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ORIGINAL ARTICLE

Miki (Mitotic Kinetics Regulator) Immunoexpression in Normal Liver, Cirrhotic Areas and Hepatocellular Carcinomas: a Preliminary Study with Clinical Relevance

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of primary malignant tumor in the liver. One of the main features of cancer survival is the generalized loss of growth control exhibited by cancer cells, and Miki is a protein related to the immunoglobulin superfamily that plays an important role in mitosis. We aim to study protein expression levels of Miki in non-tumoral liver and 20 HCCs recruited from a Pathology Department. Clinical information was also obtained. A tissue microarray was performed, and immunohistochemical techniques applied to study protein expression levels of Miki. In normal liver, Miki was weakly expressed, showing nuclear staining in the hepatocytes. Cirrhotic areas and HCCs showed a variety of staining patterns. Most HCC samples showed positive expression, with three different staining patterns being discernible: nuclear, cytoplasmic and mixed. Statistical analysis showed a significant association between grade of differentiation, Ki-67 proliferative index, survival rates and staining patterns. This study has revealed the positive expression of Miki in normal liver, cirrhotic areas and HCCs. Three different staining patterns of Miki expression with clinical relevance were noted in HCCs.

Keywords Hepatocellular carcinoma · Miki · Immunohistochemistry · Liver · Immunoglobulin superfamily

Introduction

Hepatocellular carcinoma (HCC) is the most common type of malignant tumor in the liver and it is one of the most common malignancies worldwide [1]. Despite some improvements in diagnosis and treatment in recent years, prognosis of HCC still remains poor [2]. Replicative immortality is one of the hallmarks of cancer cells, meaning that they no longer respond to many of the signals that control cellular growth and death, thus they are able to evade programmed cell death. As a result, cancer cells divide more rapidly than their progenitors and become less dependent on signals from other cells. In the late stages, cancer cells break through normal tissue boundaries and metastasize to new sites in the body [3].

Miki (mitotic kinetics regulator) is a protein related to the immunoglobulin superfamily that plays an essential role in mitosis. It is the product of the LOC253012 gene and contains three domains suggestive of a cell surface protein: an extremely hydrophobic transmembrane domain-like region, a central region with homology to immunoglobulin superfamily cell adhesion molecules, and an N-terminal putative signal peptide [4]. However, Miki does not appear to be related to cell surface, but, rather, it has been localized close to centrosomes and spindles during mitosis [4], and in the perinuclear region (Golgi apparatus) during interphase. In addition, in the late G2 phase, coinciding with the fragmentation of the Golgi apparatus, the poly(ADP-ribosylation) of Miki promotes its

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translocation to centrosomes in the cytoplasm, which may stimulate centrosome maturation in the late G2/M phase [4, 5].

Using genomic sequence analysis, Asou et al. mapped the Miki gene to chromosome 7q21.2-q21.310, and found that knockdown of *Miki* results in abnormal nuclear morphology, delay or arrest of the prometaphase with deeply disturbed chromosome alignment, including chromosome scattering, and apoptosis [6]. Moreover, chromosomal deletion which includes this gene may be associated with myeloid leukemia and myelodysplastic syndrome in human patients [7].

Miki is also called Hepacam2 because of its, albeit weak, sequence similarity to Hepacam1 (hepatocyte cell adhesion molecule) which was originally identified as a tumor-suppressor in HCCs [8]. However, the role of Miki has not, to date, been analyzed in human solid cancers. The present study aims to address this gap by examining Miki expression in normal liver, hepatic cirrhosis and HCC.

Materials and Methods

Material

The following materials were purchased from the manufacturers indicated: Ultra View universal DAB kit, from Roche Ventana. A rabbit polyclonal antibody against to residues 51–159 of human Hepacam2 (Miki synonymous),from Sigma-Aldrich (St. Louis, MO). Multiple preliminary assays were conducted using various human tissues, including liver, in order to validate the antibody's effect as specific, selective, and reproducible [9]. Proliferation index Ki67, from Roche Ventana, was also determined (30–9; rabbit monoclonal antibody).

