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OCT4 Expression in HCV-Related Hepatocellular Carcinoma

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Abstract These Incidence rates of hepatocellular cancer are rising in the United States due to increasing prevalence of cirrhosis caused by chronic hepatitis C and steatohepatatis (non-alcoholic fatty liver disease). Overall, HCC is still associated with a poor prognosis depending on delayed diagnosis, the clinical status of the patient but also tumor behavior showing a great propensity for angioinvasion. Most of the studies about OCT4 function demonstrated that OCT4 as a factor for pluripotency regulation in human embryonic stem cells, and cancer is the most studied part of OCT4-related human disease. Our study included 68 biopsy materials (tumor and non-tumor) from cases of partial hepatectomy done for patients suffering from hepatocellular carcinoma. Sections were stained with hematoxylin and eosin stain for routine histopathological examination, grading and staging of hepatitis activity and tumor grade by Masson's trichrome stain for assessment of fibrosis stage using METAVIR scoring system. Immunohistochemical staining of liver sections was done using the monoclonal antibody Oct4 (ab18976). Tumor tissue showed over-expression of OCT4 compared to non-tumor tissue. Also high grades of hepatocellular carcinoma showed higher expression of OCT4 than low grade ones. As regard hepatitis activity and liver fibrosis, it was found that high grades activity and cirrhotic livers showed higher levels of OCT4 expression. Conclusion: OCT4 expression pattern could be used in association of other tissue markers for diagnosis and follow up of hepatocellular carcinoma.

Keywords Hepatocellular carcinoma, Immunohistochemistry, HCV, OCT4

1. Introduction

Primary liver cancer is the state when the cells become abnormal in appearance and behavior, the cancer cells can destructive to adjacent normal tissue where these cells go under invasion and metastasis [1]. There are many causes for the liver cancer where people who have HCC get it in the set on chronic liver disease and increases the risk of liver cancer, where the conditions that cause liver cirrhosis are alcohol, hepatitis C and hepatitis B virus as well as cancer can be caused by environmental factors such as wrong diet way [2].

Cirrhosis of the liver due to any cause is a risk factor for liver cancer. Also, even hepatitis B infection without cirrhosis is a risk factor for liver cancer. The liver cancer can be diagnosed through many ways such as ultrasound, blood testing, imagining studies, and liver biopsy [3], but the best way to detect liver cancer is by ultrasound every 6 months for the patient. Blood testing is using alpha fetoprotein (AFP) is elevated by 70% in case of patients with liver cancer, also AFP level could be normal and not high but once it is high it indicates serious liver cancer [4].

According to the American cancer society, Stages of cancer describes how cancer is spread out, and it is very important to know the stages of cancer to select the selective treatment, also the staging system is to know how far is cancer spreading out [5].

Liver biopsy as well as imaging studies help in classifying liver cancer stages as per the American Joint

Committee on Cancer (AJCC) TNM system, the Barcelona Clinic Liver Cancer (BCLC) staging system, the Cancer of the Liver Italian Program (CLIP) system, or the Okuda system [6]. The medical treatment of cancer depends on how far the cancer spreads out as well as the general health of the liver, for example the extent of cirrhosis (scarring) of the liver can determine the treatment options for the cancer. Similarly, the spread and extent of spread of cancer beyond the liver tissue plays an important part in the types of treatment options that may be most effective [7]. There are many ways for treatment of cancer such as surgery, liver transplant, ablation therapy, embolization, radiation therapy, chemotherapy and targeted agent.

