Expression of β-Catenin in Hepatocellular Carcinoma

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Abstract: Alteration of β-catenin expression is involved in the development and evolution of hepatocellular carcinoma (HCC); β-catenin is able to influence tumor cell proliferation. We analyzed the immunohistochemical (IHC) expression of β-catenin on a group of 32 patients diagnosed with HCC using the anti-β-catenin monoclonal antibody (clone E247). We correlated the expression of β-catenin with the proliferation index of Ki-67 (PI Ki-67), the mitotic index (MI) and other clinical and pathological features. We observed an altered β-catenin expression in 58.38% of all HCC cases. This expression was insignificantly correlated with tumor size (>5 cm) (p = 0.683), histological grade G1-G2 (p = 0.307), vascular invasion (p = 0.299) and advanced pT stage (p = 0.453); we obtained a significantly higher MI in HCC with altered β-catenin expression (p = 0.018), as compared to HCC without overexpression (1.66 ± 1.37) (p = 0.038) and a PI Ki-67 of 22.49 ± 20.1 and 28.24 ± 18.2, respectively in tumors with altered β-catenin expression with insignificant differences compared to HCC without overexpression (25.95 ± 15.2) (p = 0.682 and p = 0.731, respectively). According to the results we obtained, aberrant β-catenin expression in HCC was correlated with a high mitotic index, therefore playing an important role in tumor progression by stimulating tumor cell proliferation; non-nuclear β-catenin overexpression can have a pathological significance in HCC, especially in cases of HCC associated with hepatitis B virus (HBV) infection.

Keywords: hepatocellular carcinoma, β-catenin, immunohistochemistry, PI Ki-67, mitotic index

1. Introduction

Hepatocellular carcinoma (HCC) is the most frequent hepatic malignant tumor, representing around 85–90% of primary liver cancers. The incidence of HCC is on the rise, having also a high cancer-related mortality [1].

β-catenin is a multifunctional protein subunit from the Cadherin family, playing an important role in the E-cadherin/β-catenin complex [2], involved in maintaining cellular adhesion [3, 4], its loss leading

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to tumor invasion and metastasis [5, 6]. E-cadherin and β-catenin have a close relationship with the invasion capacity of cancer, as well as with β-catenin abnormal expression in the early stages of HCC development [2].

Furthermore, β-catenin plays a key role in the Wnt signaling pathway, as transcription activator [7, 8]. Among the molecular signaling pathways implicated in the pathogenesis of HCC, the Wnt/β-catenin signaling pathway is one of the most frequently activated (up to 50% of HCC) [1]. The inadequate activation of this pathway leads to the elevation of β-catenin level that can translocate to the nucleus, stimulating cell proliferation, being involved in the process of carcinogenesis [2, 9]. The Wnt/β-catenin pathway has shown significant promise as a potential target for novel molecular therapies [1].

Alteration of β-catenin expression is also involved in the development of HCC. β-catenin immunoexpression in normal hepatic tissue presents a distinct membranous staining. Activation of β-catenin gene mutations (CTNNB1) leads to a disorder and redistribution of β-catenin protein, with a strong cytoplasmic and nuclear immunoreactivity and cyclin-D1 gene overexpression, one of the target factors of β-catenin [10, 11].

In cancer, low E-cadherin and β-catenin expressions are characteristic for the invasive phenotype, being considered as tumor suppressor genes, the partial or complete loss of E-cadherin or β-catenin expression being correlated with poor prognosis in malignant tumors. In hepatic carcinomas, E-cadherin and β-catenin expressions were inversely correlated with the degree of tumor differentiation, being considered as useful markers for the differentiation of HCC. Endo et al. [12] identified α-, β- and γ-catenin overexpression, inversely proportional with histological grade in most analyzed HCCs and a positive significant correlation between the intense β-catenin expression and vascular invasion. Ihara [13] and Endo [12] described overexpression of E-cadherin and β-catenin in most HCCs, E-cadherin being able to act as a modulator in maintaining the histological architecture of HCC.

