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Wnt/ β-catenin and CTNNB1 gene mutation in hepatocellular carcinoma, a case study in Egyptian patients

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Abstract

Background Wnt/β-catenin pathway has an important role in hepatocarcinogenesis. It has been involved in progression, growth, epithelial mesenchymal transition and metastasis of hepatocellular carcinoma (HCC). This pathway may represent a potential target for evolving treatment strategies. β-catenin gene (CTNNB1) has been identified as an important oncogene involved in hepatocarcinogenesis in previous trials to understand the pathogenesis of HCC. This study aimed to spot light on the role of Wnt/ β-Catenin and CTNNB1 gene mutation in HCC development and its relation with different clinicopathological features.

Patients and methods This study was conducted on 121 HCC cases that were obtained from liver explants from pathology laboratory at Mansoura Gastroenterology center retrospectively in the period between 2006-2017. Tissue Microarray (TMAs) were prepared. β-Catenin and Wnt immunohistochemical (IHC) staining was performed on these blocks. Detection and scoring of CTNNB1 gene mutation were done by Chromogenic In Situ Hybridization (CISH). The relation between aberrant β-Catenin, Wnt2 IHC staining and CTNNB1 mRNA expression and different clinicopathological characteristics was studied.

Results A significant association was detected between aberrent β-catenin IHC staining and larger tumor size ($p=0.011$), multiple tumor nodules ($p=0.021$), higher stages of the tumor ($p=0.03$) and with presence of lymphovascular emboli (LVE) ($p=0.034$). However, no significant association was detected with tumor site, presence of lymph node spread, distant metastasis, tumor necrosis, local recurrence and alpha-fetoprotein level. No significant association was seen between Wnt2 IHC staining with either tumor site, tumor size, number of tumor nodules, presence of LVE, tumor necrosis, tumor grade, TNM stage or presence of local recurrence. A significant association was seen between CTNNB1 mRNA expression and larger tumor size (> 5 cm) ($p=0.041$), higher tumor stages (Stages III and IV) ($p=0.005$) and presence of distant metastasis ($p=0.008$).). No significant association between CTNNB1 mRNA expression and LVE, tumor necrosis, tumor grade or occurrance of local recurrence.

Conclusion Aberrant β-catenin IHC staining and CTNNB1 gene mutation in HCC correlate significantly with tumor size, number of tumor nodules, tumor stage and presence of LVE. All of these items confer poor prognosis in HCC. A highly significant correlation was detected between CTNNB1 gene mutation and aberrant β-catenin expression in HCC cases.

Keywords β-catenin, Wnt2, CTNNB1, IHC, Gene mutation, Hepatocellular carcinoma

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Background

Hepatocellular carcinoma (HCC) represents the sixth most common cancer, the third cause of cancer related deaths worldwide (Sung et al. 2021) and the most common primary malignant tumor of the liver (McGlynn et al. 2021). In Egypt, the incidence of HCC is currently increasing, contributing to about 8% of all cancers and 12% of the malignancies of digestive organs (Gameel et al. 2017). It is considered the second cause of cancer mortality in both sexes (Zeeneldin et al. 2015).

Chronic liver disease such as Hepatitis B and C viruse (HBV and HCV) infections are major etiological risk factors in 70–90% of HCC cases (D'souza et al. 2020). HBV (in 54% of HCC cases) and HCV (in 31% of HCC cases) (Yang et al. 2019). The highest prevalence of HCV infection globally was reported in Egypt (14.7%) (El-Bendary et al. 1017) with 93% of which are of genotype 4 (Omata et al. 2015). Liver cirrhosis as a precancerous lesion is present in about 80% of hepatocellular carcinoma cases all over the world (Coral et al. 2021).

Development of HCC is a multistep process with no single dominant molecular mechanism (Huang and Niehrs 2014). Accumulation of multiple acquired genetic mutations as well as abnormal activation of signaling pathways are main factors in HCC development that cause tumor proliferation (Rahadiani et al. 2021).

Signaling pathways involved in cell proliferation, apoptosis, metabolism, splicing, and the cell cycle have an important role in the development of HCC. The signaling pathways known to be activated in HCC include the Wnt/β-catenin pathway (as much as 50% of HCC), the phosphatidylinositol-3-kinase and protein kinase B (PI3K/Akt) pathway (40–60% of HCC), the Myc pathway (30–60%), the Hedgehog pathway (50–60%), and the MET pathway (30–40%) (Vilchez et al. 2016).

The most frequently mutated genes in HCC include: TERT promoter (51%), TP53 (29%), CTNNB1 (Catenin beta 1) (28%), ALB (13%), APOB (9%), ARID1A (9%), AXIN1 (8%), RB1 (6%), and ARID2 (6%) (Chiang and Villanueva 2017).

Wnt/ β-catenin signaling pathway was discovered for about 40 years genetic analysis of drosophila melanogaster. It is involved in embryonic phase cell multiplication, stem cell proliferation, cell proliferation, differentiation, angiogenesis and in some pathological conditions, including cancer in humans (Daud et al. 2017). It is most frequently activated pathway in HCC by activating mutations of CTNNB1 (11–37%) and inactivating mutations of AXIN1 (5- 15%) or APC (1–2%) (Dhanasekaran et al. 2019).

β-catenin is encoded by the CTNNB1 gene (chromosome 3p21-p22). The commonest genetic abnormality in HCC is believed to be deletions or missense mutations in exon 3 (Rahadiani et al. 2021). CTNNB1 mutations are substitutions or in-frame deletions in a hotspot situated in the domain aimed by the APC/AXIN1/GSK3B inhibitory complex (Stratton et al. 2009). Tumors with CTNNB1 mutation have a specific transcriptomic profile with overexpression of target genes like GLUL and LGR569 (Lachenmayer et al. 2012).

