Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2021; Oct 21; 9(A):887-893.
https://doi.org/10.3889/oamjms.2021.6123
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pathology*

β-Catenin Expression and Its Association with Prognostic Factors in Hepatocellular Carcinoma: A Study on Alpha-fetoprotein, Histologic Grade, and Microvascular Invasion

Nur Rahadiani, Ignasia Andhini Retnowulan, Marini Stephanie, Diah Rini Handjari, Ening Krisnuhoni*

Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo National Referral Hospital, Jakarta, Indonesia

Edited by: Sinisa Stojanoski
Citation: Rahadiani N, Retnowulan IA, Stephanie M, Handjari DR, Krisnuhoni E. β-Catenin Expression and Its Association with Prognostic Factors in Hepatocellular Carcinoma: A Study on Alpha-fetoprotein, Histologic Grade, and Microvascular Invasion. Open Access Maced J Med Sci. 2021 Oct 21; 9(A):887-893. https://doi.org/10.3889/oamjms.2021.6123
Keywords: β-catenin; Hepatocellular carcinoma; Alpha-fetoprotein; Microvascular invasion; Tumor grade
***Correspondence:** Ening Krisnuhoni, Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo National Referral Hospital, Jakarta, Indonesia.
E-mail: ening.krisnuhoni@yahoo.com
Received: 02-Apr-2021
Revised: 21-Apr-2021
Accepted: 11-Oct-2021

Funding: This study was supported by the Ministry of Research and Technology/National Agency for Research and Innovation through SIMLITABMAS/BRIN Grant PDUPT scheme (Grant number: NKB-2763, year: 2020)
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Hepatocellular carcinoma (HCC) is the most common primary liver cancer. In addition to its high incidence, the disease burden is high due to its poor prognosis and high recurrence rate. Some of the currently known clinicopathologic prognostic factors include alpha-fetoprotein (AFP) level, histologic grade, and microvascular invasion. At the molecular level, β-catenin is one of the most common driver mutations found in HCC. The Wnt/β-catenin pathway regulates cellular processes related to initiation, growth, survival, migration, differentiation, and apoptosis. Although the underlying pathogenesis of hepatocarcinogenesis is known, clinical application warrants a greater understanding of the molecular characteristics and tumor phenotype, especially for determining the prognosis.

AIM: This study aims to analyze the expression of β-catenin and its association with AFP, histologic grade, and microvascular invasion.

METHODS: Thirty-five samples of surgically resected HCCs at Cipto Mangunkusumo National Referral Hospital were examined. Diagnoses were made based on histopathological and immunohistochemical findings followed by β-catenin staining. β-catenin expression was analyzed to determine difference between variables.

RESULTS: Here, we show that β-catenin expression is significantly associated with low serum AFP and well to moderate differentiation implications. Strong nuclear β-catenin expression implies better prognosis in HCC.

CONCLUSION: β-catenin expression is significantly associated with low serum AFP and well-to-moderate differentiation, but not with microvascular invasion. Strong nuclear β-catenin expression implies better prognosis in HCC.

Introduction

Hepatocellular carcinoma (HCC) is most common among liver malignancy [1], [2], [3], [4]. It is the sixth most common carcinoma with 841,080 new cases diagnosed and the fourth most common cause of cancer-related death with an estimated 781,631 deaths in 2018 [2]. HCC is associated with multiple risk factors, but most are related to risk factors of chronic liver disease, such as hepatitis B and C virus, alcohol abuse, and non-alcoholic steatohepatitis. Data from the Ministry of Health of the Republic of Indonesia give the prevalence of hepatitis B infection in Indonesia as 7.5% (around 17.5 million), and of these, 20–30% are estimated to progress to cirrhosis and/or liver malignancy [5]. Preliminary studies show that a majority of HCC cases are related with chronic hepatitis B.

Despite hepatitis surveillance program to reduce disease progression, incidence of HCC remains high. In addition, this burden is further increased due to poor prognosis. The short life expectancy is due to diagnosis at advanced stage [5]. Moreover, the high recurrence rate further worsens clinical outcomes despite advancements in therapeutic modalities [6].