To evaluate the relationship between β-catenin expression and tumor cell proliferation, we analyzed the immunohistochemical (IHC) expression of β-catenin in 32 patients with surgically resected HCCs; furthermore, we examined the relationship between β-catenin expression and Ki-67 proliferation index (PI Ki-67), mitotic index (MI) and other clinical and pathological parameters (associated viral infection, tumor size, histologic grade, vascular invasion and stage).

2. Materials and methods

This paper represents a retrospective analysis of a group made up of 32 HCCs, diagnosed in the Pathology Department (PD) and Surgery Clinics of the Emergency County Clinical Hospital Timișoara (ECCHT). The cases were selected after consulting the records and database of the PD and patients’ medical records. We analyzed the main information about the patients (sex, age) and the tumors (localization, dimensions, histological grade and stage).

The primary processing of surgical resection pieces was made after sectioning of the blocks, fixation in 10% formalin for 24-48 hours, paraffin inclusion using the standard technique (washing, dehydration, clearing, inclusion), followed by sectioning at 4-5 µ (multiple seriated sections being made for each case).

For the usual morphological investigation, the sections were stained with hematoxylin-eosin (HE); their microscopic examination allowed the determination of the following parameters: diagnosis of the primary tumor, tumor histological type according to the World Health Organization (WHO) classification (2010) [14], differentiation grade – G, local tumor extension – pT, status of regional lymph nodes – pN, lymphatic-vascular L-V and peri-/intraneural invasion, status of surgical resection limits – R.

Immunohistochemical staining

We examined the expression of β-catenin using the monoclonal antibody anti β-catenin (clone E247). A representative tissue block was selected from each case and additional sections were cut (4µm
thick); the sections were mounted on Superfrost Ultra Plus slides or silanized in order to avoid their detachment during pretreatment procedures. To improve antigen retrieval, sections were pretreated by boiling for 20 min in Retrieval solution at pH = 9 and then incubated with the primary anti β-catenin antibody (clone E247) (1:100 dilution) for 20 min at room temperature.

For the visualization of the reaction we used the diamino-benzidine (DAB) chromogen and the nuclei were counter-stained with Mayer hematoxylin. We obtained a membranar, cytoplasmic and/or nuclear staining pattern in shades of brown. For the positive control of the reaction, we used sections from a β-catenin positive mammary carcinoma.

β-catenin expression was observed in the membrane, cytoplasm and/or nucleus.

The patterns of β-catenin expression were divided into 3 groups [15], as follows:

1) nuclear expression, with nuclear staining in over 20% of tumor cells ± cytoplasmic expression (also called ectopic expression);

2) non-nuclear overexpression, with elevated membranar and/or cytoplasmic staining in at least 50% of tumor cells, without nuclear staining;

3) no overexpression, with a weak membranar β-catenin staining, similar to surrounding non-neoplastic hepatocytes.

We considered as HCC with altered β-catenin expression the cases with nuclear expression or non-nuclear overexpression, while the cases with normal expression were those with no overexpression.

We assessed the proliferative activity of the studied hepatic carcinomas in the LSAB immunostaining system, using the monoclonal mouse antibody anti-Ki67, clone MIB-1. After pretreatment by boiling for 60 minutes at 90°C in Retrieval solution (Dako Target Retrieval Solution) at pH 6, the analyzed tissue sections were incubated with the MIB-1 diluted antibody for 40 minutes (MIB-1, Dako, Glostrup, Denmark); we obtained a nuclear immunostaining, the final reaction product being brown when visualized with DAB.

Quantification of the reaction was made by assessing the Ki-67 proliferation index, expressed as a percentage result of the number of Ki-67 positive cells from 500 tumor cells.

The mitotic index was calculated from the number of mitotic cells per 10 random microscopic fields ("hot spot") at high magnification (x400). The mean value of mitotic cells counted on each microscopic field was defined as the mitotic index.

Statistical analysis

Data analysis was made using the SPSS software (version 10.0, SPSS INC, Chicago, IL, USA) and EpiCalc2000. The significance was defined as p<0.05.

β-catenin expression in relation with pathological parameters was examined with the significance test Pearson χ² and the comparisons between mean values of the MI were made using the unpaired t test. Standard deviation (SD) allowed the assessment of individual value repartition around the mean value.