Wnts can induce various modes of cellular signaling, either mediated by β-catenin or not. Wnts are classified into canonical (β-catenin dependent) and noncanonical (β-catenin independent) subgroups based on dependence on β-catenin for mediating cellular effects (Nusse and Clevers 2017). Wnt5a is associated with the non-canonical pathway while other Wnts are involved in the canonical pathway (Dong et al. 2019).

Activation of the Wnt/β-catenin pathway clearly contributes to early hepatocarcinogenesis as it is involved in cancer stemness, proliferation, resistance to therapy and metastasis (Liao et al. 2020). This is confirmed by presence of recurrent genetic mutations of Wnt/β-catenin signaling pathway in HCC and HCV-related tumors especially (Wang et al. 2017). Whether β-catenin mutations specifically occur late or early in hepatocarcinogenesis is not yet clearly defined (Waisberg and Saba 2015).

By immunohistochemical staining, β-catenin is usually membranous in the normal hepatocytes (Lu et al. 2014). Decreased membranous expression with increased cytoplasmic and nuclear expression (aberrant β-catenin expression) is significantly detected in liver cirrhosis and HCC. This aberrant β-catenin expression was significantly greater in HCC tissue (72.94%) in comparison with cirrhotic tissues (22.35%) (Lee et al. 2014).

Although there is no approved targeted therapy for HCC that shows abarrent β-catenin (Liu et al. 2024) and the pivotal roles of abnormal activation of the Wnt/β-catenin signaling in different types of cancer, many trials have been made to evolve therapeutic target agents such as biological agents and small molecule agents (Zhang and Hao 2015).

Few studies about the role of canonical β-catenin signaling in hepatocarcinogenesis have been done in Egypt. So, this study spotted light on this association and also studied its relation to clinicopathological and prognostic characteristics.

Patients and methods

This study was conducted on 121 HCC cases that were obtained from liver explants from pathology laboratory at Mansoura Gastroenterology center retrospectively in the period between 2006–2017. All the cases were revised for the clinicopathological data such as sex, age, tumor location, tumor size, multiplicity of the tumor, portal vein thrombosis, serum virology test (by PCR),

Alfa-fetoprotein (AFP) serum level, presence of local recurrence, tumor grade, tumor stage, presence of necrosis and lymphovascular emboli. Tumor grade based on Edmondson-Steiner grading system. Tumor stage was preformed based on TNM and the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 8th Edition (Abou Alfa et al. 2017).

Tissue microarray (TMA) construction

Microarray blocks were constructed manually using pencil tips with the use of three cores per case to minimize tissue loss and to overcome tumor heterogeneity.

β-Catenin immunohistochemical staining

Automated immunohistochemical staining of β-Catenin using Dako Omnis autostainer was done according to the manufacturer's instructions. Paraffin-embedded TMA blocks were cut at 4 μm sections, then deparaffinized using xylene, and rehydrated using graded alcohols. Antigen retrieval was done by Envision Flex-target retrieval solution pH 9. Antibody against β-Catenin (Antihuman mouse monoclonal, ready to use, code IR702, DAKO, Denmark) was used at dilution of 1:100. Sections were incubated with Envision Flex/HRP, ready to use, goat secondary antibody against mouse IgG (SM802, DAKO, Denmark). Post incubation detection reagent was DAB + Substrate Chromogen system (Agilent, Santa Clara, CA, USA).

WNT2 immunohistochemical staining

The IHC stain was carried out on a Dako Omnis autostainer (Agilent, Santa Clara, CA, USA) at AP Research Lab of Calgary Laboratory Services as a routine procedure. 4 μm sections from formalin fixed paraffin-embedded blocks were pretreated with Tris-based target antigen retrieval buffer high pH (PH9) (Agilent) followed by a series of staining and washing steps.

Rabbit polyclonal antibody to WNT2 was from Abcam, Inc (Cambridge, MA, USA) Cat#ab203225. The primary antibody was diluted at 1:100 dilution using a Dako antibody diluent. Incubation for twenty or thirty minute was done for both primary and secondary antibody. Sections were incubated with Envision Flex/HRP, for secondary antibody (Polyclonal Goat Anti-Rabbit IgG H&L (HRP) Cat# ab205718). Post incubation detection reagent was DAB + Substrate Chromogen system (Agilent, Santa Clara, CA, USA).

Interpretation of immunohistochemical staining results

β-Catenin is usually expressed as positive membranous staining of tumor cells. Interpretation of IHC results done by detection of aberrant expression of β-Catenin which was defined as loss of membranous staining with or without nucleo-cytoplasmic or cytoplasmic staining (Lee et al. 2014). Scoring of staining intensity was as follows (0 for no staining, 1 + for weak staining, 2 + for moderate staining, and 3 + for strong staining (Khalaf et al. 2018). Percentage of positive cells was also calculated.

Regarding WNT2 IHC staining, cytoplasmic and/or membranous expression was interpreted as positive. Scoring of staining intensity was as follows (0 for no staining; 1 + for weak staining; 2 + for moderate staining; and 3 + for strong staining) (Dong et al. 2019). The percentage of positive cells was also calculated.

