Journal of Current Hematology & Oncology Research

Original Article

DOI: 10.51271/JCHOR-0034

Evaluation of the relationship between immunohistochemical markers and the prognosis of patients with hepatocellular carcinoma

Elif Kuzucular[1], Figen Doran[2]

[1]Department of Pathology, Faculty of Medicine, İstanbul Medipol University, İstanbul, Turkiye
[2]Department of Pathology, Faculty of Medicine, Çukurova University, Adana, Turkiye

Cite this article: Kuzucular E, Doran F. Evaluation of the relationship between immunohistochemical markers and the prognosis of patients with hepatocellular carcinoma. *J Curr Hematol Oncol Res.* 2024;2(2):33-40.

Corresponding Author: Elif Kuzucular, elifcalis261@gmail.com

Received: 05/02/2024 ◆ **Accepted**: 26/03/2024 ◆ **Published**: 14/05/2024

ABSTRACT

Aims: This study aimed to evaluate whether prognosis and survival time in patients hepatocellular carcinoma (HCC) were associated with immunohistochemistry results for Heat Shock Protein 70 (HSP70), Glutamine synthetase (GS), Cyclase Associated Protein 2 (CAP2), Enhancer of zeste homolog 2 (EZH2) and B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1).

Methods: In this retrospective study, the medical records of 50 HCC cases evaluated at Çukurova University Faculty of Medicine, Department of Pathology between 2007 and 2012 were evaluated. Tissues were stained for the targeted antigens. Immunohistochemical stains were scored for cytoplasmic (HSP70, GS, CAP2) or nuclear (EZH2, Bmi-1) staining patterns under light microscopy.

Results: Twenty-nine (58%) of the HCC cases died and the overall survival time was 30±3 (24-37) months. Survival times were similar in terms of age (p=0.262), sex (p=0.707), cause of disease (p=0.655), tumor size (p=0.191) and degree of differentiation (p=0.280). The overall survival of HCC patients with vascular invasion was shorter (p=0.019). The frequency of EZH2 (p=0.025) and Bmi-1 (p=0.004) +/++ was higher in patients with vascular invasion. No correlation was found between overall survival time and HSP70 positivity (p=0.140) and CAP2 positivity (p=0.278); however, survival time was significantly shorter in HCC cases stained (++/+++) with EZH2 (p=0.034), Bmi-1 (p=0.008) and GS (p=0.018).

Conclusion: The results of this study showed that GS, EZH2, and Bmi-1 predicted prognosis and survival time in patients with HCC, possible due to relationships with vascular invasion. There is a need for more comprehensive, population-based studies on biomarkers that can be used in prognosis monitoring of HCC cases.

Keywords: Hepatocellular carcinoma, HSP70, EZH2, GS, CAP2, Bmi-1

INTRODUCTION

Hepatocellular carcinoma (HCC) remains a growing global health challenge, ranking as the fourth leading cause of cancer-related deaths worldwide.[1] Unfortunately, many HCC cases are diagnosed at an advanced stage, resulting in a nearly equal incidence-fatality ratio as demonstrated by data from 2018 showing that there were 841,000 newly diagnosed cases of HCC, leading to 782,000 HCC-related deaths.[1,2] More than 80% of HCC cases occur in East Asia and sub-Saharan Africa, particularly where access to medical and social care resources is limited. However, the burden of HCC has shifted over time from low-middle sociodemographic index regions to high sociodemographic index regions, reflecting the shift from viral to non-viral causes.[3]

HCC occurs mainly in patients with underlying liver disease and is considered one of the leading causes of death in this population. Several well-established risk factors are associated with HCC, including chronic Hepatitis B virus (HBV) or Hepatitis C virus (HCV) infection, alcohol abuse, non-alcoholic fatty liver disease, and exposure to dietary toxins. All these risk factors are potentially preventable and therefore the significant potential of risk prevention to reduce the global burden of HCC should not be ignored.[4] In addition to the need for surveillance, it is also crucial to understand factors that impact the pathogenesis of the disease or its emergence from precursor conditions, including clinically-assessable factors, systemic/metabolic features, and histological characteristics.

HCC precursor lesions show unique molecular alterations of Alpha fetoprotein (AFP), heat shock protein 70 (HSP70), cyclase associated protein 2 (CAP2), glypican 3, and glutamine synthetase (GS), which have proven useful in the histological diagnosis of early HCC.[5,6] These biomarkers are also reported to be associated with poor prognosis in early or advanced HCC. In addition, tumor markers have been widely used in recent years for appropriate treatment selection or response. Although new therapeutic oncological methods have been discovered, the use of biomarkers other than AFP in HCC surveillance in daily practice is limited.[6] In this context, expanding the use of some reliable biomarkers may be beneficial in terms of early diagnosis, predicting prognosis, contributing to the treatment process and positively affecting survival in HCC cases with high mortality.

