Research Article

Evaluation of SOX9 Expression in Hepatocellular Carcinoma: An Immunohistochemical Study

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Abstract
Background: Hepatic carcinoma is one of the most common cancers with a high morbidity and mortality rates worldwide. SOX9 has been described as a cancer stem cell marker and its overexpression has been associated with numerous human aggressive malignancies comprising HCC. **Aim of the study:** Is to evaluate SOX9 expression in HCC and its relationship to clinicopathological features. **Methods:** Immuonohistochemical staining of SOX9 was conducted on 50 tissue specimens of hepatocellular carcinoma by using the avidin biotin-peroxidase complex method with diaminobenizidine (DAB) chromagen detection system. **Results:** High SOX9 expression was detected in 52% of cases. SOX9 expression showed statistically significant association to high tumor grade (p= 0.017) and advanced tumor stage (p = 0.020). **Conclusions:** Overexpression of SOX9 was connected to HCC progression and tumorigenesis.
Key words: SOX9 - Immunohistochemistry - Hepatocellular Carcinoma.

Introduction
Hepatocellular cancer (HCC) is one of the deadly tumors in the world. In Egypt, HCC adds to 8% of all cancers every year. The median age is 53 years and predominates among males (El Bolkainy, 2016). HepatitisC virus (HCV) and hepatitisB virus (HBV) help promoting the oncogenesis of liver cells (Lemon and McGivern, 2012). Egypt has the highest HCV number worldwide (Elgharably et al, 2017), accounting for about half of HCC cases and thought about as the main risk factor for HCC (Parkin, 2006). The risk of HCC is rising up to 20times higher among people infected with HBV as compared to uninfected population (Donato et al., 2002). The prognosis of HCC is still poor due to the rapid rate of tumor growth, early vascular invasion and high recurrence rate (Mazzola et al., 2015). Furthermore, due to the vague symptoms, it is often discovered at an advanced stage (Park and Kim, 2005). Therefore, early diagnosis of HCC has a critical role to provide an effective treatment (Kudo et al, 2014). Studying the molecular basis of HCC is important to explain the aggressive biological behavior to promote better targeted effective therapy. The SOX genes, (SOX stands for Sry-related HMG box), family of transcription factors are well-recognized regulators of cell outcome decisions during development (Sarkar and Hochedlinger, 2013). SOX9 (Sex-Determining-Region Y-Box 9), gene located to chromosome 17q24 (Foster et al., 1994), is a member of the SOXE subgroup comprised of SOX8, SOX9 and SOX10 which plays an important part in developmental processes of mesoderm (Kobayashi et al, 2005), ectoderm; central nervous system (Stolt and Wegner, 2010), retina (Zhu et al., 2013) and endoderm; pancreas (Seymour et al, 2007), liver (Kawaguchi, 2013) and intestine (Mori-Akiyama et al, 2007). Immunohistochemical expression of SOX9 was detected in hepatic bile duct, duodenal crypt, and pancreatic duct (Furuyama et al., 2011). SOX9 is implicated in signaling pathway involved in epithelial-mesenchymal transitions. SOX9 has been considered in prostatic, CNS, skin, pancreatic, ovarian and esophageal carcinomas (Passeron et al., 2009) (Sakamoto et al., 2013) (Clemons et al., 2012). These reports present opposing roles for SOX9 in tumors, either to induce or to inhibit cell proliferation. This may be due to differences in cell lines and levels of SOX9 (Jo et al., 2014). Several studies have been performed to examine the properties of SOX9 as a cancer stem cell marker and demonstrated that SOX9+ cells hold the ability to self-renewal and differentiation into SOX9− cells. Moreover, SOX9+ cells showed a high proliferative ability, colony forming ability, and

resistance to 5-Flurouracil (5-FU) in vitro. (Kawai et al., 2016). In liver development, both hepatoblasts and hepatocytes do not express SOX9. However, SOX9 is detected in cholangiocytes (Furuyama et al., 2011). Many studies have revealed the role of SOX9 overexpression in liver cancer proliferation and metastases (Richtig et al., 2017).