Detection and scoring of CTNNB1 mutation by CISH

In situ detection of CTNNB1 mRNA transcription was performed using RNAscope 2.5 HD detection reagent kit (From Advanced cell diagnostics, Hayward, CA, USA, Cat: #322360) according to manufacturer's instructions.

Paraffin-embedded TMA blocks were cut at 4 μm sections, baked in the oven for one hour at 60c° prior to use, deparaffinized using xylene,then rehydrated using 100% alcohols. Hydrogen peroxide was added to each slide for 10 min at 3 RT. Then, heat is used for antigen retrieval with the usage of the target retrieval solution by putting it in a pan containing boiling water. Slides were left to dry, then hydrophobic barrier was drawn around each tissue section. Then protease plus was added and slides put in the HybZII tray oven for 30 min.

Then, the CTNNB1 probe was dropped on the slides and hybridized in the oven for two hours at 40c°, washed two times in washing buffer, then Amp 1 was hybridized for 30 min at 40c°, washed two times, then Amp 2 was hybridized in the oven for 15 min at 40c°, washed two times, then Amp 3 was hybridized in the oven for 30 min at 40c°, washed two times, then Amp 4 was hybridized in the oven for 15 min at 40c°, washed two times, then Amp 5 was hybridized in the tray at room temperature for 30 min, washed two times, then Amp 6 was hybridized in the tray for 15 min, washed two times.

Then, Red A solution was mixed with Red B solution at dilution of 60:1, and 180 μm was added to each slide, hybridized in the tray at room temperature for 10 min to detect the signal, then washed two times by distilled water. The slides were then counterstained with Gill hematoxylin solution, washed in water, then the slides were put in 5% ammonia solution.

Slides put in the oven at 60c° for 5 min to dry. Then, each slide was put in fresh Xylene and two drops of Ecomount solution were added before xylene dries, cover slips 24 × 50 mm were put on each slide preventing trapping of air bubbles.

Each tissue section is scored independently by two pathologists according to manufacturer's protocol (Table 1).

Interpretation of CISH staining results

The CTNNB1 gene mutation is considered positive when there are red or brown dots in the cytoplasm or the nucleus of malignant hepatocytes. The number of dots/10 cells and the percentage of the positive cells was documented. Then, the scoring is performed according to the manufacturer's protocol as mentioned before (Table 1).

Statistical analysis

The collected data was coded, processed and analyzed using GraphPad Software Prism V5 (GraphPad software Inc., San Diego, CA, USA) for Windows. Descriptive data were described using numbers and percent. Scores were presented as mean ± standard deviation (SD). Statistical analysis was done using the ANOVA test for non-parametric data and the Fisher test for data expressed as percentage. The Spearman test was used for assessment of correlations between non-parametric data. Level of significance for the above mentioned statistical tests was done, the threshold of significance is fixed at 0.05 (p-value).

Results

β catenin IHC staining in studied HCC cases

Aberrant β-Catenin staining was detected in 72 (59.5%) of HCC cases, 2 cases show mild positive nuclear staining (score 1), 13 cases show mild positive cytoplasmic staining (score 1), 9 cases show moderate positive cytoplasmic staining (score 2), 41 cases show strong positive cytoplasmic staining (score 3) and 7 cases show moderate positive nuclear and cytoplasmic staining (score2) (Fig. 1).

Regarding demographics, no significant association was found between aberrant β-Catenin IHC staining with age or sex of the patient ($p > 0.05$).

On the other side, a significant association was found between aberrant β-Catenin staining and tumor size ($p = 0.011$) being more expressed in larger tumors (> 5 cm) and also with the number of tumor nodules ($p = 0.021$) being more expressed in tumors with multiple nodules. A significant correlation between aberrant β-Catenin immunoreactivity and different TNM tumor stages ($p = 0.03$) was found being more expressed at higher stages of the tumors (Stages III and IV). However, aberrant β-Catenin was not significantly associated with tumor site, tumor grade, LN status or distant metastasis (Table 2).

As shown in Table 2, there was a significant association between aberrant β-Catenin immunoreactivity and LVE ($p = 0.034$) being more expressed in tumors with LVE. However, no significant association was seen between aberrant β-catenin and tumor necrosis, local recurrence, or alpha fetoprotein level.

All studied HCC cases were associated with cirrhosis in adjacent liver tissue. 114 cases were HCV- induced cirrhosis, 3 cases were HBV- induced cirrhosis, and 4 cases were cryptogenic cirrhosis. Aberrant β-Catenin staining was detected in 71 (62.3%) of HCC cases that are associated with HCV- induced cirrhosis, in 1 (33.3%) of HCC cases that are associated with HBV- induced cirrhosis and in 1 (25%) of HCC cases that are associated with cryptogenic cirrhosis with no significant difference.

Additionally, aberrant β-Catenin immunoreactivity was significantly associated with higher tumor stages (stages II, III and IV) according to AJCC ($p = 0.015$) (Fig. 2).

Wnt2 IHC staining in studied HCC cases

Positive Wnt2 IHC staining was detected in 118/121 (97.5%) of HCC cases (cytoplasmic and membranous). All of the positive cases ranged from mild to strong staining intensity (scores 1–3), 20 cases show mild staining (score 1), 56 cases show moderate staining (score 2) and 42 cases show strong staining (score 3) (Fig. 1).

Regarding demographics, there was no significant correlation between Wnt2 IHC staining when it came to age or sex of the patient ($p > 0.05$).