Among risk factors of HCC, high alpha-fetoprotein (AFP) levels are believed to trustily predict poor prognosis and tumor recurrence after curative treatment [7]. Histologic grade predicts survival in post-treatment HCC cases, including after surgery, liver transplantation, and ablation therapy [8]. Research by Lim *et al*. [9] states that, after resection, microvascular invasion is a better predictor of recurrence and overall survival than the Milan criteria. Significant differences in the rate of early recurrence were observed between HCC with and without microvascular invasion. Cases with microvascular invasion have a tendency to recur within the first 30 months after resection [9].

Genetic mutations and abnormal activation of signal transduction pathways play a role in HCC development and are promising targets for future therapy. In general, several signaling pathways have been observed to be activated during hepatocarcinogenesis, and specifically, the Wnt/β-catenin pathway is found to be abnormally activated in 50% of HCCs. Calderaro *et al*. [10] reported that 57% of HCC cases were correlated with either *CTNNB1* or *TP53*

mutations, where each mutation results in distinct HCC phenotypes. *TP53* mutations commonly occur in HCC with chromosomal instability and cell cycle activation. Contrarily, Wnt/β-catenin pathway activation is associated with chromosomal stability [10], [11]. Deletions and missense mutations in exon 3 of the *CTNNB1* gene are common genetic abnormalities found in HCC (20–40%) that causes aberrant activation of the Wnt/β-catenin pathway [12]. This study aims to assess expression of β-catenin in HCC and correlations to various prognostic factors, that is, AFP level, degree of differentiation, and microvascular invasion.

Materials and Methods

This study reviewed all cases of HCC treated by surgical resection in January 2013–September 2019 at Cipto Mangunkusumo National Referral Hospital, Jakarta, Indonesia. Clinical data were obtained from medical records. All pathology slides and paraffin blocks were collected from the department of anatomical pathology archive. The pathology slides were reassessed by pathologists with expertise in liver pathology. Cases with incomplete slides and paraffin blocks were excluded from the study. A total of 35 cases of HCC were finally included in the study.

Clinical and pathological variables were also obtained. Serum AFP levels were classified as either ≤100 ng/ml or >100 ng/ml. Histologic grade was classified based on the system by Sasaki *et al*. [13] wherein tumors were classified as well differentiated to moderately differentiated or poorly differentiated. Well-differentiated to moderately differentiated tumors exhibited mild-to-moderate nuclear atypia, some with nucleoli, and abundant eosinophilic to basophilic cytoplasm. Tumors without any poorly differentiated areas were categorized as well differentiated. On the contrary, poorly differentiated tumors were defined as those with pleomorphic nuclei and severe atypia, anaplastic cells, and scant basophilic cytoplasm. Tumors were defined as poorly differentiated when any poorly differentiated areas were found. Microvascular invasion was defined as the microscopic presence of tumor cells in blood and/or lymphatic vessels in non-tumor areas.

Paraffin blocks were sectioned at a thickness of 3–4 micrometers. Sections were deparaffinized in xylol and then rehydrated in a series of decreasing alcohol concentrations. The slides were incubated in TRIS-EDTA buffer (10 mM TRIS, 1 mM EDTA at pH 9.0) at 98°C for 20 min and then for 1 h at room temperature with a β-catenin antibody (BD Transduction Laboratories, San Jose, CA) at a dilution of 1:100. The slides were then incubated with secondary anti-mouse immunoperoxidase polymer antibodies for 30 min at room temperature, followed by incubation with diaminobenzidine substrate-chromogen for 5 min. Counter staining was done with Mayer's hematoxylin for approximately 1–2 min, followed by dehydration in a series of increasing alcohol concentrations, and clearing in xylol. Hepatoblastoma with positive β-catenin nuclear staining served as the positive control. β-catenin positivity was assessed in 500 cells in the tumor area. Positive immunoreactivity was defined as nuclear staining in ≥20% of tumor cells with/without cytoplasmic staining. Negative immunoreactivity was defined as either nuclear staining in <20% of tumor cells with/without cytoplasmic stain or membranous and/or cytoplasmic staining without nuclear staining.

Statistical analysis was performed using SPSS 25.0. Comparative tests were performed using either the Chi-square test or Fisher's exact test. The results were statistically significant if the $p < 0.05$ was considered with a 95% confidence interval.

Ethical approval for this study was obtained from the ethics committee of the Faculty of Medicine, Universitas Indonesia, Protocol No: 19-11-1358.