The aim of this study was to study the immunohistochemical expression of SOX9 in HCC, and to study the possible association between immunohistochemical expression of SOX9 and different clinicopathological features in Egyptian HCC patients.

Material and Methods

Tissue specimens The present study comprised 50 tissue specimens of hepatocellular carcinoma which were randomly selected from the archive of Pathology Departement, Minia University Hospital and Minia Cancer Institute in the period between 2009 to 2015. All cases were obtained by tru-cut biopsy. The available clinicopathological data were obtained from the pathology reports of the cases. These data include patients' age, gender, tumor size, tumor multiplicity, tumor histological growth pattern, hepatitis virus infection, cirrhosis, tumor grade, tumor stage, fatty change and tumor necrosis. Tumor classification was performed according to the WHO criteria (Bosman et al., 2010). Histological grading was done according to the 3-scale system; grade I, II and III (Schlageter et al., 2014). Tumors were staged according to AJCC staging system (Subramaniam et al., 2013). Tissues were fixed in 10% neutral buffered formalin processed and embedded in paraffin wax. Five µm sections were prepared and stained with haematoxylin and eosin stain to revise the histological findings of all cases. Immunohistochemistry: Four µm sections were prepared on positive charged slides for immunohistochemistry of SOX9 primary antibody utilizing the avidin-biotin-peroxidase complex method with diaminobenizidine (DAB) chromogen detection system. Primary antibody SOX9: Polyclonal rabbit antibody, 50 microgram Concentrated, Lab Vision Laboratories. Detection immunostaining kit (Lab Vision Laboratories) Antigen retrieval solution sections were treated in microwave by immersion of the slides in citrate buffer solution (pH6) for 2 times (10 minutes each). Sections were then incubated at 4ºC with SOX9 primary antibody (diluted at 1:200) overnight in a humidity chamber. Positive control tissue was the basal layer of normal stratified squamous epithelium. Negative control tissue Negative control tissue sections were processed by omitting the specific primary antibody from the staining procedure.

Scoring of immunostaining

The entire tissue sections were screened for positive tumor cells with cytoplasmic and/or nuclear staining. SOX9 expression was estimated according to (Guo et al., 2012) as the total SOX9 immunostaining, including the extent and the intensity of the staining. The intensity of positive expression was scored as: 0 (negative), 1(weak), 2(moderate) and 3(strong). The extent of positive cells was scored as: 0(0%), 1(1-10%), 2(11-50%) and 3(>50%) according to the percentage of positive tumor cells in relation to the whole carcinoma area. Scores for intensity and extent were multiplied to form a final staining score (range 0-9). The median of the total scores was calculated and used as a cut-off point to stratify cases into two groups. These include –ve/low expression score and high expression score groups corresponding to less than and equal or more than the median, respectively.

Statistical analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS software version 16). Raw data were compiled and used to determine the means ± standard deviations (SDs), median and range for some variables. The Chi- square and Fisher's exact tests were used to compare categorical features. P value of ≤ 0.05 was considered significant.

Results

Clinicopathological features

Data regarding different clinical and histopathological features for hepatocellular carcinoma patients are summarized in **Table (1).**

Clinicopathological features	No. (%)
Age at Surgery, y	
<50	**17 (34%)**
≥50	**33 (66%)**
Gender	
Male	**36 (72%)**
Female	**14 (28%)**
Tumor size	
≤5cm	**17 (34%)**
>5cm	**33 (66%)**
Tumor multiplicity	
Single	**16 (32%)**
Multiple	**34 (68%)**
Histological pattern	
Trabecular	**26 (52%)**
Acinar	**15 (30%)**
Solid	**9 (18%)**
Tumor grade	
Grade I	**8 (16%)**
Grade II	**32 (64%)**
Grade III	**10 (20%)**
Tumor stage	
T 1-2	**15 (30%)**
T 3-4	**35 (70%)**
Hepatitis	
HBV	**13 (26%)**
HCV	**33 (66%)**
None	**4 (8%)**
Cirrhosis	
Absent	**18 (36%)**
Present	**32 (64%)**
Fatty change	
Absent	**40 (80%)**
Present	**10 (20%)**
Tumor necrosis	
Absent	**35 (70%)**
Present	**15 (30%)**