Ethics surrounding the scientific research

All procedures undergone in this study (histopathological analysis and microscopic images from the slides) had patient approval and a written informed consent was signed for the use of biological material in research studies (Annex no.4 for the 112/2013 law). For the study of cases and access to the database, we had consent from the heads of the Department of Pathology and the Clinics involved in this study.

3. Results and discussions

Clinical characteristics of patients with HCC and the evaluated morphological parameters showed that 12 (37.5%) of the 32 patients with HCC were seropositive for HBs antigens and only 4 (12.5%) presented anti-hepatitis C virus (HCV) serum antibodies. 12 (37.5%) patients were men and 20 (62.5%) women and their age varied between 21 and 69 years old (mean age 56.4 years). Twelve patients (37.5%)
presented non-tumor liver with cirrhosis, 8 patients (25%) had viral chronic hepatitis, while the other 12 cases (37.5%) had other associated pathologies.

In all cases, we examined conventional pathological parameters. The histological degree of tumor differentiation was assigned according to the modified Edmondson–Steiner grading system for hepatocellular carcinoma [16]. After grading, tumors were classified into 2 groups: well differentiated (grade I+II) (n = 30) and poorly differentiated (grade III+IV) (n=2). The size of the 32 HCCs varied between 1.0 and 7.5 cm (with a mean size of 3.7 cm); according to size, the tumors were divided into HCC<5 cm (n = 4; 12.5%) and HCC ≥5 cm (n = 28; 87.5%).

The stage of the tumors was established using the criteria of the American Joint Committee on Cancer (AJCC) Staging System (2017) [17]: stage 1 (n = 3), stage 2 (n = 11), stage 3 (n = 14) and stage 4 (n = 4).

In non-neoplastic hepatic tissues, β-catenin was expressed moderately and diffusely at membrane level, with an associated light/moderate cytoplasmic staining of hepatocytes (Figure 1a). Intrahepatic bile ducts and proliferated bile canals showed a more intense β-catenin expression of the cell membrane (Fig. 1b-d). The intensity of membranar staining of β-catenin decreased gradually from the non-neoplastic tissue towards the tumor, HCC showing abnormal protein expression in the cytoplasm and the nucleus.

Figure 1. β-catenin immunohistochemistry: a. β-catenin expression in non-tumor liver showing moderate membrane and cytoplasmic staining; b-d. The bile ducts served as internal positive control; hepatocytes showing moderate cytoplasmic staining.

In HCC, altered β-catenin expression, including the two distinct patterns (nuclear expression and non-nuclear overexpression) was observed in 19/32 cases (59.38%). Of the two patterns of altered expression, non-nuclear overexpression (Figure 2a-d) was more frequently encountered (13/19 cases; 68.42%) and more significant (pathologically) than nuclear expression (6/19 cases; 31.58%) (p = 0.042) (Chart 1and Table 1).

In 13/19 cases (68.42%) with non-nuclear overexpression, β-catenin expression was observed in the cytoplasm, especially near the cell membrane (with no nuclear expression), with an evidently higher intensity than in non-neoplastic cells (Figure 2).
In 6/19 cases (31.58%) with nuclear expression, nuclear immunostaining was present in >20% of neoplastic cells and was associated with considerable cytoplasmic expression.

We did not observe β-catenin overexpression in 13 of the 32 cases (40.62%), although none of the tumors were completely negative to β-catenin. According to their associated viral status, altered β-catenin expression was noted in 9 of the 12 HCCs associated with HBV infection (75%) and 1 of the 4 tumors associated with hepatitis C virus (25%).

![Fig. 2. Aberrant expression of β-catenin in HCC: a-b. Tumor cells showing nuclear accumulation and strong cytoplasmic staining; c-d. nuclear accumulation of β-catenin protein in tumor cells of a tumor emboli within a vein](image)

**Chart 1.** Altered β-catenin expression.

We did not observe any significant correlation between nuclear β-catenin expression and the analyzed pathological features (Table 1). Tumors with altered β-catenin expression correlated insignificantly with large tumor sizes (>5cm) (p=0.683), vascular invasion (p=0.299), tumor histological grade G1-G2 (p=0.307) and advanced pT stage (p=0.453). We noted a positive but statistically
insignificant relationship between tumors with non-nuclear overexpression and invasion of the portal vein (p=0.061).