Patients and Samples

Samples from 20 patients with HCC who underwent tumorectomy were provided by the Pathology Department of the Hospital Universitario de Araba after having gained approval from the review board on ethical procedures of the same institution, and after receiving informed consent for research on the samples from each patient. Tissues obtained from biopsies were fixed in 10% formaldehyde and paraffin embedded then cut into 4 μm slices, mounted on treated slides and stained with haematoxylin-eosin (H&E). A single expert pathologist (IFV) performed the histological examination of every HCC sample following the criteria of Kondo et al., which classify HCC into 3 categories: well, moderately, and poorly differentiated [10, 11]. In addition, the proliferative index as determined by Ki-67, along with the characteristics of the patients studied and the clinicopathological features of their tumours were recorded (Table 1). Fourteen patients with HCC had a cirrhotic liver due to the hepatitis C virus. The aetiology of the remaining six HCCs arising in normal livers was unknown, and no other underlying liver disease was noted. In addition to the HCC samples, healthy tissue from six non-cirrhotic livers was also examined.

Tissue Microarray Construction

Representative tumor regions were identified and three tissue cores (diameter 1.5 mm) from each of the 20 HCCs were selected for tissue microarray (TMA). After 5 min at 60 °C the TMA blocks were cut into 4 μm thick sections in preparation for the immunohistochemical analysis. Whole tissue sections were examined to confirm negative cases. In addition, whole tissue sections were performed to analyze normal regions and cirrhotic areas.

Immunohistochemistry

The paraffin embedded tissue sections were treated with xylene to render them diaphanous (the paraffin being removed later by passing it through decreasing alcohol concentrations until water was reached). Rehydrated sections were rinsed in phosphate buffered saline (PBS) containing 1% tween-20. For the detection of Miki, sections were heated in high pH Envision FLEX target retrieval solution at 65 °C for 20 min and then incubated for 20 min at room temperature in the same solution. Endogenous peroxidase activity (3% H2O2) and non-specific binding (33% fetal calf serum) were blocked and the sections were incubated overnight at 4 °C with primary antibodies using a 1:100 dilution. Next, Ultra View universal DAB kit was used following the manufacturer's recommendations and using an automated staining procedure. Finally, samples were counterstained with haematoxylin, dehydrated and mounted. The sections were examined and photographed (×20 and ×40 objective) under a light microscope with a Leica DFC300 FX camera.

Immunohistochemistry Assessment

The protein expression levels were evaluated by two independent observers (and a third in the case of any disagreement) with no prior knowledge of each patient's clinical information or outcome. Immunohistochemical signal intensity on a scale of 0–3 and percentage of positive cells (0–100) were recorded and a score (from 0 to 300 points) resulting from the multiplication of both parameters (signal intensity x percentage of positive cells) was calculated. Any non-zero score was considered positive.

Statistical Analysis

Standard descriptive statistics were recorded for baseline demographic and clinical characteristics of each patient and the

Table 1 Patient demographics, tumor features and Miki immunoexpression in hepatocellular carcinomas classified by grades of differentiation

	Total($N = 20$)	WD ($N = 8$)	MD($N = 6$)	PD (N = 6)	p-value	Correlation coefficient
Sex, Male n (%)	15(75)	5(62.5)	5(83.3)	5(83.3)	0.367	−0.213
Age (Mean ± SD)	67 ± 13	70 ± 16	71 ± 7	61 ± 13	0.184	−0.310
Tumor size, cm (Mean ± SD)	4.5 ± 3.8	6.3 ± 4.9	2.9 ± 1.7	4.2 ± 3	0.456	−0.177
Survival (months) (Mean ± SD)	16.8 ± 12.5	24.8 ± 15.4	15.1 ± 9.6	10.5 ± 12.5	0.026	−0.553
Metastasis (%)	4(20)	1(12.5)	1(16.67)	2(33.3)	0.380	0.207
Ki-67 (Mean ± SD)	15.4 ± 4.2	11.8 ± 2.3	15.0 ± 2.5	20.6 ± 1.7	<0.001	0.838
Miki (pattern)					<0.001	0.784
-Negative	3	1	1	1		
-Nuclear	6	5	1	0		
-Cytoplasmic	9	1	3	5		
-Mixed	2	1	1	0		
Miki (mean score)					0.528	0.150
-Nuclear	148	176	117	0		
-Cytoplasmic	143	128	87	222		
-Mixed	95	73	121	0		