Immunohistochemical expression of SOX9 and its correlation with clinicopathological features: Evaluation of the immune-histo-chemical expression for SOX9 was performed and correlated with different clinicopathological features for all cases. SOX9 expression was mainly detected in the cytoplasm of neoplastic cells. Nuclear expression was combined with cytoplasmic expression and was only noticed in few sporadic nuclei in two HCC cases (the first case was grade II, stage I and the second case was grade III, stage III).

In the present study, negative SOX9 expression was observed in 4/50 (8%) of HCC cases**.** Low positive immunoreactivity was found in 20/50 (40%) of cases while high positive SOX9 expression was detected in 26/50 (52%) of HCC cases**.** The median of the total expression scores was 4. Based on the median as a cut-off point, cases were further stratified into two groups; -ve/low expression and high expression. The association between SOX9 expression and different clinicopathological features was summarized in **Table (2).**

Table (2): Association between SOX9 expression and clinicopathological features for patients

Clinicopathological features	No	SOX9 expression		*P* value
		-ve/low expression (%) (n=24)	High expression (%) (n=26)	
Age <50	17	6 (35.3)	11 (64.7)	0.161
≥50	33	18 (54.5)	15 (45.5)	
Size ≤5cm	17	8 (47.1)	9 (52.9)	0.581
>5cm	33	16 (48.5)	17 (51.5)	
Multiplicity				
Single	16	10 (62.5)	6 (37.5)	0.135
Multiple	34	14 (41.2)	20 (58.8)	
Pattern				
Trabecular	26	13 (50)	13 (50)	
Acinar	15	9 (60)	6 (40)	0.192
Solid	9	2 (22.2)	7 (77.8)	
Grade				
Grade I	8	7 (87.5)	1 (12.5)	
Grade II	32	15 (46.9)	17 (53.1)	0.017*
Grade III	10	2 (20)	8 (80)	
Tumor stage				
T 1-2	15	11 (73.3)	4 (26.7)	0.020*
T 3-4	35	13 (37.1)	22 (62.9)	
HBV	13	5 (38.5)	8 (61.5)	
HCV	33	17 (51.5)	16 (48.5)	0.725
None	4	2 (50)	2 (50)	
Cirrhosis				
Absent	18	10 (55.6)	8 (44.4)	0.306
Present	32	14 (43.8)	18 (56.2)	
Tumor necrosis				
Absent	35	15 (42.9)	20 (57.1)	0.211
Present	15	9 (60)	6 (40)	

**** P value ≤ 0.05 is considered significant.***

A statistically significant positive association was found between expression of SOX9 and tumor grade (p= 0.017). High SOX9 expression was significantly higher in grade III tumors compared to grade II and I cases. High SOX9 expression was detected in 80%, 53.1% and 12.5% of grade III, II and grade I tumors respectively. A significant association was also noticed between expression of SOX9 and tumor stage (p= 0.20). High SOX9 expression was detected in 62.9 % of cases of higher tumor stages (T3~4) while was found in 26.7% of cases with lower tumor stages (T1~2). No significant associations were found between SOX9 expression and patients' age and gender (p=0.161 and p=0.554 respectively). No significant associations were found between SOX9 expression and tumor size, tumor multiplicity or histological pattern (p= 0.581, p=0.135 and p=0.192 respectively). No significant associations were found between SOX9 expression and presence of hepatitis, cirrhosis, fatty change or necrosis (p= 0.725, p= 0. 306, p= 0.418 and p= 0.211 respectively).