Table 1
THE RELATIONSHIP BETWEEN B-CATENIN AND CLINICAL-MORPHOLOGICAL ASPECTS

| Variables                     | n (32) | Altered expression | Non-nuclear overexpression | Nuclear expression |
|-------------------------------|--------|--------------------|-----------------------------|-------------------|
|                               |        | present/absent     | present/absent              | present/absent    |
| Associated viral infection    |        |                    |                             |                   |
| HBV+                          | 12     | 9/3                | 7/5                         | 2/10              |
| HCV+                          | 4      | 1/3                | 1/3                         | 0/4               |
| *                             | 16     | 9/7                | 11/4                        | 4/12              |
| Tumor size                    |        |                    |                             |                   |
| <5 cm                         | 4      | 2/2                | 2/2                         | 0/4               |
| ≥5 cm                         | 28     | 17/11              | 11/6                        | 6/22              |
| Vascular invasion             |        |                    |                             |                   |
| Absent                        | 8      | 3/5                | 1/7                         | 2/6               |
| Present                       | 24     | 16/8               | 12/12                       | 4/20              |
| Histologic grade              |        |                    |                             |                   |
| I+II                          | 30     | 19/11              | 13/17                       | 6/24              |
| III+IV                        | 2      | 0/2                | 0/2                         | 0/2               |

The average MI in HCC was 2.4 ± 2.2 (range 0.1-9). MI showed a strong correlation with the presence of portal vein invasion (p = 0.021\textsuperscript{S}) and advanced stage (p=0.01\textsuperscript{S}), but there was no correlation with poor histological grade (p = 0.302\textsuperscript{NS}) and large tumor size (p = 0.125\textsuperscript{NS}) (Table 2).

According to the patterns of \(\beta\)-catenin expression (Table 3), mean MI (±SD) of HCC with nuclear expression (3.21 ± 3.03) and non-nuclear overexpression (2.74 ± 2.5) was significantly higher than that of tumors without overexpression (1.66 ± 1.37) (p=0.018 and p=0.038, respectively). Mean MI of HCC with nuclear expression (3.21 ± 3.03) was slightly higher than that of the cases with non-nuclear overexpression (2.74 ± 2.5), but without statistical significance (p = 0.725).

Table 2
THE RELATIONSHIP BETWEEN MI MEAN VALUES AND CLINICAL-MORPHOLOGICAL FEATURES

| Variables                     | No. cases | MI mean value | p     |
|--------------------------------|-----------|---------------|-------|
| Tumor dimension               |           |               |       |
| <5 cm                         | 4         | 1.36±1.75     | 0.125\textsuperscript{NS} |
| ≥5 cm                         | 28        | 3.25±2.29     |       |
| Portal vein invasion          |           |               |       |
| Absent                        | 8         | 1.1±1.26      | 0.021\textsuperscript{S} |
| Present                       | 24        | 3.2±2.3       |       |
| Histological grade            |           |               |       |
| I+II                          | 30        | 1.7±2.23      | 0.302\textsuperscript{NS} |
| III+IV                        | 2         | 3.41±2.16     |       |
| Stage                         |           |               |       |
| T1                            | 3         | 0.42±0.32     | T1-T3 0.006\textsuperscript{S} |
| T2                            | 11        | 1.32±1.12     | T1-T4 0.03\textsuperscript{S} |
| T3                            | 14        |               |       |
β-catenin plays an essential role in regulating the cell adhesion E-cadherin-catenin complex, as an activator of transcription [4, 19]. Normal immunoeexpression of β-catenin shows a distinct membranar staining. Activation of β-catenin gene mutations (CTNNB1) leads to deregulation and redistribution of β-catenin protein, with intense cytoplasmic and nuclear immunoreactivity and the overexpression of the targeted gene, cyclin D1 [18].