Results

HCC was more frequently found in men (80%) than in women and was more often diagnosed in individuals under 60 years old (62.9%) with average age of 54.7 ± 14.8 years. The mean age in males was 53.4 ± 13.1 years and 60.1 ± 20.8 years in females. The most common risk factor for HCC was hepatitis B infection (82%), while only 9% of patients had hepatitis C infection. Most HCC (82%) were more than 5 cm in diameter, and mean tumor size was 9.4 ± 5.3 cm (Table 1).

An analysis of β-catenin immunoreactivity revealed that 6 cases (17%) were positive for β-catenin expression, while the other 29 (83%) were negative (Figure 1). In cases with positive β-catenin expression, 83.3% of cases were above 60 years old, and 66.7% were male. Positive β-catenin expression was also

Table 1: β-catenin expression and clinicopathologic characteristics

Clinicopathologic variable (%)		β-catenin expression (%)	
		Positive β-catenin	Negative β-catenin
Sex			
Male	28 (80)	4 (66.7)	24 (82.8)
Female	7 (20)	2 (33.3)	5 (17.2)
Age (years)			
≤60	22 (62.9)	1 (16.7)	21(72.4)
>60	13 (37.1)	5 (83.3)	8 (27.6)
Hepatitis risk factor			
Hepatitis B	18 (81.8)	3 (75)	15 (83.3)
Hepatitis C	2 (9.1)	1 (25)	1 (5.6)
Non-hepatitis	2 (9.1)	0	2 (11.1)
Tumor size		5.13 ± 2.61 cm	10.34 ± 5.30 cm
Cirrhosis status			
Cirrhosis	18 (51.4)	9 (69.2)	9 (40.9)
Non-cirrhosis	17 (48.6)	4 (30.8)	13 (59.1)

more frequently found in smaller tumors (5.13 ± 2.61 cm) and in tumors with cirrhotic background (69.2%) (Table 1).

Further analysis was performed to assess expression of β-catenin in cases of HCC with hepatitis infection. No difference was found in the frequency of positive β-catenin expression between HCC tumors with hepatitis B (15%) and C infection (50%, p = 0.34). Analysis of β-catenin expression between hepatitis B (15%) and non-hepatitis HCC cases (0%, p = 1.00) also revealed no difference in frequency of positive β-catenin expression.