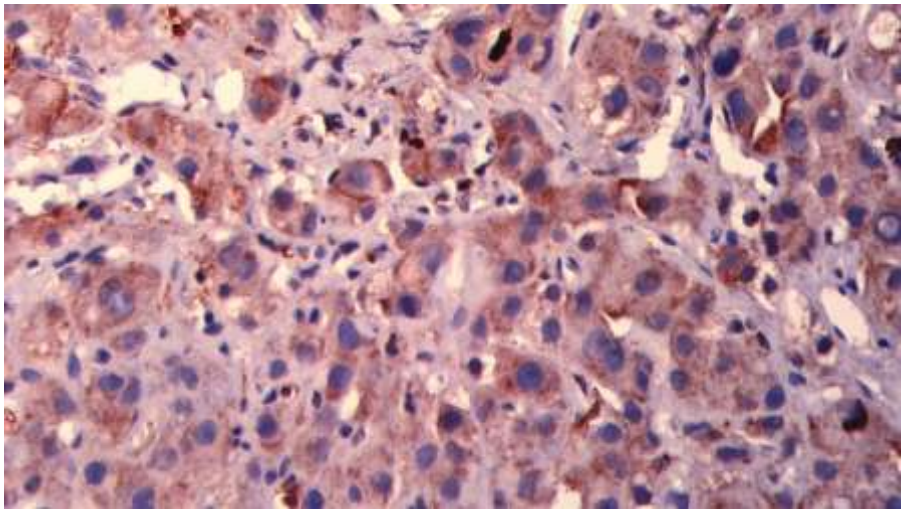

Figure 1: High SOX9 expression in grade III HCC (streptavidin-biotin-immunoperoxidase X400).

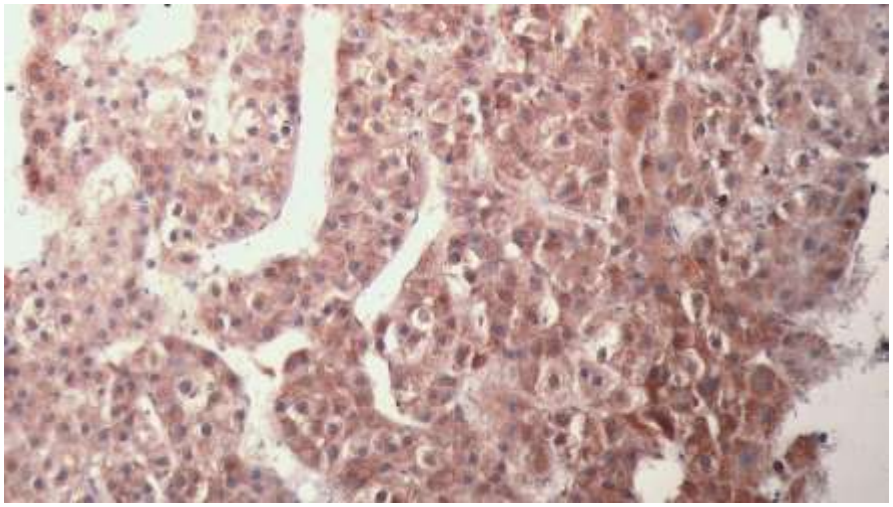

Figure 2: High SOX9 expression in grade II HCC (streptavidin-biotin-immunoperoxidase X200).

