrosis were excluded from the study.

Histopathological evaluation

Evaluation of H&E stained sections was performed by two independent pathologists. Categorization of the tumors using modified Edmondson and Steiner system into well, moderately and poorly differentiated [37], [38]. Dichotomization of the tumor according to the grade into low (well-differentiated) and high grades (moderately and poorly differentiated) was done [39], [40] and according to the size into less and greater than 5 cm [36], [41].

Immunohistochemical procedures

A section of formalin-fixed paraffin-embedded tissue block was cut at 4-μ thickness and mounted on an adhesive-coated glass slide. Immunostaining was performed using Podoplanin monoclonal antibody (DAKO, M361901-2, clone D2-40, Mouse monoclonal Ig G) pre-diluted at (1:50), manufactured by AGILENT, USA. The immunohistochemical reaction was performed in Ventana autostainer with controlled steps following Dako standard protocol, with heat mediated antigen retrieval system using the Avidin-Biotin immunoperoxidase detection system.

In each staining session, a section of the appendix served as positive control and a tumor tissue section treated with PBS instead of the primary antibody was served as a negative control.

Immunohistochemical evaluation

(I) Positive immunostaining for Podoplanin was visualized with detection of brown coloration in the cell membrane and/or cytoplasm of the malignant hepatocytes and CAFs [42].

(II) Expression of Podoplanin in CAFs was considered positive if 10% or more of spindle-shaped cells in the cancer stroma showed brownish staining intensity like that of the positive control [43].

(III) Lymphatic microvascular density (LVD) was evaluated intra and peri-tumoral, by calculating the mean value in three "vascular hot spots" at 200 magnification power. A two-tired system was used to categorize the tumor into low and high LVD, guided by the mean value [36].

(IV) Correlation of the LVD, the positively stained CAFs, and the malignant hepatocytes with the variable clinicopathological data was performed.

(VI) Correlation of the LVD with each of the positively stained CAFs and the malignant hepatocytes was performed.

Table 1: Clinicopathological parameters of studied cases

Parameter	Study Cases (n = 60)
Age, years (Mean ± SD)	57 ± 11.2
Gender, n (%)	
Male	47 (78.4)
Female	13 (21.6)
Etiology, n (%)	
Viral	51 (85)
Non-viral	9 (15)
Multifocality, n (%)	
Unifocal	49 (81.6)
Multifocal	11 (18.4)
Size, n (%)	
<5 cm in maximal diameter.	52 (86.6)
>5 cm in maximal diameter	8 (13.4)
Cirrhosis, n (%)	
Present	51 (84)
Absent	9 (15)
Grade, n (%)	
Low grade	12 (20)
High grade	48 (80)
Capsular Invasion, n (%)	
Present	11 (18.3)
Absent	49 (81.7)

Statistical analysis

Data management and analysis were performed using Statistical Package for the Social Sciences version 21. Numerical data were summarized using means and standard deviations or medians and ranges. Shapiro–Wilk normality test was used. Categorical data were summarized as frequencies and percentages. Comparisons between the two groups concerning normally distributed numeric variables were done using the t-test. For categorical variables, differences were analyzed with Chi-square test or Fisher's exact test when appropriate. p-values are two-sided. $P < 0.05$ was considered significant.

Results

The present study enrolled 60 patients; the age incidence ranged between 35 and 72 years with a mean value of 57 years. It included 47 males (78.3%) and 13 females (21.6%). Hepatic viral infection was present in 51 (85%) cases and cirrhosis was detected in 51 (85%) cases. The tumor was unifocal in 49 (81.6%) and multifocal in 11 (18.3%) cases. The tumor size was <5 cm in 52 (86.6%) cases. Either capsular and or vascular invasion was seen in 11 (18.3%) cases.

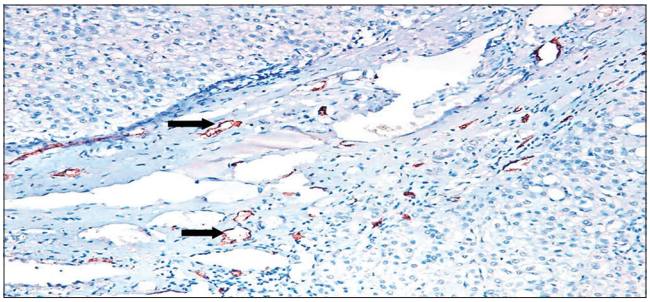

Figure 1: Low density of lymphatic vessels proliferation in hepatocellular-carcinoma (black arrow); immunostained with Podoplanin (original magnification ×200)

Low-grade anaplasia was dominating in 52 (86.7%) cases while 8 (13.3%) cases displayed high-grade features (Table 1).