β-catenin expression inside the cells is controlled by a multiproteic complex, β-catenin gene mutations leading to nuclear and/or cytoplasmic accumulation of β-catenin and consecutive transactivation of target genes TCF/LEF, a mechanism that was noted in many cancers [9, 20]. It was demonstrated IHC that the significance of subcellular localization of β-catenin is variable according to the type of tumor. In colorectal cancer, it appears that elevated cytoplasmic and nuclear expression is an independent predictor of reduced survival [21], while decreased or loss of β-catenin expression was correlated with poor prognosis in gastric or pancreatic adenocarcinoma [22, 23]. Until now, reported β-catenin gene mutations in HCC suggest an improper activation of the Wnt signaling pathway, showing a powerful association between β-catenin nuclear expression and gene mutation [24, 25].

Most of the previous studies examined primarily the pattern of β-catenin nuclear expression [10, 26]. In literature, there is contradictory data regarding the association of β-catenin with tumor progression and patient survival. β-catenin nuclear expression correlated significantly with increased Ki-67 and associated with poor prognosis [27].

The results published by Hsu [11] and Mao [24] demonstrated that mutant nuclear β-catenin expression correlated with early stage HCC and favorable prognosis. According to the results of recent studies [25, 27], nuclear β-catenin expression seems to correlate with tumor progression and poor prognosis. Aberrant Wnt/β-catenin signaling has been shown to be common in HCC tumors and to have significant clinical impact on tumor behavior, prognosis, and response to treatment. The accumulation of β-catenin in the nucleus and cytoplasm may also be associated with increased vascular invasion, cell proliferation, and more poorly differentiated tumors [1].

In our study, we obtained a moderate and diffuse β-catenin staining of non-tumor hepatocytes, with cytoplasmic and cell membrane expression and a gradual decrease in the intensity of the immunoreaction from non-tumor liver towards the tumor. Intrahepatic biliary ducts and proliferated biliary canals presented strong β-catenin expression in the cell membrane.

| β-catenin expression          | No. cases | MI mean value | p   | PI Ki-67       | p   |
|------------------------------|-----------|---------------|-----|---------------|-----|
| No overexpression            | 13        | 1.66 ± 1.3    | 0.018 | 25.95 ± 15.2 | 0.682 |
| Nuclear expression           | 6         | 3.21 ± 3.0    | 0.725 | 22.49 ± 20.1 | 0.543 |
| Non-nuclear overexpression   | 13        | 2.74 ± 2.5    | 28.24 ± 18.2 | 0.731 |

MI – mitotic index; PI Ki-67 – proliferation index Ki-67

We did not observe any relationship between β-catenin expression and PI Ki-67 (Table 3). HCC with altered β-catenin expression (nuclear expression and non-nuclear overexpression) had a PI Ki-67 of 22.49 ± 20.1 and 28.24 ± 18.2, respectively, with insignificant differences as compared to cases without overexpression (25.95 ± 15.2) (p = 0.682 and p = 0.731, respectively).

Table 3.

ASSOCIATION BETWEEN B-CATENIN EXPRESSION, MITOTIC INDEX (MI) AND Ki-67 PROLIFERATION INDEX (PI)
We identified altered β-catenin expression (with the two distinct staining patterns – nuclear expression and non-nuclear overexpression) in 19 of the 32 HCCs (58.38%), non-nuclear overexpression being found more frequently (13 cases; 68.42%) that nuclear expression (6 cases; 31.58%) (p = 0.042).

In the 13 HCCs (68.42%) with non-nuclear overexpression, altered β-catenin expression with cytoplasmic localization (without nuclear expression) had a more consistent intensity than in non-neoplastic hepatocytes. In 6 cases (31.58%), we noted nuclear β-catenin expression (accompanied by an evident cytoplasmic expression) in >20% of neoplastic cells.

We noted an altered β-catenin expression in 9 of the 12 HCCs associated with viral HBV (75%) and in 1 of the 4 carcinomas associated with hepatitis C virus (25%). We could not observe any statistically significant relationship between nuclear β-catenin expression and the evaluated pathological features. Altered β-catenin expression was more frequently encountered in large tumors (>5cm) (p = 0.683), with vascular invasion (p=0.299), grade G1-G2 (p=0.307) and advanced pT stage (p = 0.453), the results being statistically insignificant. Among tumors with non-nuclear overexpression, we observed a positive relationship with the invasion of the portal vein (p = 0.061) and tumor size (p = 0.683).