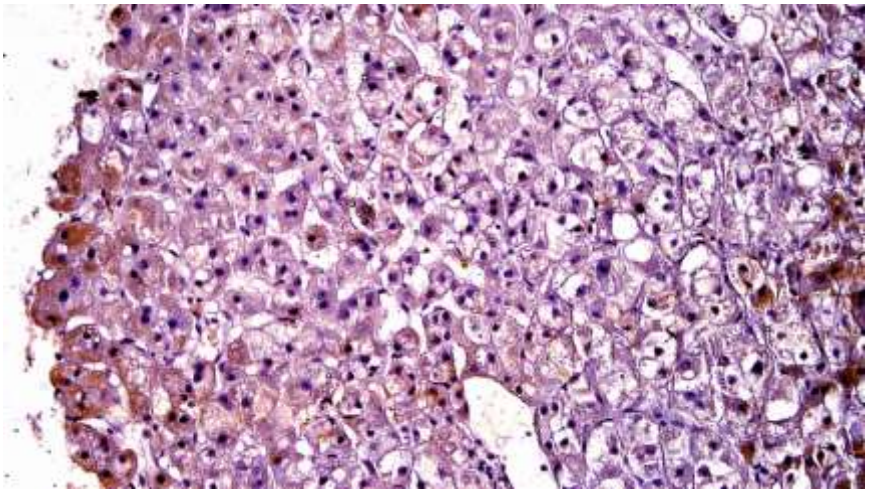

Figure 3: Low SOX9 expression in grade II HCC (streptavidin-biotin-immunoperoxidase X200).

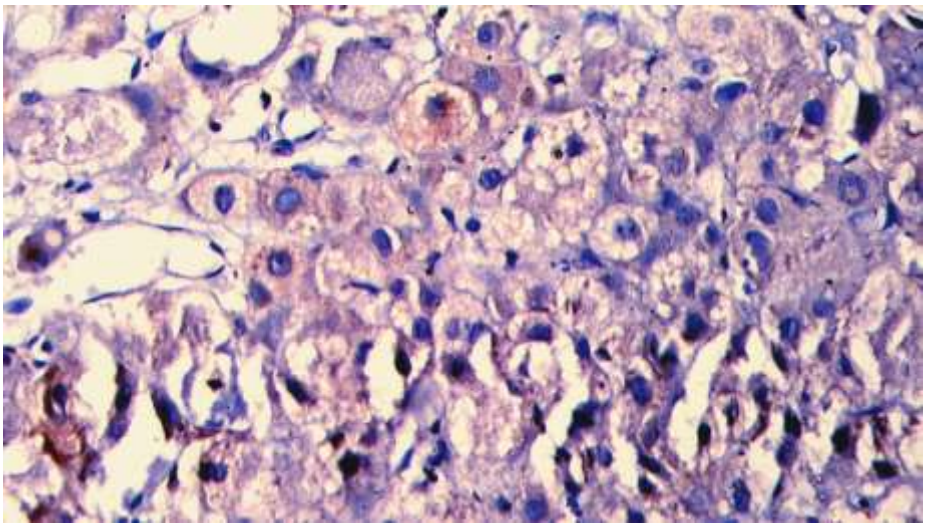

Figure 4: Low SOX9 expression in grade III hepatocellular carcinoma (streptavidin-biotin-immmunoperoxidase X400)