Table 2 : Podoplanin expression in lymphatic vessels, CAFs, and malignant hepatocytes in studied cases

Podoplanina expression	n (%)
Lymphatic vessels	
LLVD	16 (26.7)
HLVD	44 (73.3)
CAF	
Positive	41 (81.7)
Negative	19 (18.3)
Hepatocytes	
Positive	53 (88.3)
Negative	7 (11.2)

Immunohistochemical characteristics

Podoplanin-stained lymphatic vessels were used to evaluate the LVD. Its count ranged between 9 and 61, with a mean value of 37. The lymphovascular proliferation was categorized into low and high LVD guided by the statistical mean value (Figures 1 and 2).

The tabulated results of Podoplanin expression in HCC showed a predominating high lymphovascular density (HLVD) in 44 (73.3%) cases, while low lymphovascular density (LLVD) was detected in 16 (26.7%) cases (Table 2).

Podoplanin expression in CAFs and malignant hepatocytes was also detected in the majority of cases (41 cases; 81.7%) and (53 cases (88.3%), respectively (Figures 3 and 4).

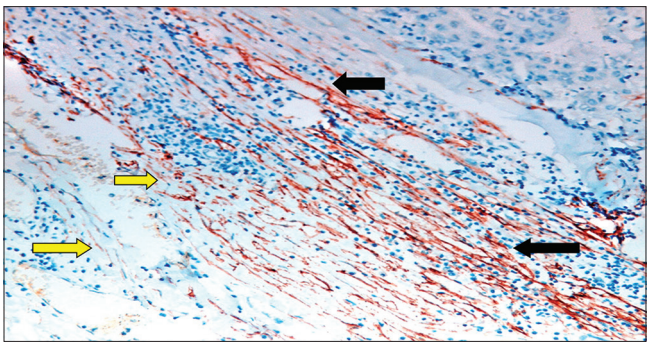

Figure 2: Hepatocellular carcinoma showing high density lymphatic vessels proliferation (black arrow) and positively stained cancer associated fibroblasts in peri-tumoral area (yellow arrow) delineated by positive Podoplanin immunostaining (original magnification ×200)

The malignant hepatocytes showed Grades 1, Grade 2, and Grade 3 in 22 cases (36.7%), 17 cases (28.3%), and 14 cases (23.3%), respectively (Table 3).

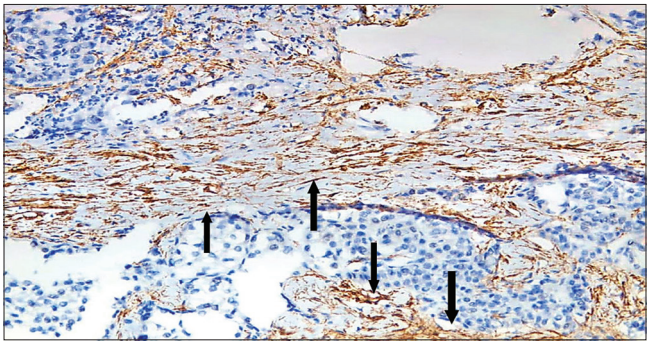

Figure 3: CAFs (black arrow) in peritumoral area showed positive staining for Podoplanin in hepatocellular carcinoma (original magnification ×200)

Age was a significant factor when correlated with Podoplanin expression as a higher mean age (58.5 years) was statistically significantly associated with HLVD-associated-HCC and the reverse for that of LLVD-associated HCC which was associated with lesser mean age (55 years) (p = 0.005) (Table 4). Both of the malignant hepatocytes and CAFs positively expressing Podoplanin were associated with a lesser mean age (55.1 years and 53.29 years, respectively). The association was statistically significant for the former (p < 0.001) (Table 6) and insignificant for the later (p = 0.1) (Table 5).