WD well differentiated, *MD* moderate differentiated, *PD* poorly differentiated

corresponding pathological data, with quantitative variables being described by mean and standard deviation and qualitative variables by frequency analysis. The association between qualitative variables (type of staining observed with Miki and degree of differentiation of HCCs) was determined by a contingency table performing Pearson chi^2 test. The score obtained in the assessment of protein expression by immunohistochemistry against Miki was compared with multiple variables using a Kruskal-Wallis test. A possible correlation between different variables, such as staining patterns and clinical data, was established by applying Spearman's correlation test. Overall survival probability was estimated by Kaplan-Meier. In all cases a p-value <0.05 was accepted as statistically significant. All analyses were performed using IBM SPSS Statistics V22.0 for Windows program.

Results

Miki in Non-tumoral Liver

Immunohistochemical studies in normal liver regions from all patients showed intense Miki expression in nerves, while biliary duct cells were negative. Furthermore, a degree of nuclear staining at hepatocytes was noted (Fig. 1) with an average staining of 110 points.

In cirrhotic areas, in contrast, nuclear, cytoplasmic and mixed staining (cytoplasmic and nuclear in the same hepatocyte) was observed. Moreover, mixed staining was predominant at the periphery of regenerative nodules (Fig. 1f). The average intensity of staining in hepatocytes from cirrhotic areas was 135.

Miki in Hepatocellular Carcinomas

A predominantly moderate positive Miki expression in the majority of HCCs was observed with only four cases (20%) being negative (score 0). No significant intra-tumour heterogeneity were noted concerning Miki expression (in only 4 cases was necessary for the third evaluator to make a definitive decision – see Methods). While average intensity of staining in malignant hepatocytes was 130, almost 40% of cases showed a positive immunorreactivity score of over 170. What is more, a three-type pattern of Miki staining in HCC was noted: nuclear, cytoplasmic and mixed, although cytoplasmic staining was the predominant pattern (45%) followed by the nuclear (30%) and then mixed (10%) (Table 1).

Statistical analysis highlighted a significant strong correlation between degree of tumour differentiation and staining pattern ($p < 0.001$) whereby nuclear staining was mainly observed in well differentiated HCCs while cytoplasmic staining appeared most frequently in those that were poorly differentiated. Staining in moderately differentiated HCCs usually displayed a mixed staining (Fig. 2). In addition, a significant positive correlation between tumor differentiation and Ki-67 was observed ($p < 0.001$). Miki analysis score did not show statistical significances with the majority of clinicopathological variables studied. As regards survival, a significant negative association between survival and tumor differentiation was observed ($p = 0.026$), and in addition, comparisons of Kaplan-Meier survival curves revealed there to be