Hepatocellular Carcinoma (HCC), ranking as the 6th position of human cancers, is the most common type of primary liver cancer, followed by cholangiocarcinoma [8]. Importantly, HCC represents the main complication of cirrhosis, and shows a growing incidence worldwide related to the increased prevalence of the various risk factors of chronic liver diseases, such as hepatitis infection with hepatitis C and B viruses, and more recently fatty Liver Diseases which are mostly associated with metabolic syndrome [10]. Liver tumor classification has recently been reviewed according to the 2015 WHO classification [10]. The increasing trend is mainly due to a cohort effect related to infection with hepatitis B and C viruses (HBV and HCV) the incidence WJG 20th Anniversary Special Issues state that Hepatocellular carcinoma of which peaked in the 1950s to 1980 was the main risk factor. By contrast, in North America, Europe, and Japan, HCV infection is the main risk factor, together with alcohol use [11]. Time trends in incidence of hepatocellular carcinoma of developed countries parallel the timing of HCV spread. In Japan and Europe, where HCV infection spread earlier than in the United States, the incidence of hepatocellular carcinoma has almost reached a plateau and in some areas it is declining; however, in the United States, incidence is still increasing and the infection could have a synergistic effect with other risk factors, such as non-alcoholic fatty liver disease [11]. Unlike other cancers, HCC usually arises on a previously damaged organ, mostly in the setting of chronic hepatopathy, cirrhosis [12]. As well as in association with hereditary diseases such as hemochromatosis, Wilson's disease and a-1-antitrypsin deficiency. However, in about 15%-20% of cases HCC may occur in the non-fibrotic liver or in livers with minimal portal fibrosis without any septal fibrosis.

The Grading and pathology of the prognostic factors of HCC, where Grading of HCC has relied for many years on Edmondson and Steiner system, which divided HCC into four grades from I to IV on the basis of histological Differentiation [13]. Importantly, grading heterogeneity inside a tumor is frequently observed and may significantly limit performance of biopsy for grading, especially when using a four-tier grade scaling [14]. Therefore, and compared to other carcinomas, there is a tendency to simplify the grading and use a three-scale system including well, moderately and poorly differentiated HCC. Finally, tumor grade appears as a weak independent predictor of the clinical course, providing little prognostic information [15]. The main prognostic factors of HCC are related to tumor stage (number and size of nodules, presence of vascular invasion and extrahepatic spread), liver function (defined by Child–Pugh's class, bilirubin, albumin, portal hypertension) and general health status [16].

The most common form of cytological atypia is small cell change/dysplasia characterized by small cells with decreased cytoplasm and moderately enlarged nuclei [17]. In these cases Oct 4 can be useful diagnostic marker where A research has asserted that cancer progenitor cells or cancer stem cells (CSC) are responsible for survival, sustained growth and recurrence of the tumor [18]. Given that the extra and intra hepatic recurrence is attributed to subgroup of stem like cells [19]. It is very important to clarify the expression and involvement of stem cell's self-renewal regulator genes in establishing new therapeutic approaches and in cancer development for HCC patients. Oct4 (also known as POU5F1) is a Pou domain, Octamer binding transcription factor expressed in both human and mouse embryonic stem cells and primordial germ cells [20]. The encoded protein plays an important role in maintaining the pluri-potent state of stem cells [21]. Where any knockdown in embryonic cancer cells and Oct4 leads to loss of pluripotency and onset of differentiation [22]. For certain somatic cells activation of Oct4 may lead to dysplastic growths in epithelial cells [22].

Oct4 has been found in recent studies to be expressed in some cancer cells such as breast, prostate and bladder cancer [23]. OCT4 expression in HCC cell lines and tumor tissues was higher than in normal hepatocytes and cirrhosis tissues. Over expression of OCT4 was significantly associated with low differentiation and tumor recurrence. Patients with elevated expression of OCT4 protein usually carried a poor overall survival and high

recurrence rate. Multivariate analysis showed that OCT4 expression was an independent predictive factor for HCC patients survival. OCT4 might serve as a promising biomarker for the diagnosis of highly recurrent cases of HCC and could be used as a valuable indicator for predicting the prognosis of HCC. Many studies on human non-small cell lung cancer have proved that OCT4 played a role in maintaining cancer cell characteristics and resistive properties [24].

2. Materials and Methodology

Groups of Patients

Our study included 68 biopsy materials (tumor and non-tumor) from cases of partial hepatectomy done for patients suffering from hepatocellular carcinoma. Tissue specimens were processed into paraffin blocks at Pathology Department, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. The specimens were histopathologically classified into: non-tumor hepatic tissue (34 sections); HCC (34 sections). All cases were proved serologically to be of hepatitis virus C etiology. This study was carried out in full accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Ethics Committee of Theodor Bilharz Research Institute. A written informed consent was obtained from each participant, in accordance with the institutional guidelines.