As shown in Table 3, Wnt2 IHC staining showed no significant association with either site, size of the tumor, or number of tumor nodules in the studied HCC cases. Also there was no significant association between Wnt2

Table 1 RNAscope scoring system for CTTNB1 detected by CISH

Staining score	Microscope objective scoring
0	No staining or less than 1 dot to every 10 cells (40X magnification)
1	1–3 dots/cell (visible at 20–40X magnification)
2	4–10 dots/cell. Very few dot clusters (visible at 20–40X magnification)
3	> 10 dots/cell. Less than 10% positive cells have dot clusters (visible at 20X magnification)
4	> 10 dots/cell. More than 10% positive cells have dot clusters (visible at 20X magnification)

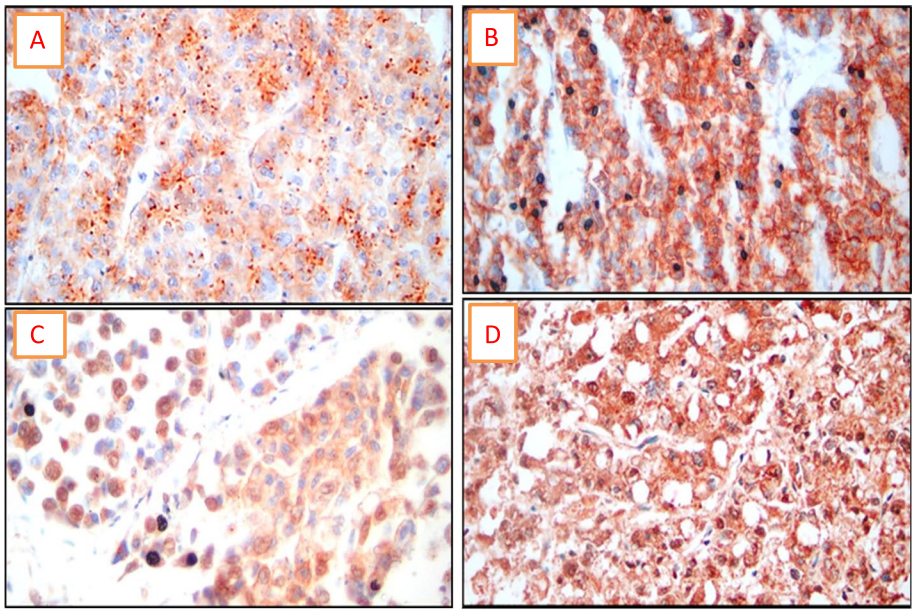

Fig. 1 Grade I HCC, β Catenin IHC staining, dot like cytoplasmic pattern, score 2, (×400) (**A**). Grade I HCC. B-Catenin staining, cytoplasmic, score 3(×400) (**B**). Grade II HCC. β Catenin staining, cytoplasmic and nuclear, score 2,(×400) (**C**). Grade II HCC, Wnt2 IHC staining, cytoplasmic, score 3 (×400) (**D**)

staining and the presence of LVE, tumor necrosis, tumor grade, TNM stage, alpha fetoprotein level and presence of local recurrence.

Positive Wnt2 staining was detected in 111/114 (97.4%) of HCC cases that are associated with HCV- induced cirrhosis and in all HCC cases that are associated with HBV- induced cirrhosis and cryptogenic cirrhosis.

CTNNB1 mRNA expression (by CISH)

Positive CTNNB1 mRNA expression was detected in 76 (62.8%) cases. Score of positivity ranged from 1 to 4 (Fig. 3).

No significant association was detected between CTNNB1 mRNA expression and either age, sex, or alpha-fetoprotein level of the studied HCC cases ($N=121$).

As shown in Table 4, a significant association was seen between CTNNB1 mRNA expression and larger tumor sizes (>5cm) ($p=0.041$). There was a significant association between CTNNB1 mRNA expression and higher stages of the tumors (Stages III and IV) ($p=0.005$). There was also a significant association between CTNNB1 mRNA and the presence of distant metastasis ($p=0.008$). No significant association between CTNNB1 mRNA expression and the presence of LVE, tumor necrosis, tumor grade or presence of local recurrence (Table 4).

Positive CTNNB1 mRNA expression was detected in 69 (60.5%) cases of HCC that are associated with HCV-induced cirrhosis, 33.3% of HCC cases that are associated with HBV- induced cirrhosis, and in 50% of HCC cases that are associated with cryptogenic cirrhosis.

There was no significant association between CTNNB1 mRNA expression and the AJCC stage of the studied HCC cases (Fig. 4).

Correlation between aberrant β-Catenin, Wnt2, and CTTNB1 expression

As shown in Table 5, aberrant β-Catenin expression showed a highly significant correlation with CTNNB1 mRNA expression ($p=<0.0001$) but not with Wnt2 expression. No significant correlation was detected between Wnt2 expression and CTNNB1 mRNA expression.

Discussion

Many signaling pathways are well established to be implicated in HCC, including the Wnt/β-catenin pathway (up to 50—60% of HCC) (Wang et al. 2015; Liu et al. 2016).

In this study, there was a great interest to clarify this relation and to understand the importance of the Wnt/β-Catenin pathway and the CTNNB1 gene mutation in hepatocellular carcinoma and its relation with different clinical and histopathological characteristics of the tumors in a cohort from gastroenterology center, Mansoura, Egypt.