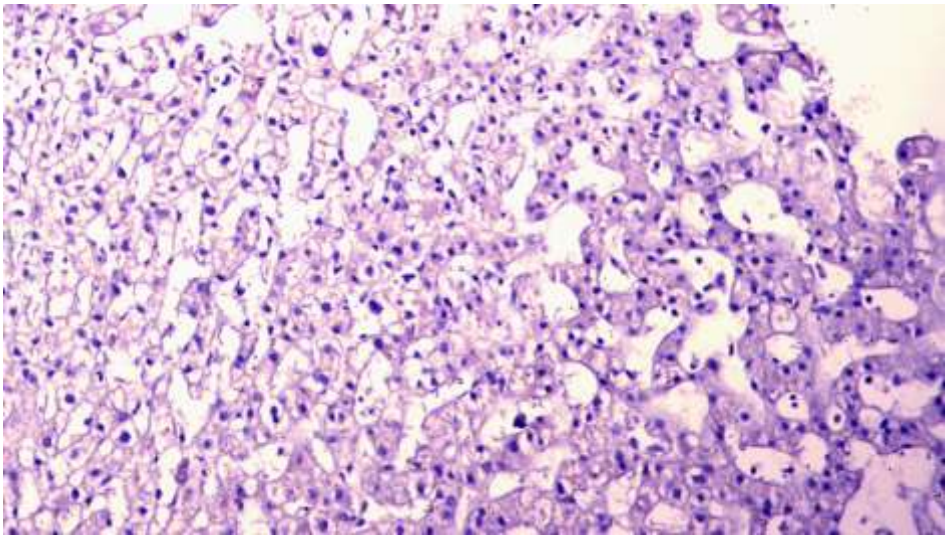

Figure 5: Negative SOX9 expression in grade I hepatocellular carcinoma (streptavidin-biotin-immmunoperoxidase X200).

Discussion

The current study investigated the expression of SOX9 in HCC cases. HCC is unique in comparison to other malignant tumors in that the presence of chronic liver diseases and cirrhosis affects the efficacy of treatment and prognosis of HCC patients (Gomaa et al., 2014). In the current study, hepatitis virus infection was found in 92% of cases mainly by HCV which is similar to other studies (Xiao et al., 2017). However, other studies reported lower rates; 40% and 37% of HCC cases with hepatitis virus infection respectively (Cioca et al., 2014; Iris et al., 2015). This can be clearly explained by the high prevalence of HCV in Egypt which has become a national epidemic. Cirrhosis was found in 64% of cases studied which was in line with (Guo, et al., 2012; Richtig et al., 2017) and (Huang et al., 2017) who reported 66.1% and 63.4% and 55% of cases with cirrhosis respectively. While (Cioca et al., 2014) reported 38% of cases with cirrhosis and 62% without cirrhosis. This can be explained by the early detection and treatment of hepatitis virus infection in developed countries and thus decreased incidence of cirrhosis.

In the present study, 16% of tumors were grade I, 64% were grade II and 20% were grade III. This is in accordance with (Guo et al., 2012; Helal et al., 2010). While (Cioca et al., 2014) reported 8.8% grade I, 44% grade II and 46.6% grade III and (Tien et al., 2005) reported 34% grade 1, 53% grade II and 12.5% grade III. As regard tumor stage, 30% were classified as T 1~2 and 70% were T 3~4. This is in agreement with (Yang et al., 2017). However, this wasn't in accordance with (Yu et al., 2017) who reported that 31.1% of his cases and (Kuo et al., 2017) who reported that 24.4% of his HCC cases were in higher tumor stage (T3~4). This difference may be attributed to difference in time of detection of tumor in different populations and also different number of cases included in each study.

Generally, the frequencies regarding different clinicopathological features including elderly patients, male sex predilection, history of viral hepatitis infection, cirrhosis and large tumor size found in this study were in agreement with those reported by several Egyptian large studies in HCC (Gomaa et al., 2014; Ziada et al., 2016).