Histological Study

Paraffin blocks were made and 4 microns thick sections were stained with hematoxylin and eosin stain (H and E stain) for routine histopathological examination, grading and staging of hepatitis activity and tumor grade. Sections were also stained by Masson's trichrome stain for assessment of fibrosis stage using METAVIR scoring system [22] .

Grade of hepatitis activity; based on amount of inflammation:

A1:- mild activity, A2:- moderate activity, A3:- severe activity.

Stage of fibrosis; representing amount of fibrosis or scarring:

F1:-portal fibrosis without septa, F2: portal fibrosis with few septa, F3: numerous septa without cirrhosis, F4: cirrhosis. In our study, for simplicity, we have grouped A1 and A2 as low hepatitis activity and a separate A3 as high hepatitis activity. We have also grouped F1, F2 and F3 as fibrosis and F4 as cirrhosis.

HCC grade was done according to the WHO classification of tumors of the liver and intrahepatic bile ducts [23] into:

Grade 1: (well differentiated)

Grade 2: (Moderately differentiated)

Grade 3: (Poorly differentiated)

Immunohistochemical Technique

I. Formalin-fixed paraffin sections (5µm in thickness) were cut. Sections underwent deparaffinization and rehydration. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide. Heat induced epitope retrieval was performed at 100°C for 30 min. Antigen retrieval was performed by microwaving the sections in citrate buffer, pH 6.0. The slides were allowed to cool in room temperature and washed 3 times by immersing them in TBST (Tris Buffered Saline having 0.05% Tween 20). Immunohistochemical labeling WITH OCT-4 (Abcam, USA) (ab18976) at 1/100 to 1/200 dilution. Slides were then kept overnight in closed humid box in 4^{o}C.

II. Next day, the slides were washed 4 times, 5 minutes each with TBST. The sections were then incubated for 30 minutes with the secondary biotinylated antibody followed by avidin peroxidase complex for another 30 minutes according to the manufacturer's instructions (Universal Detection Kit, Dako, Denmark). The antigen was localized by the addition of DAB (Diaminobenzidine) substrate chromogen solution. Finally, slides were counterstained with hematoxylin, dehydrated in alcohol, and mounted.

For each setting, negative controls were carried out in which the primary antibody was omitted and replaced by PBS. Positive controls were sections of testicular seminoma.

Interpretation of Immunostaining

All immunostained slides were assessed and scored. The sections were examined by using light microscope (Scope A1, Axio, Zeiss, Germay). Photomicrographs were taken using a microscope-camera (AxioCam, MRc5, Zeiss, Germany).

The expression level of these antibodies was judged according to the percentage positive cells in each tumor tissue. Specifically, a percentage of ≤10 % was judged negative and >10 % was positive [24-26], regardless of the color intensity.

Statistical Analysis

SPSS for Windows, version 20 was used for statistical analysis (IBM corporation, Armonk, new York, USA). The comparisons of quantitative variables were performed between two groups using Mann Whitney U-test. Associations between OCT4 expressions, tumor grade and fibrosis stage were evaluated by chi square test and fisher test. The P value < 0.05 was considered statistically significant.

3. Results

Our study included 68 biopsy specimens from tumor (34) and non-tumor (34) hepatic tissue specimens resected from patients suffering of hepatocellular carcinoma (HCC) of HCV pathogenesis. Our patients were 23 males (67.65%) and 11 females (32.35%). The mean age for males was (55.31) and the mean age for females was (56.12 years). No statistically significant difference was detected between males and females in their mean ages ($p > 0.1$). The studied specimens were categorized into 2 main groups based on histopathological examination; non-tumor hepatic tissue (34 cases) and hepatocellular carcinoma (HCC) (34 cases). We found that HCC showed significantly high percentage of positive OCT4 expression (76.47%) compared to non-tumor hepatic tissue (11.75%) [Table 1].