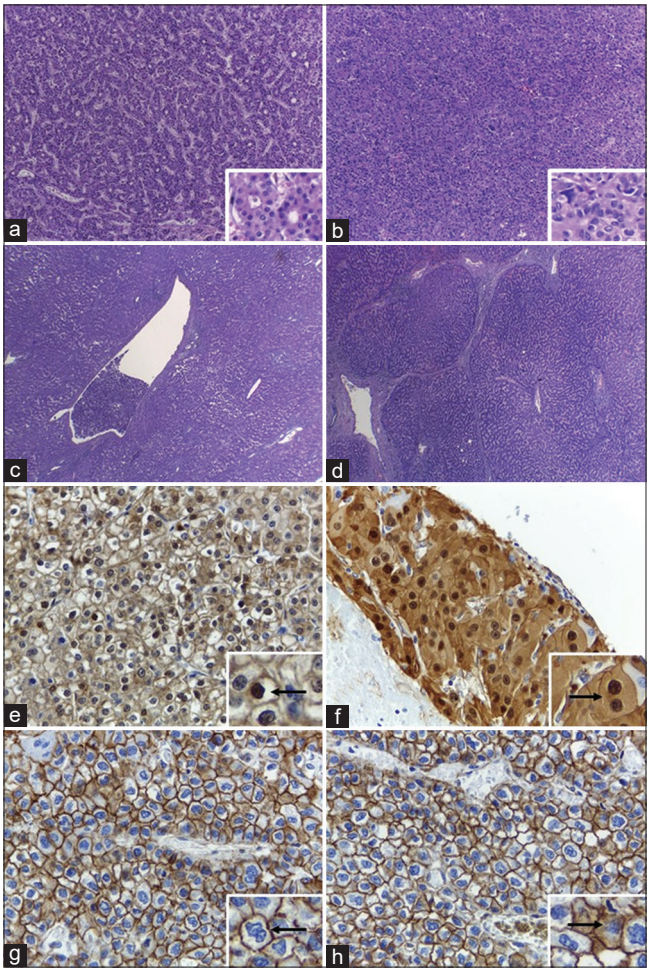

Figure 1: Histomorphological features of HCC (H&E) and β-catenin expression. (a) Well-to-moderate differentiation with microtrabecular and pseudoglandular patterns (×100). (b) Poor differentiation featuring a solid pattern (×100). (c) Microvascular invasion (×100). (d) Liver cirrhosis in the non-tumor area showing the formation of bridging fibrosis and regenerating nodules (×100). (e) Positive expression of β-catenin in the nucleus (inset) with no cytoplasmic expression (×400). (f) Positive expression of β-catenin in the nucleus with cytoplasmic expression (inset) (×400). (g) Negative expression of β-catenin in the cell membrane (inset) with no nuclear expression (×400). (h) Negative expression of β-catenin in the cytoplasm (inset) with no nuclear expression (×400)

Fisher's exact comparative test was performed to assess the differences in β-catenin expression on various known prognostic factors of HCC. The expression of β-catenin was significantly different between well-to-moderately differentiated tumor and poorly differentiated tumor. Positive expression of β-catenin was more commonly found in well-to-moderately differentiated HCC (66.7%, p = 0.043). Positive β-catenin expression was more frequent in HCC cases with AFP levels of ≤100 ng/ml (100%, p = 0.037). In contrast, no significant differences were found between HCC cases with or without microvascular invasion (Table 2).

Discussion

β-catenin is a multifunctional protein encoded by the *CTNNB1* gene that can be found in the cell membrane, cytoplasm, and nucleus [14]. The Wnt/β-catenin pathway regulates cellular processes related to initiation, growth, survival, migration, differentiation, and apoptosis [13]. Several pathways induce aberrant activation of the Wnt/β-catenin pathway and cause initiation and progression of HCC. Deletions and missense mutations in exon 3 of the *CTNNB1* gene are common genetic abnormalities in HCC (20–40%). Other causes of activation of the Wnt/β-catenin pathway are mutations in the proteasome degradation complex, including APC and Axin, overexpression of Wnt ligands or frizzled receptors, and various factors that cause disruption of adherent junctions. This results in the accumulation of β-catenin and transcription of oncogenes [12].

Positive expression of β-catenin is found in 12%–80% of cases in various studies [15]. Various criteria are used for β-catenin expression in literature, including nuclear expression, cytoplasmic expression, expression intensity, and percentage of cells stained [15], [16], [17], [18]. Many studies suggested nuclear β-catenin expression to be stronger correlated with *CTNNB1* mutations [10], [16], [19], [20], while a meta-analysis by Chen *et al*. [17] reported β-catenin expression in the cytoplasm alone as not related to Wnt/β-catenin activation and with inconclusive implications. Nuclear expression of β-catenin is more important in assessing tumor progression than membranous and cytoplasmic expression [21]. Based on this information, this study only defines β-catenin nuclear expression as meaningful.

This study found positive β-catenin expression in a minority of cases, agreeing with various studies reporting nuclear β-catenin expression in 11–43% of HCC cases; β-catenin mutations are also found in 13–34% of cases of HCC [18], [19], [22], [23], [24], [25]. Wong *et al*. [25] reported the accumulation of nuclear β-catenin in 17% of their cases. Variations in β-catenin expression exist among ethnic groups. For example, higher β-catenin expression is found in Asians, while lower expression is found in Africans [15].

This study showed that β-catenin expression was more commonly observed in older patients. Hsu

Table 2: β-catenin expression and prognostic factors of HCC

β-catenin expression	Prognostic factors								
	AFP level (ng/ml)			Histologic grade			Microvascular invasion		
	≤100	>100	p-value	Well to moderate	Poor	p-value	Yes	No	p-value
Positive	4 (100)	0	0.037[a,*]	4 (66.7)	2 (33.3)	0.043[a,*]	4 (66.7)	2 (33.3)	1.000[a]
Negative	7 (36.8)	12 (63.2)		6 (20.7)	23 (79.3)		21 (72.4)	8 (27.6)	
Total	11 (49.7)	12 (52.2)		10 (28.6)	25 (71.4)		25 (71.4)	10 (28.6)	

[a]Fisher's exact test, *Statistically significant (p-value ≤ 0.05)

et al. [20] also reported similar results. A predilection for HCC to be found earlier is also in agreement with El-Serag [26] which states that HCC is more often diagnosed at younger age in areas with high prevalence, such as Indonesia. This is related to risk factors such as viral infection or alcoholic cirrhosis and age of exposure to such factors. Hepatitis B is generally seen in younger patients, whereas hepatitis C and alcoholic cirrhosis are generally seen in older patients [26].