This study was conducted on 121 explanted HCC cases. In the current study, aberrant β-catenin immunoreactivity was detected in 59.5% of HCC cases. All of the cases (100%) were equal to or above 40 years old,

Table 2 Relation between aberrant β-Catenin expression with different clinicopathological parameters of studied HCC cases

	Right lobe		**Left lobe**		***P* value**
Site	**Number**	**SD ± Mean**	**Number**	**SD ± Mean**	
β-Catenin	90	1.01 ± 0.93	31	0.90 ± 1.01	0.47
	< = 5 cm		> 5 cm		
Size	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	94	0.85 ± 0.90	27	1.43 ± 0.98	0.011*
	Single		Multiple		
Number of nodules	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	75	0.81 ± 0.87	46	1.26 ± 1,03	0.021*
	Grade I		Grade II		
Tumor grade	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	88	1.02 ± 0.93	33	0.93 ± 1.02	0.31
	Stage I&II		Stage III&IV		
T	Number	SD ± Mean	Number	SD ± Mean	
β-Catenin	116	0.95 ± 0.92	5	2.05 ± 1.01	0.03*
	Positive LN		Negative LN		
N	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	3	0.43 ± 0.75	118	0.99 ± 0.95	0.29
	Metastasis		No metastasis		
M	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	4	0.98 ± 0.95	117	0.98 ± 0.87	0.92
	Present		Absent		
Tumor necrosis	Number	SD ± Mean	Number	SD ± Mean	
β-Catenin	41	0.98 ± 0.88	80	0.98 ± 0.99	0.86
	Present		Absent		
LVE	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	30	1.24 ± 0.92	91	0.85 ± 0.89	0.034*
	Absent		Present		
Local recurrence	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	114	1.06 ± 0.69	7	0.98 ± 0.96	0.71
	< = 15 ng/ml		> 15 ng/ml		
AFP level	Number	Mean ± SD	Number	Mean ± SD	
β-Catenin	51	0.98 ± 0.93	70	1.00 ± 0.99	0.99

* Significant association, *p* value < 0.05

with no significant relation with either age or sex. Lee and his colleagues found that aberrant β-catenin expression in HCC cases was about 72.9% (Lee et al. 2014). In another study on 35 HCC cases, they found that aberrant β-Catenin is expressed in 17% of HCC cases, with most cases (83.3%) above the age of 60 (Rahadiani et al. 2021). The frequency was lower mostly because of the different number of cases in that study. An Egyptian study on 30 HCC cases compared IHC staining of β-Catenin in HCCs to normal liver tissue and found cytoplasmic and/or nuclear β-catenin immunostainig in 25/30 (83%) of the cases (Ashmawy et al. 2017). The frequency in this study was higher than the current study, mostly because of the lower number of cases in their study.

In this study, a higher frequency of aberrant β-Catenin expression in tumors with higher AFP levels (> 15 ng/ml) was noticed, but without a significant difference ($p = 0.99$). Against the results of the current study, Rebouissou & Nault concluded that HCC cases with β-catenin mutations showed lower levels of serum α-fetoprotein (Rebouissou and Nault 2020).

In the current study, aberrant β-Catenin expression was significantly higher with tumors of larger size (> 5 cm) ($p = 0.01$ respectively), multiplicity of tumor nodules ($p = 0.02$), lymphovascular invasion ($p = 0.03$) and tumors with distant metastasis ($p = 0.008$). In agreement with current results, Khalaf et al. reported that HCC cases with nuclear expression of β-catenin showed increased microvascular and macrovascular

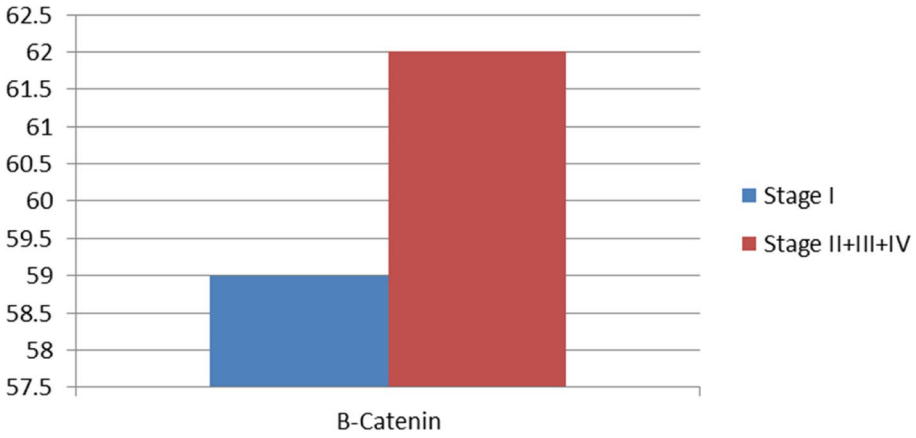

Fig. 2 Relation between aberrant β -Catenin expression and AJCC staging of the tumor

invasion (78%) compared with HCC without β-catenin mutation (38%). Also these tumors have larger tumor size, multiple tumor nodules and a higher proliferation rate in comparison with tumors lacking β-catenin mutation (Khalaf et al. 2018; Zhang et al. 2021). Another study showed no significant correlation with vascular invasion (Rahadiani et al. 2021).

The current results showed that aberrant β-Catenin is not significantly correlated with tumor grade. In contrast, Ingawa and his colleagues found that aberrant β-catenin expression is higher in poorly differentiated HCC (Inagawa et al. 2002). This can be explained by the fact that all the current study`s cases are well and moderately differentiated. So, the relationship between aberrant β-catenin expression and poorly differentiated as well as undifferentiated tumors needs to be further studied. Others reported that aberrant β-Catenin staining was associated with smaller tumors and well to moderately differentiated tumors (66.7%) (Rahadiani et al. 2021). This may be explained on the basis of ethnic diversity, tumor heterogeneity, and the different number of cases.