The aim of the present study was to evaluate whether the prognosis and survival of HCC cases could be associated with various histological markers, including EZH2, Bmi-1, HSP70, GS and CAP2.

METHODS

This retrospective study was authorized in 2013 by the institution of Çukurova University Faculty of Medicine. However, ethics committee approval was not required at that time. Within the scope of the research, liver section data of 50 cases who underwent liver resection for HCC between 2007 and 2012 were evaluated. Clinical data of the cases were obtained from the detailed medical records of the patients in the hospital. For cases with insufficient clinical information, additional information was obtained by contacting the patient or their relatives.

Immunohistochemical Evaluations

The tissues fixed in 10 percent formaldehyde were blocked after the tissue tracking process and hematoxylin and eosin (HE) stained preparations were obtained from 5 micron serial sections. It was examined under a light microscope and suitable blocks were selected. Histological sections were taken on special polylysine slides for immunohistochemical staining. Strept Avidin-Biotin complex immunoperoxidase method was applied to the sections prepared from paraffin blocks of the cases included in the study group (SensiTek HRP, Anti-Polyvalent RTU; HSP70=SPRING BIOSCIENCE, Rabbit Anti-Human HSP70 polyclonal Antibody, CAP2=BIOSS, Rabbit Polyclonal Antibody, EZH2=SPRING, Rabbit Polyclonal Anti–Human, Bmi-1=BETHYL Laboratories Inc, Rabbit polyclonal, Glutamine Synthetase=NOVUS BIOLOGICALS, Rabbit Polyclonal).

Preparation of Tissues For Streptavidin-Biotin Staining

Sections taken from paraffin blocks with a thickness of 5 microns were kept in the oven at 60°C for 30-45 minutes until the paraffin on them melted. The sections were kept in xylene chalk in the same oven for 10 minutes. The sections taken out of the oven were kept in three separate chalks containing xylol at room temperature, then in three separate chalks containing 95% alcohol for five minutes each, and then washed thoroughly in distilled water and the deparaffinization process was completed. To block endogenous peroxidase activity, it was incubated in a solution of 3% Hydrogen Peroxide (H2O2) in distilled water for five minutes. The stages of painting the sections are as follows:

- Antigen retrieval was performed by turning the slides 3 times for five minutes in appropriate solutions in special microwave-resistant chalks (HSP70: Citrate Buffer, pH=6; CAP2: Citrate Buffer, pH=6; EZH2: EDTA Buffer, pH=8, Bmi-1: Tris-EDTA Buffer, pH=9, GS: Citrate Buffer, pH=6). Then, samples were left to cool at room temperature for 50 minutes.
- The sample was washed in Phosphate Buffer Saline (PBS 0.01M) at pH 7.2-7.4 for 3-5 minutes.
- The tissues were placed horizontally in a humid environment, and primary antibody (diluted as required) was added. Incubation was performed at room temperature for approximately 90 minutes (HSP70: 1/150, CAP2: 1/200, EZH2: 1/100, Bmi-1: 1/250, GS: 1/700).
- Wash with PBS (5X).
- Sensi Tek Anti Polyvalent (Scytek) with secondary antibody biotin was added and incubated at room temperature for 15 minutes.
- Washed 5 times with PBS.
- Sensi Tek HRP (ScyTek Laboratories) was added and incubated at room temperature for 20 minutes.
- PBS wash (5X).
- AEC chromogen was added to the sample and incubated for 5-20 minutes, and the tissues were placed in tap water and checked for staining under a microscope.
- The floor was stained with Mayer Hematoxylin for 1-3 minutes and washed with tap water for 3-5 minutes.
- The area around the section was wiped and sealed with water-based sealing agent (Thermo Scientific - Shandon).

Immunohistochemical stains were evaluated according to cytoplasmic or nuclear staining patterns under a light microscope. Nuclear markers Bmi-1 and EZH2 were evaluated with an immunohistochemical score based on the percentage and intensity of staining.7 Staining intensity was scored between 0 and 3 (<5% staining=negative=0, 5–25% staining=sporadic=1, 25–50% staining=focal=2, >50% staining=diffuse=3). Staining intensity was scored between 0 and 3 (No staining=0, Slight staining=1, Moderate staining=2, Severe staining=3). The final immunohistochemical score was calculated with the following formula=(Percentage of Bmi-1 positive tumor area)x(Staining intensity of tumor cells). Accordingly, scoring was made between 0-9 ["-"=(score 0-1), "+"=(score 2-3), "++"=(score 4-6), "+++"=(score >6)]. The same method was used in EZH2. Semiquantitative evaluation was made according to the prevalence and intensity of staining for cytoplasmic markers HSP70, GS and CAP2 (No staining=negative, mild staining=weak positive=+, moderate staining=moderate positive=++, strong staining=strong positive=+++).