Table 1: Difference in OCT4 expression in the studied groups

			***OCT4* expression**		**Total**
			Negative	**Positive**	
Diagnosis	Non-tumor	Count	30_a	4_b	34
		%	88.24%	11.75%	100.0%
	HCC	Count	8_a	26_b	34
		%	23.53%	76.47%	100.0%

Each subscript letter denotes a subset of *OCT4* categories whose column proportions do not differ significantly from each other at the .05 level. (Pearson Chi-Square test)

Most cases of HCC showed significantly higher values in mean percentage and intensity of OCT4 expression compared to non-tumor cases. Student t-test showed significant difference between examined groups ($p<0.001$) [Table 2].

Table 2: Difference in mean expression of OCT4 percentage and intensity between studied groups

Diagnosis		***OCT4* percent**	***OCT4* intensity**
Non-tumor	Mean	7.15	0.40
	N	34	34
	Std. Deviation	2. 47	0.18
HCC	Mean	61.11	2.34
	N	34	34
	Std. Deviation	29.95	0.54
P value (student t-test)		< 0.001	< 0.001

Positive OCT4 expression was higher in high grade tumors (93.3%) compared to low grade tumors (63.16%), however this difference was statistically non-significant ($p>0.05$) [Table 3].

Table 3: Difference of OCT4 expression in high and low grade tumors

Tumor grade		***OCT4* expression**		**Total**
		-Ve	**+Ve**	
High	N	1	14	15
	%	6.7%	93.3%	100.0%
Low	N	7	12	19
	%	36.84%	63.16%	100.0%

Total	N	8	26	34
	%	23.53%	76.47%	100.0%

Fisher's exact test: The two-tailed P value equals 0.0529

As regard the relation of OCT4 mean percentage and OCT4 mean intensity to tumor grade, we found that high grade tumors showed statistically significant higher OCT4 mean percentage and higher OCT4 mean intensity compared to low grade tumors ($p<0.05$ and $p<0.001$ respectively) [Table 4].

Table 4: Relation of mean OCT4 percent and mean OCT4 intensity to tumor grade

Tumor grade		***OCT4* percent**	***OCT4* intensity**
	Mean	74.13	2.8
High	N	15	15
	Std. Deviation	31.83	0.5
	Mean	51.66	1.7
Low	N	19	19
	Std. Deviation	22.40	0. 3
P value (t-test)		<0.05	< 0.001

No significant difference in percentage of OCT4 expression between low and high grades of hepatitis activity ($p>0.05$). On the other, liver cirrhosis showed higher percentage of positive OCT4 expression than with fibrosis. The difference was statistically significant ($p<0.05$) [Figure 1].

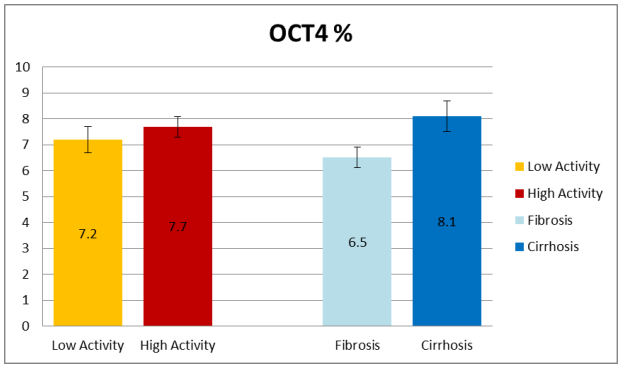


Figure 1: Relation of grade of hepatitis activity and stage of fibrosis with OCT4 expression percent in non-tumor liver tissue

No significant difference in intensity of OCT4 expression was achieved between either low and high grades of hepatitis activity or between liver fibrosis and cirrhosis ($p>0.05$). ($p<0.05$) [Figure 2].