The stem cell marker; SOX9 has been recently reported to be involved in HCC development and progression (Kawai et al., 2016). The previous immunohistochemical studies were so variable regarding SOX9 expression rates and subcellular localization in different types of malignant tumors (Chakravarty et al., 2011; Liu et al., 2016). In the present study, high SOX9 expression was detected in 52% of HCC cases. While (Guo et al., 2012) found that 75.4% of their HCC cases showed high SOX9 expression, (Richtig et al., 2017) reported positive SOX9 expression in 34.1% of the studied HCC cases. This wide range of SOX9 expression rates is attributed to variation in the clinicopathological characters of HCC cases included in the previous studies, different SOX9 clones used and different cut-off points used to identify the positivity. In the current study, SOX9 expression was combined cytoplasmic and nuclear expression in a few neoplastic cells in only two cases. This was in agreement with a previous study that reported cytoplasmic expression of SOX9 in breast carcinoma cases (Chakravarty et al., 2011), or combined nuclear and cytoplasmic expression in another study of HCC cases (Liu et al., 2016). Proteins function optimally in a specific subcellular localization. Therefore, revealing the subcellular distribution of a specific protein often provides important information for the elucidation of its function. Chakravarty and his co-authors reported that SOX9 protein, which was normally found in the nuclear component in atypical ductal hyperplasia, was instead localized in the cytoplasm in about one third of invasive duct breast carcinoma cases and lymph node metastases. This was explained by the inability of SOX9 to be translocated to the nucleus in response to suppressed growth arrest signals that promote cancer cells indefinite proliferation. However, its nuclear expression might slow down the proliferation of cancer cells by inducing growth arrest and differentiation (Chakravarty et al., 2011). These reports together with our findings imply that depletion of nuclear SOX9 and its cytoplasmic accumulation promote neoplastic progression and cancer development while nuclear SOX9 localization may promote differentiation. On the other hand, two studies reported nuclear SOX9 expression in HCC cases (Guo et al., 2012; Richtig et al., 2017). These differences could be explained by interaction with other co-regulatory proteins and histone deacetylase activity which act in concert for regulation of SOX9 nucleocytoplasmic shuttling during malignant transformation. Tumors with cytoplasmic SOX9 expression were derived from earlier progenitors with a more dedifferentiated phenotype compared with those displaying nuclear SOX9 expression (Chakravarty et al., 2011).

On studying the association of SOX9 expression with different clinicopathological features, the present study showed that high SOX9 expression was significantly associated with high tumor grade in which high. SOX9 expression was detected in 80% of grade III cases. This was in the line with (Guo et al., 2012) who reported that high SOX9 expression was detected in 95.6% of grade III cases, 76.3% of grade II cases and 58% of grade I cases.

The present study showed that high SOX9 expression was significantly associated with high tumor stage in which high SOX9 expression was found in 62.9% of cases in higher tumor stage (T3~4). This was in line with (Guo et al., 2012). However, this results

were contradictory to (Richtig et al., 2017) who found that 31.7% of high tumor stage cases (T3~4) showed positive expression for SOX9. These findings together with the results found in the current study were supported by previous two experimental studies on HCC. The first study showed that silencing of SOX9 in HCC cells inhibits cell proliferation, tumor sphere formation, migration, invasion and metastasis (Leung et al., 2016). The other study reported that overexpression of SOX9 is involved in liver cancer proliferation and stem cell capabilities (Richtig et al., 2017).

On studying SOX9 expression in relation to other clinicopathological features, the current study revealed no significant association between expression of SOX9 and either patients' age, gender, tumor size, tumor multiplicity, histological pattern, hepatitis, cirrhosis, necrosis or fatty change. These findings were also reported by (Guo et al., 2012; Leung et al., 2016). However, (Richtig et al., 2017) found a significant association between SOX9 expression and male gender.

The current study suggests SOX9 as a molecular protein with an oncogenic function and biological value, being involved in HCC development and progression as well as its association with poor prognostic features such as high tumor grade and advanced tumor stage.

Conclusions

SOX9 overexpression was detected in a substantial proportion of HCC cases. High SOX9 expression was significantly associated with high tumor grade and advanced tumor stage, indicating its association with poor prognostic features in HCC. The expression features of SOX9 in HCC are assumed to play a role in tumor aggressiveness and progression.

Recommendations

Studying the expression of SOX9 and its association with follow up data on a large scale of HCC cases might elucidate its role as a prognostic marker in HCC. Future molecular and genetic studies are recommended to demonstrate the role of SOX9 as a regulator of stemness properties in HCC and as a possible therapeutic target for HCC cases. Studying the molecular regulators of SOX9 and its subcellular localization could provide important information regarding its function.

References

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