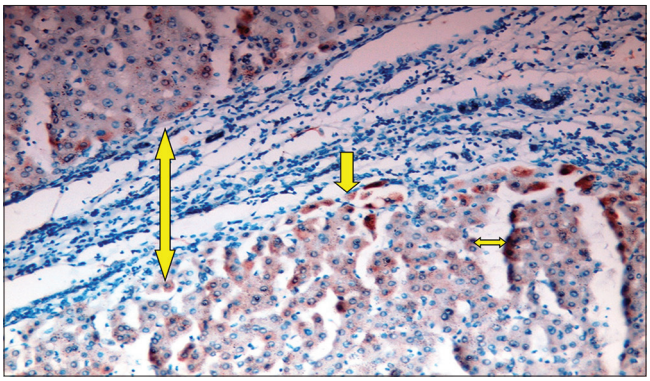

Figure 4: Malignant hepatocytes showing positive Podoplanin staining with cytoplasmic pattern (yellow arrow) (original magnification ×200)

The association between Podoplanin expression and gender was statistically significant; HLVD-associated HCC was detected in 38 males (86.4%) and 6 females (13.6%), while LLVD-associated HCC was detected in 9 males (56.25%) and 7 females (43.75%) (p = 0.01) (Table 4).

Table 3: Degree of Podoplanin expression in malignant hepatocytes

The degree of staining		n (%)
Negative	<1%	7 (11.7)
Positive	1–10% (Grade 1)	22 (36.7)
	≥10–<50 (Grade 2)	17 (28.3)
	≥50% (Grade 3)	14 (23.3)
Total		60 (100)

Positively expressed CAFs were detected in 36 males (87.8%) and 5 females (12.2%) (p = 0.008) (Table 5).

Table 4: Association between Podoplanin expression in lymphatic vessels and other clinicopatholgical variables

Factors	Podoplanin expression in lymphatic vessels		p-value
	LLVD n = 16	HLVD n = 44	
Mean age, years	55 years	58.53 years	*0.005
Gender, n (%)			
Male	9 (56.25)	38 (86.4)	*0.01
Female	7 (43.75)	6 (13.6)	
Etiology, n (%)			
Viral	11 (68.8)	40 (91)	*0.03
Non-viral	5 (31.2)	4 (9)	
Size (cm), n (%)			
<5 cm	14 (87.5).	38 (86.4)	0.9
>5 cm	2 (12.5)	6 (13.6)	
Focality, n (%)			
Unifocal	12 (75)	37 (84.1)	0.4
Multifocal	4 (25)	7 (15.9)	
Grading, n (%)			
Low	8 (50)	4 (9)	*<0.001
High	8 (50)	40 (91)	
Capsular invasion, n (%)			
Negative	13 (81.3)	36 (81.8)	0.95
Positive	3 (18.7)	8 (18.1)	
Cirrhosis, n (%)			
Present	11 (68.8)	40 (91)	*0.03
No Cirrhosis	5 (31.2)	4 (9)	
Podoplanin in CAFs, n (%)			
<10% 0f tumor area	11 (68.8)	8 (18,2)	*<0.001
>10% of tumor area	5 (31.2)	36 (81.8)	
Podoplanin in malignant hepatocytes, n (%)			
Negative	5 (31.25)	2 (4.5)	*<0.001
Positive	11 (68.75)	42 (95.6)	

Positively stained malignant hepatocytes were detected in 44 males (83%) and 9 females (17%) (p = 0.01) (Table 6).

Table 5: Association between Podoplanin expression in CAFs and other clinicopathological variables

Factors	Podoplanin expression in CAFs		p-value
	Negative, n = 19	Positive, n = 41	
Age (years)			
Mean	57.39 years	55.29 years	0.115
Gender, n (%)			
Male	11 (58)	36 (87.8)	*0.008
Female	8 (42)	5 (12.2)	
Etiology, n (%)			
Viral	13 (68.4)	38 (92.7)	*0.01
Non-viral	6 (31.6)	3 (7.3)	
Size (cm), n (%)			
<5 cm	15 (79).	37 (80)	0.2
>5 cm	4 (21)	4 (20)	
Focality, n (%)			
Unifocal	16 (84.2)	33 (80.5)	0.7
Multifocal	3 (15.8)	8 (19.5)	
Grading, n (%)			
Low	7 (36.8)	5 (12.2).	*0.02
High	12 (63.2)	36 (87.7)	
Capsular invasion, n (%)			
Negative	11 (57.9)	38 (92.7)	*0.001
Positive	8 (42.1)	3 (7.3)	
Cirrhosis, n (%)			
Present	12 (63.1)	39 (95.1)	*0.001
Absent	7 (36.9)	2 (4.8)	
Podoplanin in lymphatic vessels, n (%)			*<0.001
LLVD	11 (68.8)	5 (31.2)	
HLVD	8 (18.2)	36 (81.8)	
Podoplanin in malignant hepatocytes, n (%)			
Negative	4 (21)	3 (7.3)	*0.12
Positive	15 (79)	38 (92.7)	