Additionally, a significant relation between aberrant β-Catenin expression and higher tumor stages according to TNM (StagesIII and IV) ($p=0.03$) and AJCC staging (Stages II, III, and IV versus Stage I) ($p=0.015$) was observed in the current study.

Another study showed positive correlations between the invasive character of HCC, high metastatic potential, poorer cellular differentiation, poor prognosis, and shorter survival present with nuclear or cytoplasmic expression of β-catenin (Deldar Abad Paskeh et al. 2021). Aberrant β-catenin expression is considered a negative prognostic factor for overall survival and may represent a promising therapeutic target (Han and Lim 2020).

In another context, of the reported 19 Wnt ligands, Wnt1, Wnt2, Wnt3a, Wnt5a, and Wnt10 were implicated in liver carcinogenesis. Wnt5A through a non-canonical pathway and the rest through a canonical pathway (Dong et al. 2019). Upregulation of Wnt2 is likely to be an early event during tumorigenesis (Park et al. 2009). Wnt2 expression is associated with cell survival, metastasis, and tumor invasion through the canonical pathway. In cancer cells, Wnt2 reprograms tumorigenic liver progenitor cells to replicate fibrogenic myofibroblast-like cells. These cells display stem and invasive features in HCC (Bravo et al. 2013; Désert et al. 2018).

In the current study, positive Wnt2 staining is detected in 97.5% of HCC cases. Wnt2 protein is more expressed in old age patients, mostly males, in tumors at the right lobe, in tumors of large size, in tumors with multiple nodules, in tumors with negative LN and no metastasis, in advanced stages of the tumors, and in tumors associated with lower levels of alpha-fetoprotein and hence associated with poor prognosis of the tumor. But all the aforementioned relationships did not reach a statistical significance.

Dong and his colleagues conducted a study on 360 HCC cases, which reported Wnt2 protein expression in 164/360 (45%). All of these positive cases were associated with older age of the patient, larger tumor size, multiple tumor nodules, advanced TNM stage of the tumor, and poor prognosis of the tumor, which goes with the current results (Dong et al. 2019).

CTNNB1 is the gene coding for β-catenin and is one of the most commonly mutated genes in HCC. It is mutated in about 11–37% of HCC cases (Chiang and Villanueva 2017; Zucman-Rossi et al. 2015).

CTNNB1 mRNA expression was detected in 62.9% of the current study`s HCC cases. Javanmard et al. reported that CTNNB1 mutation was detected in 18.1% of HCC cases (Javanmard et al. 2020). The results of the current study were significantly higher. This may be

Table 3 Relation between Wnt2 expression and different clinicopathological parameters of studied HCC cases

	Right lobe		**Left lobe**		***P* value**
Site	**Number**	**SD ± Mean**	**Number**	**SD ± Mean**	
Wnt2	90	1.65 ± 0.61	31	1.69 ± 0.55	0.9
	< = 5 cm		> 5 cm		
Size	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	94	1.68 ± 0.61	27	1.79 ± 1.02	0.98
	Single			Multiple	
Number of nodules	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	75	1.70 ± 0.61	46	1.60 ± 0.58	0.37
	Grade I		Grade II		
Tumor grade	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	88	1.66 ± 0.60	33	1.62 ± 0.58	0.73
	Stage I&II		Stage III&IV		
T	Number	SD ± Mean	Number	SD ± Mean	
Wnt2	116	1.67 ± 0.59	5	1.26 ± 0.43	0.13
	Positive LN		Negative LN		
N	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	3	1.20 ± 0.34	118	1.67 ± 0.58	1.69
	Metastasis		No metastasis		
M	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	4	1.10 ± 0.17	117	1.68 ± 0.58	0.09
	Present		Absent		
Tumor necrosis	Number	SD ± Mean	Number	SD ± Mean	
Wnt2	41	1.58 ± 0.64	80	1.70 ± 0.55	0.3
	Present		Absent		
LVE	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	30	1.79 ± 0.52	91	1.61 ± 0.60	0.14
	Absent		Present		
Local recurrence	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	114	1.65 ± 0.60	7	1.71 ± 0.41	0.89
	< = 15 ng/ml		> 15 ng/ml		
AFP level	Number	Mean ± SD	Number	Mean ± SD	
Wnt2	51	1.75 ± 0.56	70	1.60 ± 0.60	0.25

explained by the fact that this study`s cases were HCCs associated with cirrhosis, mainly related to viral infection while cases in their study were non-cirrhotic non-viral HCCs, and CTNNB1 mutations were described as prevalent in cirrhotic HCCs, also due to ethnic diversity and tumor heterogeneity.

Some studies reported that the CTNNB1 mutation was more likely to occur in older than younger age groups (> 40 years) and with male predominance (Lu et al. 2014; Ang et al. 2017), which supports current results.

On the other hand, another study performed in Italy reported a significant relationship between CTNNB1 mRNA expression and younger patients (< 40 years) (Tornesello et al. 2013), This may be due to genetic and ethnic diversity.

In this study, a higher frequency of the CTNNB1 mRNA expression was noticed in tumors with lower AFP levels (≤ 15 ng/ml) (63%), but without the presence of a significant difference. Other studies reported a higher frequency of CTNNB1 mutation in tumors with lower AFP levels, with no significant difference (Rebouissou and Nault 2020; Pezzuto et al. 2016).