Statistical Analysis

All analyses were performed on IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp., Armonk, NY, USA). Shapiro-Wilk test was used to determine whether variables

are normally distributed. Data are given as mean±standard deviation or median (1st quartile - 3rd quartile) for continuous variables according to normality of distribution and as frequency (percentage) for categorical variables. Continuous variables were analyzed with the Student's t test or Mann-Whitney U test / Kruskal-Wallis test depending on normality of distribution and group count. Categorical variables were analyzed with the chi-square test. The statistical significance value was accepted as $p<0.05$.

RESULTS

The research group consisted of 41 (82%) males and 9 (18%) females, with a mean age of 59.1±14.8 (range: 18-94) years. During the examined time period, 29 (58%) patients died. Cause of disease was HBV in the majority of HCC patients (52.0%). The majority of lesions were 4–9 cm in size (60%), 44% of the patients had vascular invasion, and 48% of lesions were well differentiated according to the Edmondson-Steiner grading system. There were no cases with early HCC diagnosis, or were found to have dysplastic focus or dysplastic nodule (Table 1).

Table 1. Distribution of the study group according to descriptive characteristics

	n (%)
Sex	
Male	41 (82)
Female	9 (18)
Cause of disease	
HBV	26 (52)
HCV	10 (20)
HBV+HCV	5 (10)
Unknown cause	9 (18)
Tumor size, cm	
≤3 cm	10 (20)
4-9 cm	30 (60)
≥10 cm	10 (20)
Vascular invasion	
Present	22 (44)
Absent	28 (56)
Degree of differentiation	
Good	24 (48)
Middle	19 (38)
Little	7 (14)
Mortality	
Present	29 (58)
Absent	21 (42)

HBV: Hepatitis B virus, HCV: Hepatitis C virus

The overall survival time of patients was 30±3 months (24-37). Overall survival was not associated with age group (p=0.262), sex (p=0.707), disease cause (p=0.655), tumor size (p=0.191) and differentiation degree (p=0.280). The overall survival of HCC patients without vascular invasion was significantly longer than those with invasion (p=0.019, Table 2).

Table 2. Survival times according to the descriptive characteristics of the study group

	Mortality/n	Survival, month Mean±SD (95% CI)	p
Age groups, year			
≤54 year	9/13	37±6 (26-47)	0.262
55-64	14/18	39±5 (29-50)	
≥65 year	6/19	21±5 (12-30)	
Sex			
Male	24/41	30±4 (22-37)	0.707
Female	5/9	34±7 (20-49)	
Cause of disease			
HBV	16/26	25±6 (13-37)	0.655
HCV	5/10	19±4 (11-27)	
HBV+HCV	3/5	19±6 (8-30)	
Unknown cause	5/9	36±6 (24-49)	
Tumor size, cm			
≤3 cm	8/10	45±5 (36-54)	0.191
4-9 cm	15/30	26±4 (17-34)	
≥10 cm	6/10	19±4 (11-27)	
Vascular invasion			
Present	10/22	16±3 (11-22)	0.019
Absent	19/28	36±4 (28-44)	
Degree of differentiation			
Good	15/24	23±2 (18-28)	0.280
Middle	9/19	24±6 (13-35)	
Little	5/7	37±9 (20-54)	

CI: Confidence interval, HBV: Hepatitis B virus, HCV: Hepatitis C virus

Among the markers, the frequency of strong positivity (+++) was 8% for EZH2, 10% for Bmi-1, 24% for HSP70, 28% for GS, and 34% for CAP2 (Table 3, Figure 1, Figure 2).