Positive expression of β-catenin was more common in men than in women, related to a more common prevalence of HCC in men. The male-to-female incidence ratio ranges from 2:1 to 4:1, with greater difference seen in areas with high HCC incidence [27]. This study confirmed a predilection of HCC in men, which might be explained by higher prevalence of risk factors in men, such as viral hepatitis and alcohol abuse. Sex hormones can also relate to hepatocarcinogenesis, with androgens being pro-tumorigenic, and estrogens being anti-inflammatories to IL-6 [28]. However, multiple studies have not shown an association between β-catenin mutations and sex [16], [20].

Both hepatitis B and C infections increase β-catenin expression [29], [30], [31]. However, a higher frequency of β-catenin mutations is found in cases with hepatitis C infection than those with hepatitis B infection [20], [23]. Verma *et al*. [15] mentioned that Axin1 mutations are associated with hepatitis B infection, while β-catenin mutations are related to non-hepatitis B infections.

The frequency of HCC with hepatitis B infection in this study explained the low positivity of β-catenin expression. Although many studies have reported an association between β-catenin activation and hepatitis C infection, the results of this study did not show any difference in β-catenin expression in cases with hepatitis B and C infection or between cases with hepatitis B and without hepatitis [20], [25], [30]. Only two cases of HCC with hepatitis C infection were included in this study, with only one mutated case. Larger sample size is needed to provide conclusive data of β-catenin expression in HCC with various viral infections.

This study showed that HCC tumors were more likely to be large. This size is due to the advanced stage of disease at discovery in populations with higher incidence [4]. In addition, non-cirrhotic HCC was found in around half of all cases, which are generally found at a more advanced stage than cirrhotic HCC because of less surveillance [32]. In cases of cirrhosis, a strict surveillance program is followed so that malignant transformation is diagnosed earlier [33].

CTNNB1 mutations are associated with small tumor size and a good prognosis [12]. HCC with β-catenin mutations has a lower rate of cell proliferation than those without [34]. This study agreed with these correlations.

Although the literature states that the prevalence of cirrhosis in HCC is 80–90%, this study revealed a balance between the frequency of cirrhosis and non-cirrhosis [35], [36], [37]. This may be due to high prevalence of hepatitis B in this study, which causes malignant progression either with or without cirrhosis. Furthermore, only surgically resected HCC is included, and thus, the proportions in inoperable HCC are unknown.

This study showed that positive expression of β-catenin was more common in HCC with prior cirrhosis. In cirrhosis and HCC, membranous β-catenin expression is decreased, while cytoplasmic and nuclear expression is increased. Khalaf *et al*. [12] reported a significant difference in nuclear and cytoplasmic β-catenin expression in HCC (72.9%), non-tumor tissue (22.35%), and cirrhotic liver tissue (26.67%). Ge *et al*. [38] mentioned that activation of Wnt/β-catenin signaling increases fibrogenesis in the liver. Higher β-catenin expression is found in cases in which the liver has higher fibrosis [38].

AFP is a tumor marker physiologically secreted by the yolk sac and fetal hepatocytes. In adults, it is increased in cirrhosis, malignancies, and other liver pathologies. High AFP is correlated with malignant progression, poorer differentiation, early recurrence, and poor prognosis. The regulation of AFP gene expression occurs through a complex process and is mediated by various transcriptional activators and repressors that bind to the promoter complex in the AFP gene. Increased AFP in HCC is more common in young patients and in those with positive HBsAg titers [39], [40]. AFP in the serum binds to AFP receptors in the HCC cell membrane, which activates the PI3/AKT and Ca2+ pathways, and upregulates Ras, Src, c-jun oncogene, and c-fos, thus increasing cancer proliferation [39].

Peng *et al*. [40] reported a rarity of high AFP level in HCC with β-catenin mutations. In contrast, an increase in AFP 5 times higher than the normal level is found in HCC with p53 mutations [40]. Agreeing with the literature, this study indicated differences in AFP levels in cases with positive and negative β-catenin expression. Positive β-catenin expression is more frequently found in cases with low AFP, while negative β-catenin expression was more often found in cases with high AFP.