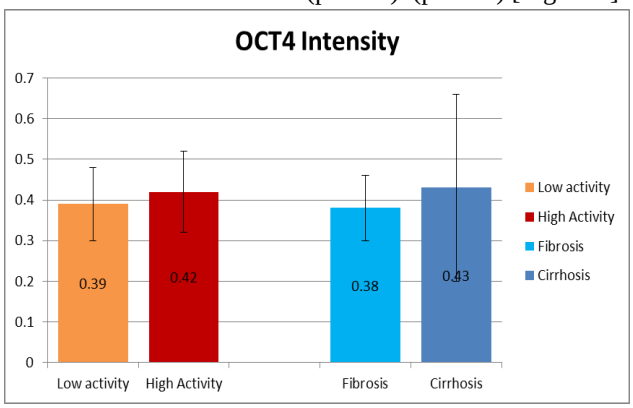


Figure 2: Relation of Stage of fibrosis with intensity of OCT4 expression in non-tumor liver tissue

In all studied cases, OCT4 expression percentage showed significant positive correlation with grade of tumor differentiation and with intensity of expression ($p <0.05$ and $p < 0.001$ respectively). No significant correlations were achieved between hepatitis activity or the stage of fibrosis with the different parameters of OCT4

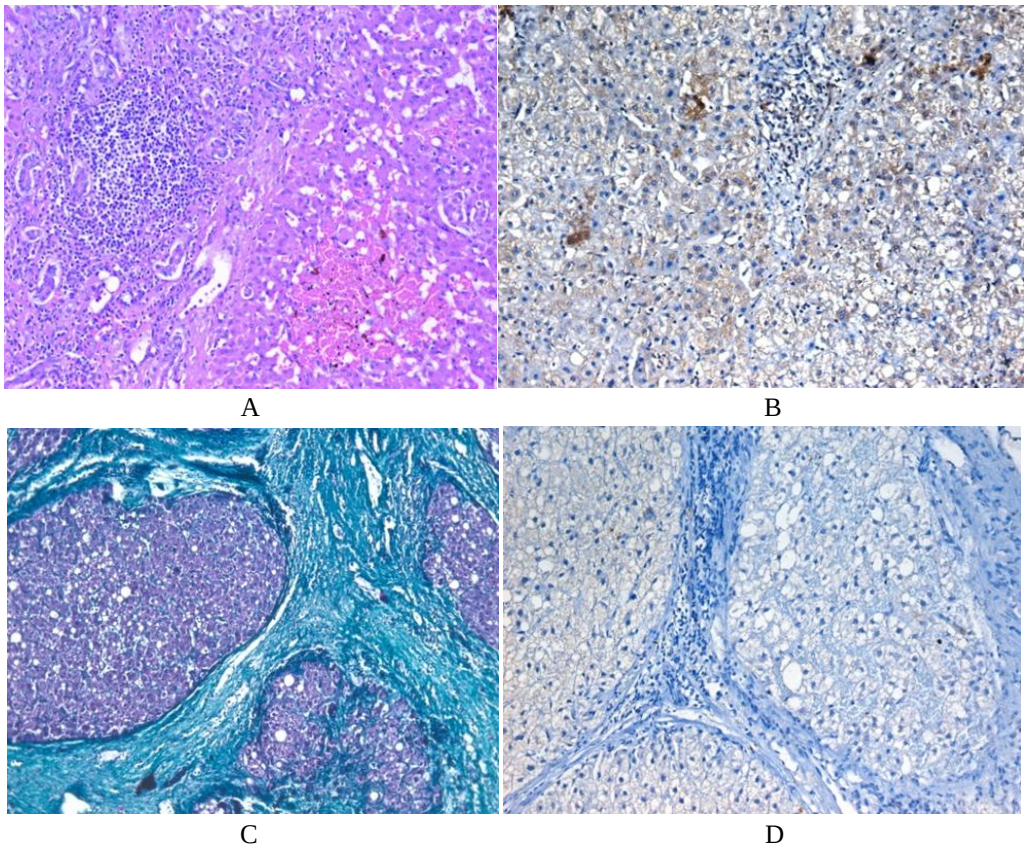
expression (p > 0.05) [Table 5].