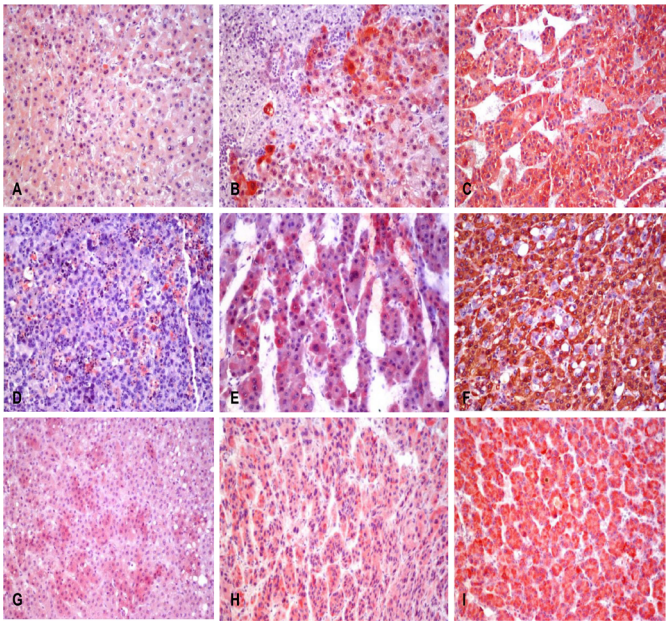

Figure 1. Immunohistochemical evaluations of HSP70, GS and CAP2 [A: HSP70 weakly positive (X200), B: HSP70 moderately positive (X200), C: HSP70 strongly positive, D: GS weakly positive (X200), E: GS moderately positive (X200), F: GS strongly positive, G: CAP2 weakly positive (X200), H: CAP2 moderately positive (X200), I: CAP2 strongly positive (X200)]

Table 3. Distribution of immunohistochemical markers

	n(%)
EZH2	
Negative (-)	29 (58)
+	7 (14)
++	10 (20)
+++	4 (8)
Bmi-1	
Negative	29 (58)
+	3 (6)
++	13 (26)
+++	5 (10)
HSP70	
Negative	4 (8)
+	12 (24)
++	22 (44)
+++	12 (24)
GS	
Negative	16 (32)
+	6 (12)
++	14 (28)
+++	14 (28)
CAP2	
Negative	6 (12)
+	8 (16)
++	19 (38)
+++	17 (34)

EZH2: Enhancer of zeste homolog 2, Bmi-1: B-cell-specific moloney murine leukemia virus integration site 1, HSP70: Heat shock protein 70, GS: Glutamine synthetase, CAP2: Cyclase associated actin cytoskeleton regulatory protein 2

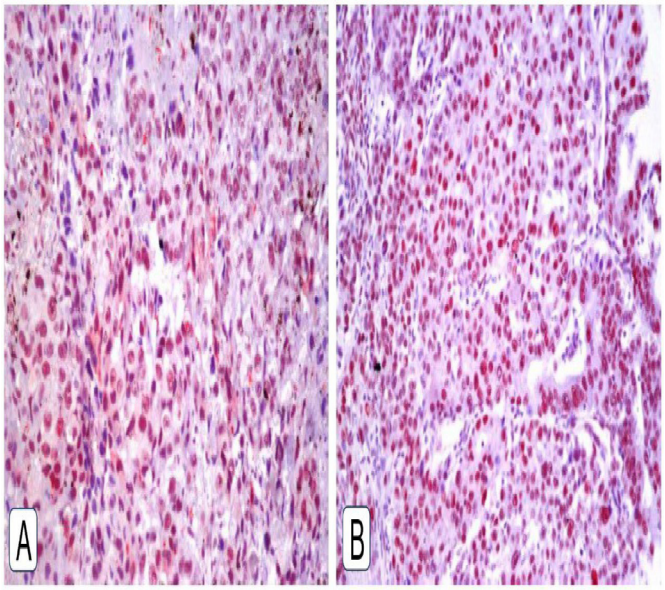

Figure 2. Immunohistochemical evaluations of EZH2 and Bmi-1 [(A: EZH2 nuclear positivity results (X200), B: Bmi-1 nuclear positivity results (X200)]

HSP70 positivity was not associated with age (p=0.122), sex (p=1.000), disease cause (p=0.566), tumor size (p=0.154), vascular invasion (p=0.373) and differentiation degree (p=0.570, Table 4). GS positivity was not associated with age (p=0.375), sex (p=0.481), disease cause (p=0.231), tumor size (p=0.897), vascular invasion (p=0.158) and differentiation degree. (p=0.610, Table 4). CAP2 positivity was not associated with age group (p=0.137), sex (p=1.000), disease cause (p=0.726), tumor size (p=0.589), vascular invasion (p=0.215) and differentiation degree (p=0.892.