AFP is a growth regulator in HCC. Increased *in vitro* proliferation due to AFP has been reported in embryonic and HCC cells [40]. Accumulation of β-catenin activates the p53 pathway, causing an anti-proliferative response to p53 that can inhibit growth and is correlated with senescence. P53 induction by β-catenin deregulation is dependent on ARF tumor suppressors [41]. β-catenin deregulation stimulates ARF expression and p53 and then suppresses AFP gene expression [40], [41]. The p53 protein will bind to the AFP repressor region, replace the HNF-3 bond, and cause changes in the chromatin structure of the core promoter, which ultimately represses AFP gene expression [40].

A cohort study performed by Cavard *et al*. [34] who reported that HCC with β-catenin mutations tends to be well differentiated, has a microtrabecular to acinar arrangement, with rare steatosis [34]. β-catenin expression is, therefore, related to histologic grade, with positive β-catenin expression generally correlating with well-to-moderate differentiation.

Well differentiation is connected to an increase of genes expressing hepatocyte polarity markers, such as BCRP/ABCG2 and Claudin2, in HCC cases with β-catenin mutations. Aberrant activation of the Wnt/β-catenin pathway, therefore, maintains epithelial polarity, and expression of genes related to hepatocyte differentiation and function, such as APOB, CYP1A1, CYP1A2, CYP3A4, CYP2E1, CYP2C9, HNF1A, and HNF4A [10], [34].

Well differentiation is also supported by low cell proliferation. HCC with β-catenin mutations is usually equally proliferative to non-tumor areas and much lower than in HCC without β-catenin mutations. The expression of *MKI67* gene significantly decreases in HCC with β-catenin mutations, as well as other genes playing a role in cell cycle control, such as CCNA2, CCNE2, CDC2, CDC20, CDC6, CDC7, and CDK4 [10], [34]. This study revealed dramatically higher mitotic counts between HCC with negative β-catenin expression than in HCC with positive β-catenin expression.

In addition, deregulation of genes that play a role in the architecture and stability of chromosomes, such as BIRC5/SURVIVIN, BUB1, CDCA5, CDCA8, NUF2, and CENPF, is not seen in HCC with β-catenin mutations. This condition causes HCC with β-catenin mutations to have low chromosomal instability and proliferation status [34].

The results of this study did not show a significant difference of microvascular invasion between HCC cases with positive and negative β-catenin expression. Agreeing with these results, other studies have also not reported a relationship between microvascular invasion and β-catenin expression [25], [42]. In contrast, Zulehner *et al*. [43] reported that nuclear β-catenin expression is significantly associated with microvascular invasion in advanced HCC. Data on the clinical stage of cases in this study were insufficient for a statistical analysis, and thus, association between β-catenin expression and advanced-stage HCC is inconclusive.

Variations in studies of β-catenin expression in HCC, as recorded in Verma *et al*. [15], can be explained by the differences of criteria used to assess β-catenin expression, as well as the population size [15], [17]. Chen *et al*. [17] reported significant relationship between cytoplasmic and/or nuclear β-catenin expression and microvascular invasion, which may indicate that non-nuclear expressions of β-catenin is associated with worse pathologic features and prognoses [12], [25]. Wong *et al*. [25] stated that non-nuclear β-catenin expression may be independent of the Wnt signaling pathway. Thus, cytoplasmic and/or nuclear expression assessed using other criteria may provide different results than the ones obtained in this study, and must be further elucidated. Moreover, nuclear β-catenin expression might not always be related to mutations of β-catenin gene [12]. Therefore, the different results obtained in this study should be reconfirmed by further research.

Other factors affecting the results of this study might be confounders such as secretion of vascular endothelial growth factor promoting microvascular invasion, or cytokeratin-16, associated with a more aggressive HCC phenotype. Hepatocarcinogenesis is a gradual process with further mutations as tumors grow in size and acquire further mutations. This remains a further avenue to be explored [44], [45], [46].

Conclusion

β-catenin expression is significantly associated with low serum AFP and well-to-moderate differentiation, but not with microvascular invasion. Strong nuclear β-catenin expression implies better prognosis in HCC.

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