Viral infection was statistically significantly associated with Podoplanin expression in HCC. HLVD-associated HCC was detected in 40 viral-caused cases (91%) and LLVD-associated HCC was detected in 11 viral-caused HCC (68.6%) (p = 0.03) (Table 4). Positively stained CAFs were detected in 38 viral-caused HCC (92.7%) (p = 0.01) (Table 5). The positively stained malignant hepatocytes in viral-caused HCC were detected in 49 cases (92.5%) (p = 0.17) (Table 6).

The presence of hepatic cirrhosis in HCC and Podoplanin expression revealed a statistically

Table 6: Association between Podoplanin expression in malignant hepatocytes and other clinicopathological variables

Factors	Podoplanin expression in malignant hepatocytes		p-value
	Negative, n = 7	Positive, n = 53	
Age (years)			
Mean	57 years	55.12 years	*<0.001
Gender, n (%)			
Male	3 (42.9)	44 (83)	*0.01
Female	4 (57.1)	9 (17)	
Etiology, n (%)			
Viral	5 (71.4)	46 (86.8)	0.2
Non-viral	2 (28.6)	7 (13.2)	
Size (cm), n (%)			
<5 cm	2 (28.6).	50 (94.3)	*<0.001
>5 cm	5 (71.4)	3 (5.7)	
Focality, n (%)			
Unifocal	4 (57.1)	45 (84.9)	0.07
Multifocal	3 (42.9)	8 (15.1)	
Grading, n (%)			
Low	4 (57.1)	8 (15.1).	*0.008
High	3 (42.9)	45 (84.9)	
Capsular invasion, n (%)			
Negative	5 (71.4)	44 (83)	0.4
Positive	2 (28.4)	9 (17)	
Cirrhosis, n (%)			
Present	2 (28.4)	49 (94.3)	0.2
Absent	5 (71.4)	4 (5.7)	
Podoplanin in CAFs, n (%)			
<10% of tumor area	4 (57,1)	15 (28.3)	0.12
>10% of tumor area	3 (42.9)	38 (71.7)	
Podoplanin in lymphatics, n (%)			
LLVD	5 (71.4)	11 (20.75)	*0.004
HLVD	2 (28.4)	42 (79.25)	

significant association with LVD and CAFs and an insignificant one with the malignant hepatocytes. HLVD was detected in 40 cirrhosis-associated HCC (91%) and LLVD was detected in 11 cirrhosis-associated HCC (95.2%) (p = 0.03) (Table 4).The positively stained CAFs were detected in 39 cirrhosis-associated HCC cases (95.2%) (p = 0.001) (Table 5). Podoplanin positively stained malignant hepatocytes were detected in 46 cirrhosis-associated HCC cases (86.6%) (p = 0.2) (Table 6).

The association of Podoplanin expression with unifocal HCC was significant as regards malignant hepatocytes and insignificant with LVD and CAFs. The malignant hepatocytes positively expressing Podoplanin were detected in 45 unifocal HCC (84.9%) and 8 multifocal HCC (15.1%) (p = 0.07) (Table 6). LLVD was detected in 12 unifocal HCC (75%) and in 4 multifocal HCC (25%), while HLVD was detected in 37 unifocal HCC (84.1%) and in 7 multifocal HCC (15.9%) (p = 0.4) (Table 4). Positive CAFs were detected in 33 unifocal cases (84.2%) and in 8 multifocal cases (15.8%) (p = 0.7). (Table 5).