In the present study, a significant correlation was discovered between the CTNNB1 mRNA expression and larger tumor size (> 5cm), the presence of LVE, distant metastasis, higher tumor stages according to TNM (Stages III and IV) ($p = 0.005$) and AJCC staging (Stages II, III, and IV versus Stage I). These findings support the

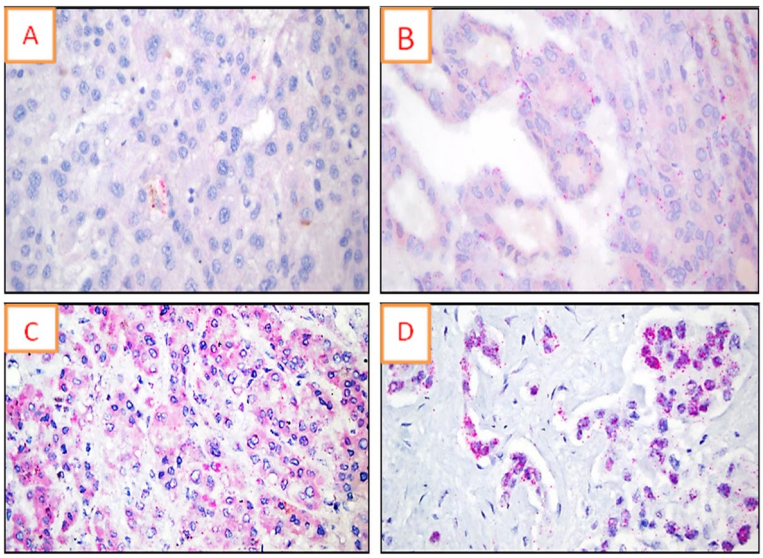

Fig. 3 Grade II HCC. CTNNB1 staining, score 1 (×400) (**A**) Grade I HCC. CTNNB1 staining, score 2 (×400) (**B**) Grade II HCC. CTNNB1 staining, score 3 (×400) (**C**) Grade II HCC. CTNNB1 staining, score 4 (×400) (**D**)

idea that the CTNNB1 mutation is associated with a poor prognosis in HCC.

The CTNNB1 mRNA expression was higher in tumors at the right lobe, in tumors with negative LNs, in low grade tumors, specifically in well differentiated tumors than in moderately differentiated tumors and in tumors with no local recurrence after transplantation. But all these differences did not reach statistical significance.

Some studies reported that the CTNNB1 gene mutation was associated with well differentiated tumors, small sized tumors, less local recurrence, and a good prognosis (Hoshida et al. 2009; Yuan et al. 2011; Tien et al. 2005). This discrepancy in some of the results might be explained by the ethnic diversity and biological heterogeneity of HCCs.

In accordance with current results, several studies have reported that HCC with CTNNB1 gene mutation in has been linked to a higher prevalence of HCV infection, an increased incidence of vascular invasion, a doubled tumor size, a multiplicity of nodules, and a poor prognosis (Khalaf et al. 2018; Cieply et al. 2009; Rebouissou et al. 2016).

Tornesello et al., reported that the CTNNB1 mutation was significantly associated with poorly differentiated tumors (Tornesello et al. 2013). However, all of the HCC cases in this cohort were of low grade (Grade I and II). So, this study could not predict association with grading and further studies on CTNNB1 mutation in poorly differentiated and undifferentiated HCC cases among Egyptian patients need to be performed.

Zucman-Rossi and his colleges have classified HCCs molecularly into two groups: the proliferative group (Class I and III) and the non proliferative group (Class II) (Zucman-Rossi et al. 2015 Oct). The proliferation group (Class I) is characterized by chromosomal instability, global DNA hypomethylation, TP53 mutations, overexpression of cell cycle-associated genes, poor histological differentiation, frequent LVE, high serum AFP levels, and a more aggressive prognosis (Calderaro et al. 2019). The non proliferative group (Class II) tumors are mostly caused by HCV, enriched in CTNNB1 mutation, associated with low AFP levels and less vascular invasion, chromosomal stability and carry better prognosis (Rebouissou and Nault 2020). CTNNB1-mutated cases in the current study are similar to those of non-proliferative (Class II) tumors except for being associated with the presence of LVE and poor prognosis. So, the HCC cases in this study have fallen into a gray zone that requires more studies and clarifications.

On the other hand, another study has classified WNT-activated HCCs according to their target downstream effector gene into two classes: the CTNNB1-activated class and the WNT-TGFB class (Lachenmayer et al. 2012). The CTNNB1 mutated class revealed CTNNB1 mutation in 62.5%, showed nuclear and/or cytoplasmic β-Catenin expression in 74% by IHC, and was associated with tumors > 5 cm (Lachenmayer et al. 2012). The current study reported CTNNB1 mutation in 62.9% of HCC cases, nuclear and/or cytoplasmic β-catenin expression in 80.2% of these cases, and was associated with a larger tumor size > 5 cm. So, WNT-activated HCC cases in the

Table 4 Relation between CTNNB1 mRNA expression and different clinicopathological parameters of studied HCC cases