Table 5: Correlation between OCT4 expression of different parameters and Spearman's rho

			***OCT4* %**	***OCT4* intensity**	**Activity**	**Fibrosis**
Spearman's rho	Tumor Grade	Correlation Coefficient	0.444*	0.383	0.258	0.408*
		Sig. (2-tailed)	0.030	0.064	0.223	0.048
		N	34	34	34	34
	OCT4 %	Correlation Coefficient	1.000	0.963**	-0.023	-0.009
		Sig. (2-tailed)	.	0.000	0.864	0.949
		N	64	64	34	34
	*OCT4*intensity	Correlation Coefficient	0.963**	1.000	0.052	-0.088
		Sig. (2-tailed)	0.000	.	0.700	0.512
		N	64	64	34	34
	Activity	Correlation Coefficient	-0.023	0.052	1.000	-0.047
		Sig. (2-tailed)	0.864	0.700	.	0.726
		N	34	34	34	34

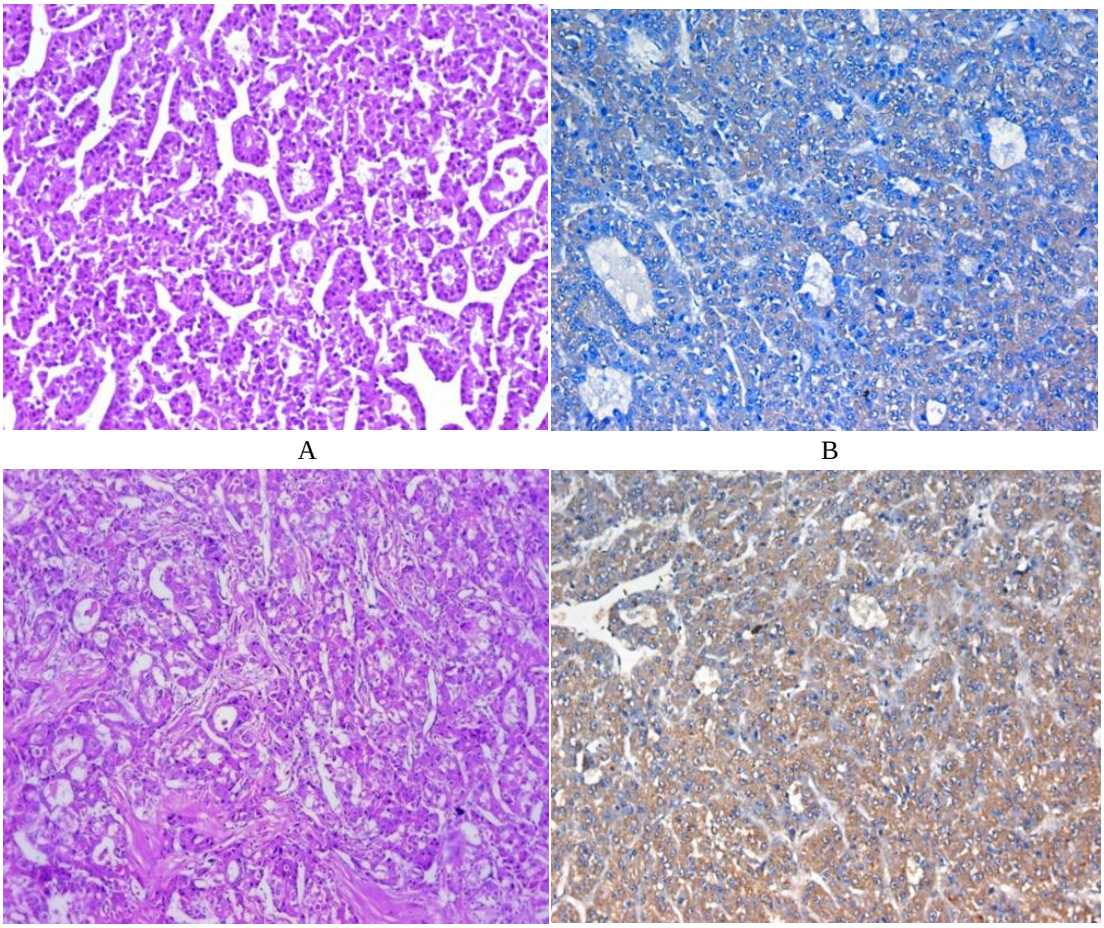
*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).