The association between Podoplanin expression with HCC of <5 cm size revealed significance with malignant hepatocytes and insignificance with LVD and CAFs. The positively stained malignant hepatocytes were detected in 50 cases with size of <5 cm (94.3%) (p < 0.001) (Table 6). LLVD was detected in 14 HCC sized <5 cm (87.5%), while HLVD was detected in 38 HCC sized <5 cm (86.4%) (p = 0.9) (Table 4). CAFs showed positive expression in 37 HCC sized <5 cm (80%) (p = 0.2) (Table 5). Higher tumor grade was significantly associated with Podoplanin expression. LLMVD was detected equally in both grades (8/16; 50% for each), while HLVD was detected in 40 high-grade HCC cases (91%) (p < 0.001).CAFs were detected in 36 high-grade cases (81.8%) (p = 0.02). Positively stained malignant hepatocytes were detected in 45 high-grade HCC cases (85%) (p = 0.008).

Podoplanin expression in HCC showed a significant association with CAFs and insignificant correlation with LVD and malignant hepatocytes in HCC cases associated with invasion (vascular and or capsular). The positively stained CAFs were detected in 3 cases (7.3%) (p = 0.001). LLVD-HCC was associated with invasion in 3 cases (18.7 %), while HLVD- HCC was detected in 8 cases (18.1%) (p = 0.95). Malignant hepatocytes positively expressing Podoplanin were detected in 9 invasion-associated cases (17%) (p = 0.4).

The association between Podoplanin expression in CAFs and the degree of lymphovascular proliferation revealed a statistically significant relationship; as LLVD-HCC associated with positive expression in CAFs was detected in 5 cases (31.2%), while HLVD-HCC was detected in 36 cases (81.8%) (p < 0.001). Increased degree of LVD is significantly associated with Podoplanin expression in malignant hepatocytes, as malignant hepatocytes positively immunostained for Podoplanin were associated with LLVD in 11cases (68.75%) and HLVD in 42 cases (95.6%) (p = 0.004).

Grade 2 Podoplanin expression in malignant hepatocytes was the most frequently encountered in association with the presence of positively stained CAFs. HCC cases associated with negatively stained CAFs showed absence of staining in malignant hepatocytes in 4 cases (12.1%) and Grade 1 in 15 cases (45.6%), Grade 2 in 9 cases (27.3%), and Grade 3 in 5 cases (15.1 %), while those associated with positively stained CAFs showed absence of staining in 1 case (14.3%) and Grade 1 in 1 case (14.3%), Grade 2 in 3 cases (42.8%), and Grade 3 in 2 cases (28.6 %). The correlation was statistically insignificant (p = 0.477).

Discussion

HCC is an aggressive worldwide malignancy, with rapid death of untreated cases [44]. This aggressive behavior could be partly attributed to its high, yet, complex angiogenic potentialities that it is a corner stone in tumor growth and spread [45]. In the present study, the mean value of lymphatic vessels proliferation in HCC was estimated to be 37. These results were within the range reported by Abd El-Fattah *et al*., whose mean expression value was 34.70 ± 8.655 [46] and approaching those of Thelen *et al*., which was 22.9 ± 22.1 [47], but it was much less than that reported by He *et al*., whose mean value was 63.40 [48]. In the present study, positive Podoplanin expression in cancer-associated fibroblasts was seen in the majority

of cases which showed approval with other studies like Yoshida *et al*., [49] who reported 53.7%, Andreea *et al*., [38] who documented 57%, and Abd El-Fattah *et al*. [46], that documented positivity in 73.3 %. Studying the Podoplanin expression in malignant hepatocytes revealed its predominant presence which showed agreement with Ciurea *et al*., who reported expression ranging between 15% and 70% [50], Andreea *et al*., and Cioca *et al*., who reported 32% [38], and (55%) Podoplanin positivity of the malignant hepatocytes [36].

In the present study, a higher prevalence was detected in Grade 1 (22 cases; 36.7%), which showed approval with Cioca *et al*., who reported a predominance of Grade 1+ in 7 cases (64%) [36].