Table 4. Distribution of cytoplasmic immunohistochemical markers HSP70, GS and CAP2 positivity according to descriptive characteristics

	HSP70		GS		CAP2	
	-/+ n (%)	++/+++ n (%)	-/+ n (%)	++/++n (%)	-/+ n (%)	++/+++ n (%)
Age groups, year						
≤54 year	3(18.8)	10 (29.4)	7 (31.8)	6 (21.4)	3 (21.4)	10(27.8)
55-64	9(56.3)	9 (26.5)	9 (40.9)	9 (32.1)	8 (57.1)	10 (27.8)
≥65 year	4(25.0)	15 (44.1)	6 (27.3)	13 (46.4)	3 (21.4)	16(44.4)
p	0.122		0.375		0.137	
Sex						
Male	13(81.3)	28 (82.4)	17 (77.3)	24 (85.7)	12(85.7)	29(80.6)
Female	3 (18.8)	6 (17.6)	5 (22.7)	4 (14.3)	2 (14.3)	7 (19.4)
p	1.000		0.481		1.000	
Cause of disease						
HBV	6 (37.5)	20 (58.8)	12 (54.5)	14 (50.0)	6 (42.9)	20(55.6)
HCV	4 (25.0)	6 (17.6)	2 (9.1)	8 (28.6)	4 (28.6)	6 (16.7)
HBV+HCV	2 (12.5)	3 (8.8)	2 (9.1)	3 (10.7)	7 (7.1)	4 (11.1)
Unknown cause	4 (25.0)	5 (14.7)	6 (27.3)	3 (10.7)	3 (21.4)	6 (16.7)
p	0.566		0.231		0.726	
Tumor size, cm						
≤3 cm	5 (31.3)	5 (14.7)	5 (22.7)	5 (17.9)	4 (28.6)	6 (16.7)
4-9 cm	10(62.5)	20 (58.8)	13 (59.1)	17 (60.7)	7 (50.0	23(63.9)
≥10 cm	1 (6.3)	9 (26.5)	4 (18.2)	6 (21.4)	3 (21.4)	7 (19.4)
p	0.154		0.897		0.589	
Vascular invasion						
Present	6 (37.5)	16 (47.1)	7 (31.8)	15 (53.6)	4 (28.6)	18 (50.0)
Absent	10(62.5)	18 (52.9)	15 (68.2)	13 (46.4)	10 (71.4)	18(50.0)
p	0.373		0.158		0.215	
Degree of differentiation						
Good	6 (37.5)	18 (52.9)	9 (40.9)	15 (53.6)	6 (42.9)	18 (50.0)
Middle	7 (43.8)	12 (35.3)	9 (40.9)	10 (35.7)	6 (42.9)	13 (36.1)
Little	3 (18.8)	4 (11.8)	4 (18.2)	3 (10.7)	2 (14.3)	5 (13.9)
p	0.570		0.610		0.892	

HBV: Hepatitis B virus, HCV: Hepatitis C virus, HSP70: Heat Shock Protein 70, GS: Glutamine synthetase, CAP2: Cyclase Associated Actin Cytoskeleton Regulatory Protein 2

EZH2 positivity was not associated with age (p=0.804), sex (p=1.000), disease cause (p=0.956), tumor size (p=0.081) and differentiation degree (p=0.892). Notably, the frequency of EZH2 ++/+++ staining was higher in patients with vascular invasion (p=0.025, Table 5). Bmi-1 positivity was no associated with age (p=0.288), sex (p=0.459), cause

of disease (p=0.287) and degree of differentiation (p=0.865). However, the frequency of Bmi-1 ++/+++ staining was higher in patients with a tumor size of 4-9 cm compared to other values (p=0.037), and in patients with vascular invasion compared to those without (p=0.004) (Table 5).No significant relationship was found between overall survival time and HSP70 positivity (p=0.140) or CAP2 positivity (p=0.278). Overall survival time was significantly shorter in HCC cases stained (++/+++) with EZH2 (p=0.034), Bmi-1 (p=0.008) and GS (p=0.018) (Table 6).

Table 5. Distribution of nuclear immunohistochemical markers Bmi-1 and EZH2 positivity according to descriptive features

	EZH2		Bmi-1	
	-/+ n (%)	++/+++ n (%)	-/+ n (%)	++/+++ n (%)
Age groups, year				
≤54 year	10 (27.8)	3 (21.4)	6 (18.8)	7 (38.9)
55-64	12 (33.3)	6 (42.9)	13 (40.6)	5 (27.8)
≥65 year	14 (38.9)	5 (35.7)	13 (40.6)	6 (33.3)
p	0.804		0.288	
Sex				
Male	29 (80.6)	12 (85.7)	25 (78.1)	16 (88.9)
Female	7 (19.7)	2 (14.3)	7 (21.9)	2 (11.1)
p	1.000		0.459	
Cause of disease				
HBV	19 (52.8)	7 (50.0)	14 (43.8)	12 (66.7)
HCV	7 (19.4)	3 (21.4)	7 (21.9)	3 (16.7)
HBV+HCV	4 (11.1)	1 (7.1)	3 (9.4)	2 (11.1)
Unknown cause	6 (16.7)	3 (21.4)	8 (25.0)	1 (5.6)
p	0.956		0.287	
Tumor size, cm				
≤3 cm	10 (27.8)	0 (0.0)	8 (25.0)	2 (11.1)
4-9 cm	20 (55.6)	10 (71.4)	15 (46.9)	15 (83.3)
≥10 cm	6 (16.7)	4 (28.6)	9 (28.1)	1 (5.6)
p	0.081		0.037	
Vascular invasion				
Present	12 (33.3)	10 (71.4)	9 (28.1)	13 (72.2)
Absent	24 (66.7)	4 (28.6)	23 (71.9)	5 (27.8)
p	0.025		0.004	
Degree of differentiation				
Good	18 (50.0)	6 (42.9)	16 (50.0)	8 (44.4)
Middle	13 (36.1)	6 (42.9)	12 (37.5)	7 (38.9)
Little	5 (13.9)	2 (14.3)	4 (12.5)	3 (16.7)
p	0.892		0.865	