A B C D

Figure (3-A): Section in case of chronic hepatitis showing lymphocytic aggregate and focal hepatocellular necrosis (Hematoxylin and Eosin stain, X200). Figure (3-B): Section in case of chronic hepatitis showing mild expression of OCT4 in scattered hepatocytes (IHC for OCT4, using DAB, X200). Figure (3-C): Section in case of post-hepatitic cirrhosis, showing a regenerating nodule surrounded by fibrous tissue (Masson's trichrome stain, X100). Figure (3-D): Section in case of post-hepatitic cirrhosis showing negative expression for OCT4 (IHC for OCT4, using DAB, X100)



A B

C D

Figure (4-A): Section in case of low grade hepatocellular carcinoma, with acinar pattern (Hematoxylin and Eosin stain, X200). Figure (4-B): Section in case of hepatocellular carcinoma showing mild focal expression of OCT4 (IHC for OCT4, using DAB, X200). Figure (4-C): Section in case of high grade hepatocellular carcinoma, showing irregular trabeculae of malignant hepatocytes (Hematoxylin and Eosin stain, X200). Figure (4-D): Section in case of high grade hepatocellular carcinoma showing dense positive expression of OCT4 (IHC for OCT4, using DAB, X200)

4. Discussion

The risk factors for liver cancer in cirrhosis are being male, age 55 years or older, Asian or Hispanic ethnicity, family history in a first-degree relative, obesity, hepatitis B and C, alcohol use, and elevated iron content in the blood due to hemochromatosis [25].

Our study included 68 biopsy specimens from tumor (34) and non-tumor (34) hepatic tissue specimens resected from patients suffering of hepatocellular carcinoma (HCC) of HCV pathogenesis. Our patients were 23 males (67.65%) and 11 females (32.35%). The mean age for males was (55.31) and the mean age for females was (56.12 years). No statistically significant difference was detected between males and females in their mean ages. The studied specimens were categorized into 2 main groups based on histopathological examination; non-tumor hepatic tissue (34 cases) and hepatocellular carcinoma (HCC) (34 cases). We found that HCC showed significantly high percentage of positive OCT4 expression (76.47%) compared to non-tumor hepatic tissue (11.75%).

OCT4 is a type of POU transcription factor that is over expressed in undifferentiated embryonal carcinoma, reproductive stem cells, and embryonic stem cells. It has a very important role in maintaining the self-renewal and pluripotency ability of embryonic stem cells. Several studies have found that OCT4 expression in the healthy population is low, whereas OCT4 expression is high in gallbladder carcinoma, pancreatic cancer, and

liver cancer [26]. Specially, its expression level is up regulated following clinical upstaging. In pancreatic cancer, OCT4 sensitivity was higher, but no significant correlation was found with the differentiation state of pancreatic cancer.

Most of our cases of HCC showed significantly higher values in mean percentage and intensity of OCT4 expression compared to non-tumor cases.

Also Saxe JP [27], stated that the positive rate of OCT4A mRNA expression was significantly higher in the HCC tissues than in the non-neoplastic liver tissues (72.0% vs. 30.8%. No OCT4A mRNA expression was found in the normal liver tissues. OCT4A mRNA expression was correlated with the tumor size, vascular invasion, and TNM stage. Kaplan-Meier survival curves showed that patients with positive expression of OCT4A mRNA had lower overall survival and disease-free survival rates [27]. Kim et al [28] shows in his studies on the expression of Oct4 in cervical cancer that OCT4 expression was higher in cervical cancer than normal cervix.

We found that positive OCT4 expression was higher in high grade tumors (93.3%) compared to low grade tumors (63.16%) , however this difference was statistically non-significant ($p>0.05$).

Jeanette et al [29] mentioned in their study, that Oct4 was expressed in all tumors, and they observed a grade-dependent increase in expression, with the highest expression in glioblastoma multiformis (GBMs). However, no association was found between Oct-4 protein expression and overall survival, it was demonstrated that Oct-4 is expressed in human astrocytic brain tumors [30] where it was identified that Oct-4 expression in the nucleus as well as a weak cytoplasmic staining of most astrocytic brain tumors, Du et al. found expression of Oct-4 in the nuclei, but not in the cytoplasm of glioma cells [31]. However, cytoplasmic staining has been observed in other cancers such as oral cancer [32]. The detection of both nuclear and cytoplasmic staining in our study may be explained by a sensitive staining and is in line with Oct-4 been a transcription factor been synthesized in the cytoplasm followed by transportation to the nucleus. Furthermore, we demonstrated a significant increase in Oct-4 tumor cell fraction with increasing tumor grade. Du et al. found that the fraction of Oct-4 increased in a grade dependent manner [31].