In the present study, there was significant association between LVD and the age; as HLVD-associated-HCC was more detected with higher mean age (58.5 years) than that of LLVD-associated HCC; that showed (55 years) (p = 0.005). These results showed disagreement with that published by Andreea *et al*., who detected that HLVD was more in patients >60 years with insignificant p-value (>0.05) [38] and showed disapproval with He *et al*., as there were no statistically significant differences in the age, between the high-MVD group and low-MVD group (p > 0.05) [48], and Sha *et al*., who concluded that patient age was not significantly different between high and low LVD with significant p = 0.35 [51]. Malignant hepatocytes positively expressing Podoplanin were significantly associated with a younger mean age(p = 0.001). These results contradicted those of Ciurea *et al*. and Andreea *et al*., who both stated that no statistically significant correlation exists between Podoplanin expression and age (p = 0.35 and p > 0.05, respectively) [38], [50]. This discrepancy may be attributed to wider range of age of subjects enrolled in the present study. Positively stained CAFs showed a younger mean age incidence (53.29 years) than that of the negatively stained cases (57.39 years), the relation was statistically insignificant (p = 0.1).These results showed agreement with that obtained by Yoshida *et al*. and Obulkasim *et al*., who showed a statistically insignificant correlation between Podoplanin expression in CAF and age (p = 0.59 and p = 0.06, respectively) [49], [52]. Both high and low LVD showed more prevalence in males than females and attained a statistically significant relation with gender (p = 0.01). These findings showed disapproval with other studies that documented an insignificant relationship between gender and MVD of the lymphatic vessels in HCC that performed by Andreea *et al*. (p > 0.05) and Sha *et al*., who documented similar insignificant results (p 0.27) [48]. Podoplanin expression in CAFs was highly detected in males (87.8%) than females (12.2%), conferring a statistically significant relationship with gender (p = 0.008). These results showed disapproval with those of Yoshida *et al*. and Obulkasim *et al*. who concluded an insignificant gender effect when evaluated Podoplanin expression in CAF of HCC and reported p-values of 0.55 and >0.05, respectively [52]. Podoplanin expression in malignant hepatocytes was more significantly detected in males than females (p = 0.01). These results were approaching those of Andreea *et al.* who observed more Podoplanin expression in male cases of HCC but they could not achieve a significant correlation (p > 0.05) [38]. Does hepatotropic viral infection influence Podoplanin expression in HCC? The results in the present study may contribute to yes. Regarding LVD, HLVD was detected in most cases, while LLVD was detected in lesser percentage achieving a statistically significant relationship (p = 0.03), but unfortunately, no published comparable research delineated this item. Positively stained CAFs were significantly detected in viral-caused HCC (p = 0.01). No previous research was done regarding this item.

Podoplanin expression in malignant hepatocytes showed more prevalent expression in viral-caused HCC; however, an insignificant statistical correlation was attained (p = 0.13). These results showed concordance with those obtained by Cioca *et al*., who also attained a statistically insignificant correlation (p > 0.05) [36]. The expression of Podoplanin in CAFs was statistically significant (p = 0.01), but no comparable results could be attained. LLVD was a little bit more associated with cirrhosis than HLVD with a significant correlation (p = 0.03). These results were not in concordance with those obtained by Thelen *et al.* who stated that “high LVD” tumors were more frequently and significantly detected in cases associated with liver cirrhosis than those with “low LVD” tumors (p = 0.001) [47] and Cioca *et al.* who also noted a similar statistically significant results (p = 0.006) [36]. This could be attributed to the difference in the sample size. The positively stained CAFs were significantly detected in cirrhosis-associated HCC cases (p = 0.001). These results showed approval with those of Abd El-Fattah *et al*. who detected higher expression in HCC-associated cirrhosis with a statistically significant correlation (p = 0.05) [46]. Despite that, Podoplanin showed more prevalent expression in malignant hepatocytes associated with cirrhosis (86.6%), the correlation was insignificant (p = 0.2). These results are approved with those obtained by Cioca *et al*., who declared a statistically insignificant correlation between Podoplanin expression and cirrhosis (p =0.85) [36]. Podoplanin showed more frequent expression in unifocal HCC as but without a statistically significant correlation (p = 0.4), which is going in concordance with that of Thelen *et al*. who showed more Podoplanin expression in unifocal tumor but without statistically significant level (p = 1.0) [46], and Sha *et al*., who documented similar results as well (p = 0.11) [51]. Unifocal HCC showed more prevalent Podoplanin expression in CAFs, and in malignant hepatocytes but the correlation was statistically insignificant (p = 0.7 and p = 0.07, respectively). These pieces of the thesis have not yet a comparable research subject. When