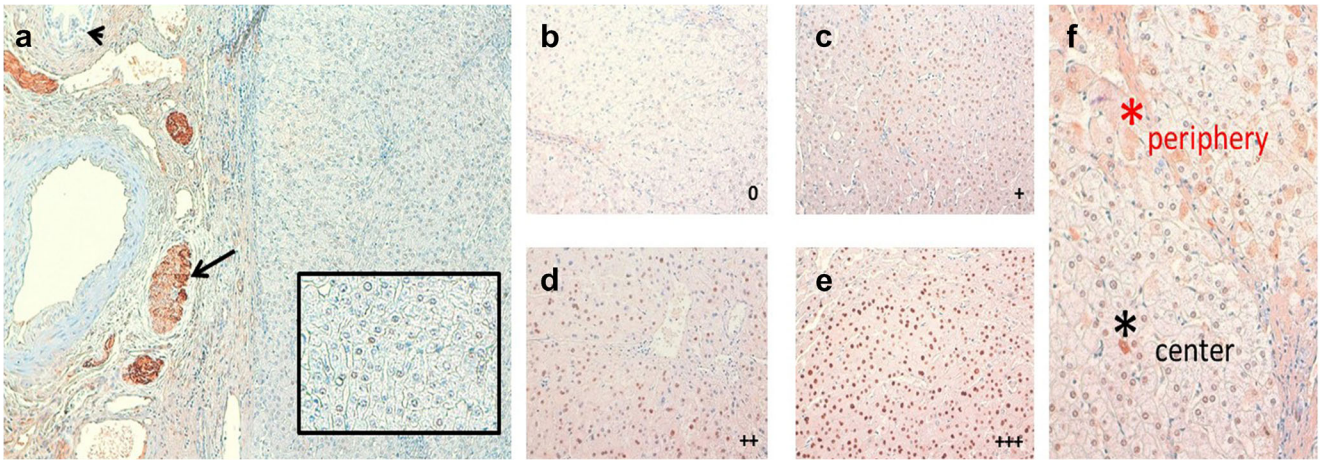


Fig. 1 Immunostaining for Miki protein in non-tumoral liver. **a**) Intense Miki expression was observed in nerves (arrow). Weak staining at hepatocyte nucleus was found (insert). Duct cells from biliary ducts were negative (arrowhead); ×100. **b-e**) Progressive degrees of nuclear intensity at hepatocytes from negative (0) to mild (+), moderate (++) and intense expression (+++); ×200. **f**) Cirrhotic areas. X400

significantly better prognosis for those HCCs with nuclear, as opposed to cytoplasmic, staining for Miki. At twenty months of follow-up after diagnosis of HCC, the difference is about 35% (80% vs. 45% for the nuclear and cytoplasmic groups, respectively ($p = 0.03$) (Fig. 3).