HBV: Hepatitis B virus, HCV: Hepatitis C virus, EZH2: Enhancer of zeste homolog 2, Bmi-1: B-cell-specific Moloney murine leukemia virus integration site 1

Table 6. Distribution of survival times according to immunohistochemical markers

	Mortality/n	Survival, month Mean±SD (95% CI)	p
EZH2			
Negative/+	23/36	34±4 (26-41)	0.034
++/+++	6/14	13±2 (8-18)	
Bmi-1			
Negative/+	22/32	37±4 (29-44)	0.008
++/+++	7/18	12±2 (7-16)	
HSP70			
Negative/+	10/16	35±5 (26-47)	0.140
++/+++	19/34	21±3 (15-26)	
GS			
Negative/+	15/22	38±4 (29-46)	0.018
++/+++	14/28	15±2 (11-19)	
CAP2			
Negative/+	9/14	36±5 (25-42)	0.278
++/+++	20/36	29±4 (21-37)	

HBV: Hepatitis B virus, HCV: Hepatitis C virus, EZH2: Enhancer of zeste homolog 2, Bmi-1: B-cell-specific Moloney murine leukemia virus integration site 1, HSP70: Heat Shock Protein 70, GS: Glutamine synthetase, CAP2: Cyclase Associated Actin Cytoskeleton Regulatory Protein 2

DISCUSSION

HCC is a serious malignant tumor in the world due to its complex molecular and cellular heterogeneity. Besides, HCC incidence continues to increase.[1,2] Over 200 genes related to HCC proliferation, invasion and metastasis have been reported. However, the specific prognostic biomarkers and therapeutic targets are insufficient.[3] Therefore, the screening of HCC molecular biological markers could improve prognosis and reduce mortality.

In this study, we assessed whether some nuclear and cytoplasmic biomarkers could be associated with survival or various other prognostic features in patients with HCC. Our data showed that survival was shorter in patients with EZH2, GS and Bmi-1 positivity; whereas there were no relationships for HSP70 or CAP2. This impact on survival was likely associated with vascular invasion, which was more common in subjects with EZH2 and Bmi-1 positivity.

Possible curative treatments for HCC are liver transplantation, radiofrequency ablation, and resection. However, the effect of these approaches is limited in advanced HCC cases.[8] Advanced stages of HCC can be treated alone or in combination with chemotherapy, immunotherapy and oncolytic viruses. Despite these efforts, high mortality rates are evidence that current treatment options do not achieve the desired therapeutic goals.[9] In the 2015 results of the Global Burden of Disease Study, it was reported that

HBV was the leading cause of liver cancer, death and DALY cases at the global level, followed by alcohol.[2] Similarly, in the current study, the disease cause in the majority of patients diagnosed with HCC was HBV (52.0%). More efforts on HBV vaccination may be beneficial in reducing the prevalence of HCC.

In the Surveillance Epidemiology, and End Results (SEER) database, young age, female sex, Hispanic ethnicity and being married were the determinants that prolonged disease-related survival. Additionally, disease-related survival was worse in patients with greater tumor size (>5 cm), vascular invasion, and lymph node involvement.10 According to the study of Sakamoto et al.[8] in HCC cases, serosal invasion, preoperative AFP, presence of invasion into hepatic veins, and liver cirrhosis were independent predictors of overall survival in multivariate analyses. Another study reported factors that independently affected five-year survival in HCC cases as tumor size >3 cm, involved lymph nodes >2, metastasis, combination therapy with surgery and chemotherapy and coinfection with HBV and HCV.[11] In the current study, overall survival time did not vary according to age group, sex, cause of disease, tumor size and degree of differentiation. The overall survival of HCC patients without vascular invasion was significantly longer than those without. When evaluating factors affecting survival, differences between studies in the factors included in the analyses, clinicopathological status of the cases, treatment regimens, and access to prevention efforts may explain the diversity of results.