talking about LVD and CAFs in the present study, tumor size constituted an insignificant factor in Podoplanin expression. LVD (Low and high) was detected more in HCC sized <5 cm than that of ≥ 5cm but it could not attain a statistically significant value (p = 0.9), which was supported by Andreea *et al*., who documented higher expression of Podoplanin in lymphatic vessels coupled with tumors <5 cm and it was also statistically insignificant (p > 0.05) [38]. Meanwhile, CAFs showed more prevalent expression in smaller HCC cases (<5cm), but the correlation was statistically insignificant (p = 0.2), that showed concordance with other authors such as Obulkasim *et al*. and Abd El-Fattah *et al*., who noticed increased expression in CAFs associated with HCC sized<5 cm with a statistically insignificant value (p =0.53 and p > 0.05, respectively) [46], [52]. On the contrary, Podoplanin expression in malignant hepatocytes in HCC cases sized <5 cm was coupled with higher expression and a significant correlation (p = 0.001). This finding showed contrast with those obtained by Aishima *et al*. and Andreea *et al*., who both stated a statistically insignificant relationship(p = 0.76 and p > 0.05, respectively) [38], [53].

Podoplanin expression showed a statistically significant correlation with the tumor grades; Regarding LVD, HLVD was significantly detected in high-tumor grade (p = 0.001). These results come in concordance with that of Cueni *et al*. and Andreea *et al*. who both concluded a statistically increased Podoplanin expression in lymphatic vessels in moderately and poorly differentiated tumors (p = 0.06 and p = 0.05, respectively) [53], but disapproved with those of Thelen *et al.* who stated that histological grade was not significantly different between low and high grade of Podoplanin expression (p = 1.00) [47].

Regarding CAFs, they were more significantly detected in high-grade HCC (p = 0.02), in line with Abd El-Fattah *et al*. that documented a positive correlation between Podoplanin expression in CAFs and tumor grade (p < 0.001) [46] and showed disapproval with Obulkasim *et al*., who noticed a higher expression in moderately differentiated HCC cases but it was statistically insignificant (p = 0.87) [52]. Malignant hepatocytes of high-grade HCC showed higher Podoplanin expression, with a statistically significant correlation (p = 0.008). These findings were similar to Ciurea *et al*., Andreea *et al*., and Cioca *et al*., who found increased Podoplanin expression in high-grade tumors when compared with low-grade ones (p = 0.014, 0.040, and 0.036,respectively) [36], [38], [50]. Podoplanin expression in lymphatic vessels showed a nearly similar incidence in both LLVD and HLVD, but it was statistically insignificant (p = 0.95). This showed approval with Thelen *et al*. and Abd El-Fattah *et al*., who documented that vascular invasion was not significantly different between low- and high-grade Podoplanin expression (p = 0.79 and 0.92, respectively) [45], [46], [47]. These findings were different from those of Andreea *et al*., who found a significantly increased LVD in cases associated with vascular invasion (p = 0.018) [38]. CAFs positively expressing Podoplanin were detected in a minority of invasion associated HCC and the correlation was statistically significant (p = 0.001). These results come in agreement with those obtained by Abd El-Fattah *et al*., who stated that positive Podoplanin expression in CAFs was significantly related to the vascular invasion (p < 0.05) [46]. These results showed disapproval with those of Obulkasim *et al*., who noticed a non-statistically significant correlation between Podoplanin expression and the presence of tumor invasion (p = 0.56) [52].

Malignant hepatocytes expressing Podoplanin and associated with invasion were detected in nine cases, but despite this increased expression, it showed a statistically insignificant level (p = 0.4), which approves with Andreea *et al*., who observed increased Podoplanin expression in cases associated with invasion and also with statistically insignificant value (p > 0.05) [38].

The correlation between Podoplanin expression in the lymph vessels (LVD) and CAFs was investigated and revealed a statistically significant correlation (p = 0.001), which come in concordance with that performed by Andreea *et al*., who also postulated a significant correlation with LVD (p = 0.019) [38].

The correlation between LVD and Podoplanin expression in malignant hepatocytes was assessed and revealed a statistically significant correlation (p = 0.004). No previous research was done regarding this subject.

Several significant study limitations should be mentioned. The small sample size may have an impact on the statistical results. Longer follow-up studies could be considered to assess the prognostic significance as well as the effect of anti-lymphangiogenesis therapy.

Conclusion

Podoplanin can be used to detect the density of the tumor associated lymphovascular proliferation and hence a prognostic significance. It is also detected within the malignant hepatocytes and CAFs suggesting a role in hepatocellular tumorigenesis through an epithelial mesenchymal link. This triple expression could be used as a target for anti-lymphangiogenesis therapy or to abort the epithelial mesenchymal loop.

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