	Right lobe		**Left lobe**		***P* value**
Site	**Number**	**SD ± Mean**	**Number**	**SD ± Mean**	
CTNNB1	90	1.09 ± 1.01	31	0.99 ± 0.89	0.76
	< = 5 cm		> 5 cm		
Size	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	94	1.01 ± 0.96	27	1.41 ± 0.89	0.04#
	Single		Multiple		
Number of nodules	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	75	0.98 ± 1.02	46	1.19 ± 0.89	0.16
	Grade I		Grade II		
Tumor grade	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	88	1.10 ± 0.96	33	0.95 ± 10.03	0.44
	Stage I & II		Stage III & IV		
T	Number	SD ± Mean	Number	SD ± Mean	
CTNNB1	116	1.02 ± 0.94	5	2.42 ± 0.75	0.005#
	Positive LN		Negative LN		
N	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	3	0.63 ± 0.55	118	1.07 ± 0.98	0.53
	Metastasis		No metastasis		
M	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	4	2.52 ± 0.83	117	1.01 ± 0.94	0.008#
	Present		Absent		
Tumor necrosis	Number	SD ± Mean	Number	SD ± Mean	
CTNNB1	41	0.94 ± 0.92	80	1.12 ± 1.00	0.35
	Present		Absent		
LVE	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	30	0.86 ± 0.76	91	1.13 ± 1.03	0.3
	Absent		Present		
Local recurrence	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	114	1.20 ± 0.99	7	1.05 ± 0.98	0.59
	< = 15 ng/ml		> 15 ng/ml		
AFP level	Number	Mean ± SD	Number	Mean ± SD	
CTNNB1	51	1.08 ± 1.02	70	1.05 ± 0.95	0.9

[#] Significant association, p value < 0.05

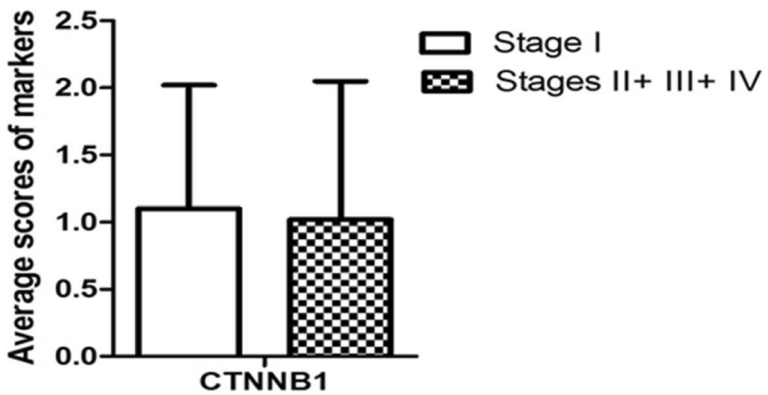

Fig. 4 Relation between CTNNB1 mRNA expression and AJCC staging of studied HCC cases

Table 5 Correlation between aberrant β-Catenin, Wnt2, and CTNNB1 in HCC cases

		β-Catenin	Wnt2	CTNNB1
β-Catenin	Rs		0.006	0.4701
	P		0.94	< 0.0001 *
Wnt2	Rs	0.006		0.1015
	P	0.94		0.2261
CTNNB1	Rs	0.4701	0.1015	
	P	< 0.0001 *	0.2261	

Rs Spearman correlation, *P p*-value

*Significant association, *p* value <0.05

current study belong to the CTNNB1 category according to Lachenmayer`s classification. The CTNNB1 mutation in Wnt2 activated HCCs was also associated with advanced stages of cancer (Stages III and IV) (Wang et al. 2018) which is consistent with current results.

Additionally, among the aberrant β-Catenin HCC cases in this cohort (59.5%), 61 cases (84.7%) had a concomitant CTNNB1 mRNA expression by CISH, and the remaining 11 cases (15.3%) showed no concomitant CTNNB1 mRNA expression, suggesting its activation by tyrosine phosphorylation or other pathways that need further studies. There was a highly significant correlation between the CTNNB1 mRNA expression and aberrant β-Catenin expression in both all HCC cases and in HCC cases assocaited with HCV-induced cirrhosis. This can be explained logically by the fact that the CTNNB1 gene mutation is the main driver causing activation of the canonical Wnt/β-catenin pathway with subsequent accumulation of β-catenin in both the cytoplasm and nucleus of malignant hepatocytes.

Conclusion

The aberrant β-Catenin expression in hepatocellular carcinoma correlates significantly with number of tumor nodules, tumor stage, and the presence of LVE.

The CTNNB1 gene mutation is highly correlated with larger tumor size (> 5cm), the presence of LVE, distant metastasis, and higher tumor stages. All of these items confer poor prognosis. A highly significant correlation was found between CTNNB1 gene mutation and aberrant β-Catenin expression in HCC cases. It can be concluded from current results that the CTNNB1 mutation and aberrant β-Catenin expression are associated with poor prognosis in patients with HCC.

Authors' contributions

Ramy A. Abdelsalam M.D, Ibrahim El-Shawwaf M.D, Azza Abdel-Aziz M.D, Tarek A.Bismar Ph.D, Shaimaa M. Yussif M.D. The authors confirm contribution to the paper as follows: study conception and design: Ibrahim M El-Shawaf, Azza Abdel-Aziz, Ramy A. Abdelsalam; data collection: Ramy A. Abdelsalam, Shaimaa M. Yussif; analysis and interpretation of results: Tarek A.Bismar, Shaimaa M. Yussif, Ramy A. Abdelsalam; draft manuscript preparation: Azza Abdel-Aziz, Ramy A. Abdelsalam. All authors reviewed the results and approved the final version of the manuscript.

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Data availability

The data and materials that support the findings of this study are available from the corresponding author, upon request.

Declarations

Ethics approval and consent to participate

Obtained from Institutional Research Board (IRB) at Faculty of Medicine, Mansoura University,Egypt.This research was done on tissue optained from liver explant of HCC cases that went for liver transplantation after accepatance of the consent for operation.

Competing interests

The authors declare that no relevant financial affiliations or conflicts of interest to disclose.

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