Discussion

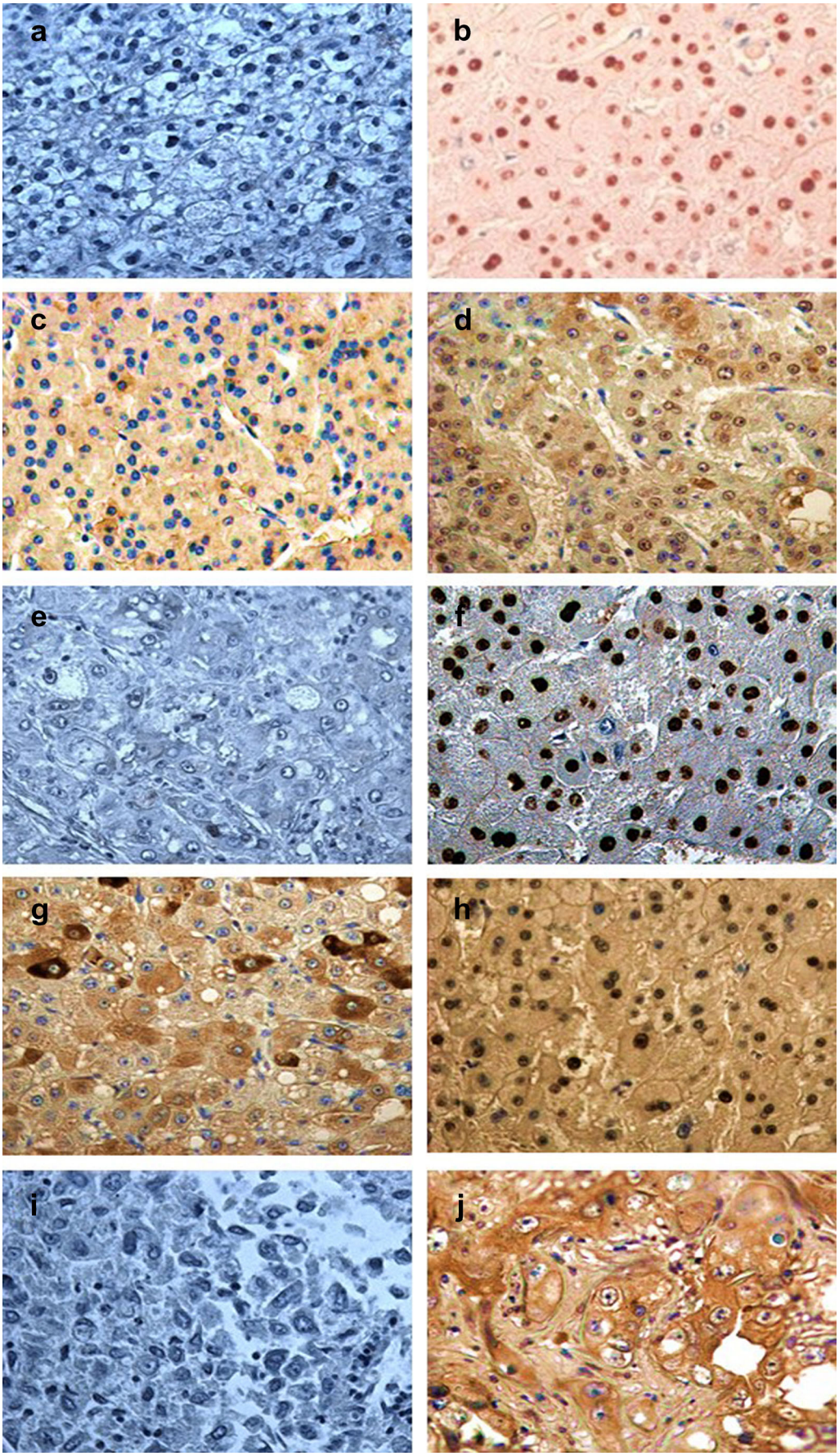
In this paper, we carried out a characterization of Miki, a member of immunoglobulin superfamily (IgSF) cell adhesion molecules that participates in biological processes such as nuclear division (mitosis), driving chromosomes organization [4]. Immunohistochemistry screening with an anti-Miki antibody revealed the differing expression of Miki in normal liver, cirrhotic areas and HCC samples. Additionally, we characterized the location of Miki in hepatocytes from normal regions, cirrhotic areas and HCCs, finding that Miki expression in non-tumoral regions, at the center of regenerative nodules in cirrhotic tissue and in most of well differentiated HCCs occurred at the hepatocyte nucleus. However, at the periphery of the regenerative nodules in cirrhosis and most other HCC samples, Miki expression was either cytoplasmic or mixed. The above findings speak in support of the principal functions recently attributed to Miki, such as its participation in nuclear division and the organization of centrosomes. In fact, other research findings have reported that mutations in this gene disrupt the formation of the microtubules which generate alterations in the prometaphase [4]. In addition, alterations in the expression of Miki have also been associated with the appearance of abnormal mitosis in myelodysplasia [6].

We also identified a significant association between expression patterns of Miki in HCCs, Ki-67 proliferation index and tumor differentiation. In this light, well differentiated tumors (i.e. those with a lower proliferation index) mostly showed a nuclear staining pattern, while poorly differentiated HCCs (i.e. with a higher proliferation index) presented a predominantly cytoplasmic pattern. To the best of our knowledge, there are no previous articles in the literature reporting Miki immunoexpression in cancer, although some works have studied cytoplasm protein translocations, p27 in glioblastomas, for example, where cytoplasmic expression was also associated with poor prognosis [12]. Furthermore, the existence of at least two variants of Miki (Miki-alpha and Miki-beta), resulting from different splicings of the gene, might account for the mixed pattern observed at the periphery of regenerative nodules in cirrhosis and in some cases of HCCs [4].

Moreover, the nuclear staining described for Miki in this article corresponds to the accumulation of Miki in the Golgi apparatus, which is very close to the nucleus, during interphase, before Miki translocates from the Golgi apparatus to the mitotic centrosomes/spindles in late G2/M phase [6]. Although most of the molecular mechanisms underlying atypical mitosis in cancer cells that pathologists routinely see, such as chromosome scattering, multipolar mitosis and pseudometaphase phenotypes, are still unknown, our results support the notion that Miki is one of the possible mediators [13–15].