HCC with nuclear expression and non-nuclear overexpression of β-catenin had a MI significantly higher (3.21 ± 3.03 and 2.74 ± 2.5, respectively) than cases without overexpression (1.66 ± 1.37) (p=0.018 and p = 0.038, respectively). In HCC with nuclear expression, mean MI was slightly higher (3.21 ± 3.03) than in cases with non-nuclear overexpression (2.74 ± 2.5), but without statistical significance (p = 0.725).

We did not observe any relationship between β-catenin expression and PI Ki-67, tumors with nuclear expression and non-nuclear overexpression of β-catenin having a PI Ki-67 of 22.49 ± 20.1 and 28.24 ± 18.2, respectively, with insignificant differences as compared to cases without overexpression (25.95 ± 15.2) (p = 0.682 and p = 0.731, respectively).

Joo M. [15] found altered β-catenin expression (non-nuclear overexpression and nuclear expression) in 58.4% of HCCs and described significant correlations with large tumor dimensions, low histological grade and advanced disease stage, but did not note any relationship between β-catenin nuclear expression and pathological features of HCC; between the two patterns of altered expression, the authors identified non-nuclear overexpression as being more frequent than nuclear expression (37.7% vs. 20.8%).

Regarding proliferative activity, both nuclear expression and non-nuclear overexpression of β-catenin correlated significantly with high PI. Non-nuclear overexpression of β-catenin, as well as other immunohistochemistry differentiation patterns or tumor morphology related biochemical markers have been previously mentioned in many studies [10, 12, 27-34].

Wong et al. [10] reported that in HCCs associated with HBV, the non-nuclear pattern of β-catenin overexpression correlated significantly with poor prognosis factors, as we also observed in our study. According to our results, β-catenin non-nuclear overexpression seems to be more frequently encountered in HCCs with HBV and it can contribute to tumor progression by stimulating tumor cell proliferation.

Previous studies stated that nuclear expression of β-catenin stimulates tumor cell proliferation or tumor progression [27], while information regarding the effects of cytoplasmic β-catenin are little and unclear. Although the molecular mechanism of non-nuclear overexpression pattern stays uncertain, it is possible that it differs from that of the nuclear expression pattern for the following reasons: 1) it is known that subcellular distribution of β-catenin regulates its function; membrane-linked β-catenin mediates cell-cell adhesion, while cytoplasmic and nuclear protein expression is associated with an oncocgenic function; 2) in our study we reported a pattern of non-nuclear overexpression with predominantly cytoplasmic staining and an association between HCC with non-nuclear overexpression and vascular invasion (p=0.061). Similar results were also described by other studies, Joo M. et. al. [35] stating that this phenomenon can be related to the alteration of cell-cell adhesion, thus facilitating vascular invasion.

Other authors reported the presence of vascular invasion in HCC with elevated β-catenin expression, showing that dynamic decrease and increase of this cell adhesion molecule can be necessary for HCC progression [28].
The majority of the previous studies focused on the pattern of nuclear expression, showing a strong relationship between β-catenin nuclear expression in HCC associated with HCV infection [25, 26]. The HCCs examined in our study associated predominantly with HBV infection and non-nuclear β-catenin overexpression, similar aspects being noted by Wong CM [10] and Joo M [35]. Although this data is insufficient to suggest a close relationship between non-nuclear overexpression and HCC associated with HBV infection, non-nuclear overexpression can be frequently associated with HCC related with HBV.

4. Conclusions

Non-nuclear overexpression of β-catenin seems to have pathological and prognostic significance. The results we obtained showed a significantly higher mitotic index in HCC with altered β-catenin expression; β-catenin can play an important role in tumor progression by stimulating tumor cell proliferation. Although our results are not sufficient to prove a close relationship between non-nuclear overexpression of β-catenin and hepatic carcinomas associated with HBV, we consider that non-nuclear overexpression can be frequently found in HCC associated with HBV infection. The accumulation of β-catenin in the nucleus and cytoplasm may also be associated with increased vascular invasion, cell proliferation and more poorly differentiated tumors.

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