As regard the relation of OCT4 mean percentage and OCT4 mean intensity to tumor grade, we found that high grade tumors showed statistically significant higher OCT4 mean percentage and higher OCT4 mean intensity compared to low grade tumors.

In another recent IHC study using tissue microarrays Elsir et al. showed that Oct-4 fraction among other TSC-related markers were up-regulated in 98 high-grade gliomas compared with 80 low-grade gliomas of oligodendroglial and astrocytic type [33]. However, in both studies semi-quantitative pathologist-based scoring systems was used and no statistical comparison was made. To provide a more unbiased quantification approach we used blinded quantitative stereology and systematic random sampling [34]. Although this may influence the TCGA mRNA results and the IHC results by Du et al [31] and Elsir et al [33], an increase of Oct-4 with grade seems to be a robust finding. Similar results have been obtained in oral cancer [32] and urothelial cancer [35] suggesting an association of Oct4 with tumor malignancy in cancer in general.

In a study on prostatic cancer, research showed that the positive rate of OCT4 expression in prostate cancer was 77.38%, markedly higher than that in normal and hyperplastic tissues. The OCT4 positive rate in prostate cancer stages III and V was 86.67 and 94.12%, respectively, significantly higher than in stage I. This demonstrated that OCT4 has a close relationship with TNM staging, consistent with previously published research. In addition, it was also found that OCT4 expression rates of tissues at different degrees of differentiation were similar, suggesting that OCT4 expression has no close relationship with prostate cancer differentiation.

OCT4 over expression was associated with lympho vascular space invasion ($p = 0.045$). Notably, OCT4 was significantly low-expressed in premalignant cervical lesions, but not in malignant cervical tumor. OCT4 over expression showed worse 5-year disease-free and overall survival rates when compared to the low-expression group, Cox regression analysis showed that OCT4 was an independent risk factor (hazard ratio = 11.23, 95 % CI, 1.31 - 95.6; $p = 0.027$) [36].

In our study, no significant difference in percentage of OCT4 expression between low and high grades of hepatitis activity. On the other, liver cirrhosis showed higher percentage of positive OCT4 expression than with fibrosis. The difference was statistically significant. No significant difference in intensity of OCT4 expression was achieved between either low and high grades of hepatitis activity or between liver fibrosis and cirrhosis.

In all studied cases, OCT4 expression percentage showed significant positive correlation with grade of tumor differentiation and with intensity of expression ($p < 0.05$ and $p < 0.001$ respectively). No significant correlations were achieved between hepatitis activity or the stage of fibrosis with the different parameters of OCT4 expression.

One recent study suggests a role for OCT4 in sustaining the self-renewal capacity of adult stem cells in the liver [37], When the liver is damaged, replacement of damaged liver cells was occurred through the trans-differentiation of liver epithelial cells (hepatocytes to biliary cells) which is the mainmechanism for liver regeneration [38] Considering the liver regeneration was not completely occurred when blocking the OCT4 expression in hepatocytes [37], OCT4 may play a role in such processes during liver regeneration.

5. Conclusion

Our results showed also that all parameters of OCT 4 expression were correlated inversely with high statistical significance to both the grade and stage of hepatocellular carcinoma.

In conclusion, OCT 4 expression is not just a research tool. In Liver cancer, OCT4 expression pattern could be used in association of other tissue markers for diagnosis and follow up of hepatocellular carcinoma. Our results showed OCT4 expression percentage is significant positive correlation with grade of tumor differentiation and with intensity of expression ($p < 0.05$ and $p < 0.001$ respectively). No significant correlations were achieved between hepatitis activity and the stage of fibrosis with the different parameters of OCT4 expression. In the future, the OCT 4 gene and/or protein may be a site for immunotherapy. Before such treatments are possible, the full significance of this molecule in different variants of liver cancer needs to be examined.

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