In response to the stressful cancer microenvironment, HCC tumor cells can increase the expression of chaperone proteins for cytoprotective function, such as HSP70, GS, CAP2, EZH2 and Bmi-1, resulting in tumor growth and metastasis.[12] Members of the HSP70 family have important roles in protein folding, prevention of protein aggregation, and transport of proteins across membranes under physiological conditions. In environmental (irradiation, chemotherapy), physiological (cell growth, differentiation) and pathophysiological (infection, malignancy) stress situations, the synthesis of the HSP family increases, while protein synthesis generally decreases. Unlike normal cells, the presence of tumors such as HCC causes overexpression of HSP70.[13] It is reported that HSP70 causes the differentiation of tumor cells by stabilizing Cyclin D1 and suppresses the apoptosis of tumor cells by inhibiting the p53 pathway.[14,15] Similar to numerous studies reporting overexpression of HSP70 in HCC cases[12,13,16,17], our results also suggest that HSP70 can be used in the diagnostic process of HCC cases. In addition to being a useful diagnostic marker for HCC, HSP70 also appears to be a predictor of prognosis. High expression of HSP70 was associated with portal vein invasion, but no relationship could be detected between HSP70 and Edmons grade.[18] Joo et al.[16] reported that HSP70 positivity in HCC tissues was positively correlated with tumor size, portal vein invasion, and tumor stage. It has also been reported that HSP70 shows a close relationship with tumor progression, prognostic factors, and pathological parameters.[17] However, Luk et al.[12], reported that HSP70 was unassociated with any of the pathological features examined in their study. In the current study, HSP70 positivity in patients diagnosed with HCC did not vary according to tumor size and differentiation degree, but HSP70 positivity was more frequent in patients with vascular invasion. There was no relationship between survival time and HSP70 positivity.

Another biomarker useful for early diagnosis and prognostication in HCC patients is GS, which causes β-catenin activation. GS-mediated glutamine synthesis in the liver is an important mechanism for ammonia detoxification. In liver malignancy, GS is highly expressed as a transcriptional target of oncogenic β-catenin. Therefore, there is a strong positive correlation between β-catenin activation and GS expression in HCC patients.[19] However, contrary to the widely recognized pro-tumorogenic role of GS, a recent study also reported that GS has a tumor suppressor role in liver cancer by maintaining nitrogen homeostasis through ammonia detoxification.[20] Another study reported that strong positivity for GS was a sensitive marker for HCC in the presence of cirrhosis, independent of tumor differentiation.[21] In the current study, GS positivity was detected in 68% of patients diagnosed with HCC, but was not associated with age, sex, disease cause, tumor size, vascular invasion and differentiation degree. Similarly, there are also results reporting high frequency of GS positivity (53.7%) in HCC cases, but no relationship with clinicopathological parameters. In the study of Morita et al.[22], it was reported that negative staining of β-catenin/GS was associated with both progression-free survival and prolongation of overall survival. Similarly, in this study, we found GS positivity to be associated with a shortened survival time.

In a study by Fu et al.[23], CAP2 expression (53.3%) was reported to be associated with poor overall survival, disease-free survival, and the possibility of relapse. It has been reported that CAP2 was an independent predictive factor for overall survival in multiple Cox regression analysis. Another study reported that CAP2 was a more sensitive biomarker than AFP for early-stage HCC cases, with implications on clinicopathological parameters.[24] In the present study, CAP2 positivity was present in 88% of HCC samples, but no relationship was found between CAP2 and clinicopathological data indicating HCC prognosis and survival.

Bmi-1 and EZH2, which are thought to have active roles in the oncogenic process because they are limited in normal tissue and overexpressed in tumor tissues, are reported to be promising target antigens for cancer immunotherapy.[25] EZH2 plays a role in the cell cycle, DNA damage repair, cell differentiation, autophagy, apoptosis and immunological modulation. The main function of EZH2 is to catalyze the methylation of histone H3K27Me3, which inhibits the transcription of target genes such as tumor suppressor genes. EZH2 also regulates gene transcription by forming complexes with transcription factors or by directly binding to the promoters of target genes. For these reasons, EZH2 inhibition has become an important target for cancer treatment and potential targeting drugs have been developed.[26] A study to identify novel tumor-associated antigens in patients with primary hepatocellular carcinoma reported serological responses to the polyethylene group (PcG) protein Bmi-1, which is overexpressed in a number of different tumor types. In the same study, it was reported that EZH2-derived peptides

caused more significant interferon-γ release than Bmi-1.25 In the study of Li et al.[27], it was reported that the expression of Bmi-1 was increased in HCC tissues and its expression was positively associated with tumor size, metastasis, venous invasion and TNM stage. Additionally, high Bmi-1 expression has been reported to be an independent prognostic factor for overall survival. In the present study, the frequency of EZH2 and Bmi-1 positivity was 42%, and the positivity of both biomarkers was significantly higher in patients with vascular invasion. Additionally, Bmi-1 positivity was higher in patients with larger tumors. When EZH2 and Bmi-1 positivity increased, overall survival time was significantly shortened.