In the long term, Miki could prove be helpful in routine biopsies of liver cancer as an additional parameter which enhances the accuracy of both diagnosis and prognosis. In addition, Miki may be a potential therapeutic target in a

Fig. 2 Immunostaining for Miki protein in hepatocellular carcinomas. **a-d**) Well differentiated HCCs showing negative nuclear type, cytoplasmic type and mixed type expression; ×400. **e-h**) Moderately differentiated HCCs showing negative expression of nuclear type, cytoplasmic type and mixed type expression; ×400. **i-j**) Poorly differentiated HCCs showing negative cytoplasmic type expression; ×400 ▶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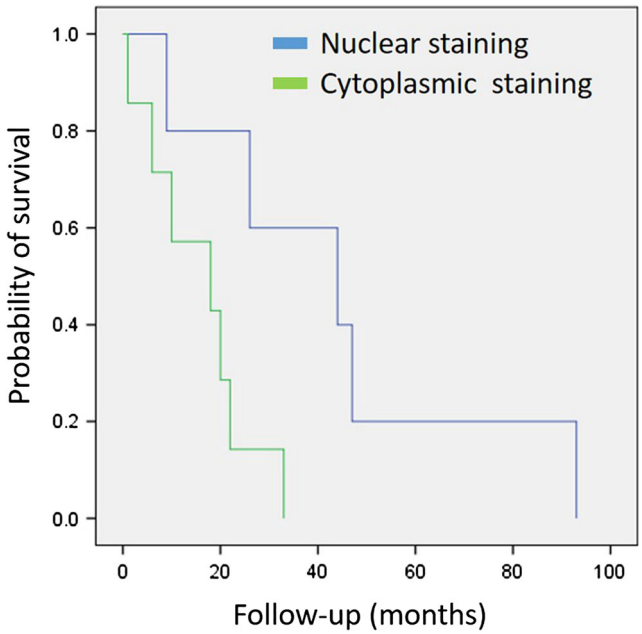

Fig. 3 Kaplan-Meier estimates of overall survival for Miki staining pattern in HCCs. Those HHCs with nuclear immunoexpression of Miki showed significantly better prognosis

variety of malignancies due to its role as a regulator of mitotic kinetics, or it may be able to tell us which types of tumor are most susceptible to mitosis-targeted anti-cancer therapies [16–18].

That said, we are well aware that our study has a number of limitations. First, there are potential biases due to the retrospective nature of our study. Second, these findings are based on patients treated in a university hospital, which have a higher percentage of poor prognostic tumors than other hospitals because of referrals. Third, we used TMAs, and because the antibody expression pattern are sometimes heterogeneous, our scoring may not have reflected the situation across the entire tumor. Nevertheless, we observed that antibody expression was highly concordant in the three representative tissue cores selected from each tumor. Even in the case of analysis of diagnostic sections, this heterogeneity could also influence the scoring. Four, we are aware that with a low number of tumors our study may lack associations due to small sample size (type II error). Fifth, the study was performed at only one centre. Sixth, the study did not include a second group of samples to validate the hypothesis generated for each antibody. Finally, while our results are clearly interesting, further controlled studies are needed to validate our results in a prospective study with a greater number of HCC patients.

In conclusion, this preliminary study provides an overview of Miki expression in non-tumoral regions and HCCs from twenty patients, the results of which significant clinical relevance. To that end we extend the state of the art in terms of knowledge of cancer biology, contributing to the multidisciplinary coordination against the multiple faces of this disease.

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Author Contributions IFV made the diagnoses of hepatocellular carcinomas, carried out the TMA preparations together with ECU, and coordinated the study. Immunohistochemistry was carried out by FBGC. Data analyses were performed by JSJ, and JJA. LLG, ECU and IFV carried out and interpreted the staining, contributed to data analyses and drafted the manuscript. BG, IGM and LMQ provided technical support and critically reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest in the research.

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