Although it is awaiting standardization, HCC screening is performed every 6 months with AFP levels and ultrasonography.[28] According to the results of the SEER database, there was a significant improvement in the survival rate of HCC patients from 1988 to 2015, which can be attributed to screening efforts, early diagnosis, therapeutic advances, including HBV vaccination.10 Our results indicate that some of these biomarkers may offer unique potential for HCC assessment. Research results supporting this idea continue to be added to the literature. A recent study reported that the EZH2 inhibitor (EZH2i) GSK126 combination was associated with an increase in the number of upregulated genes in HCC cell lines, long-lasting anti-proliferation effects, and increased nucleosome accessibility.[29] In another previous study, it was reported that Hotair silence activates P16(Ink4a) and P14(ARF) signaling by increasing miR-218 expression and suppressing Bmi-1 expression –thereby suppressing HCC tumor formation.[30] There are also results reporting that shRNA-mediated inhibition of Bmi-1 can reduce the invasiveness of HCC (in vitro).[27]

Limitations

The present study includes a small population from a single center and reports results that may have limited generalizability. This is compounded by the fact that the follow-up period was short, and therefore, prognostic or survival analyses may not apply for the mid or long term. The study results could have been strengthened with long-term assessment of HCC prognosis, metastasis and survival. Although our purpose was to examine relationships with clinical data, it could also be valuable to determine relationships between immunohistochemical results and other laboratory data (for instance, AFP levels). In addition, the lack of an evaluation in terms of regimen and treatment duration differences between the patients may have affected the results. Despite all these limitations, this study is valuable in that it provides comprehensive analyses for several crucial cytoplasmic and nuclear biomarkers in HCC cases.

CONCLUSION

The results of this study showed high ratios of positivity for HSP70, GS, CAP2, EZH2, and Bmi-1, indicating their utility as diagnostic markers for HCC. More importantly, EZH2, GS and Bmi-1 were associated with survival, indicating potential use as predictors for prognosis and overall survival. There is a need for more comprehensive, population-based research on biomarkers that can be used in the diagnosis and prognostication of HCC.

ETHICAL DECLARATIONS

Ethics Committee Approval

Since this thesis research was authorized in 2013 by the institution of Çukurova University, ethics committee approval does not require.

Informed Consent

Written consent was obtained from the patient participating in this study.

Referee Evaluation Process

Externally peer-reviewed.

Financial Support

It was also supported by Çukurova University Research Fund (no: TF2012LTP22).

Conflict of Interest

The authors declare that they have no conflict of interests regarding content of this article.

Author Contributions

All of the authors declare that they have all participated in the design, execution, and analysis of the paper, and that they have approved the final version.

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68(6):394-424.
2. Akinyemiju T, Abera S, Ahmed M, et al. The burden of primary liver cancer and underlying etiologies from 1990 to 2015 at the global, regional, and national level: results from the global burden of disease study 2015. *JAMA Oncol*. 2017;3(12):1683-1691.
3. Toh MR, Wong EYT, Wong SH, et al. Global epidemiology and genetics of hepatocellular carcinoma. *Gastroenterol*. 2023;164(5):766-782.
4. Llovet JM, Castet F, Heikenwalder M, et al. Immunotherapies for hepatocellular carcinoma. *Nat Rev Clin Oncol*. 2022;19(3):151-172.
5. Sakamoto M, Mori T, Masugi Y, Effendi K, Rie I, Du W. Candidate molecular markers for histological diagnosis of early hepatocellular carcinoma. *Intervirol*. 2008;51(Suppl 1):42-45.
6. Pinero F, Dirchwolf M, Pessoa MG. Biomarkers in hepatocellular carcinoma: diagnosis, prognosis and treatment response assessment. *Cells*. 2020;9(6):1370.
7. Wang H, Pan K, Zhang HK, et al. Increased polycomb-group oncogene Bmi-1 expression correlates with poor prognosis in hepatocellular carcinoma. *J Cancer Res Clin Oncol*. 2008;134(5):535-541.
8. Sakamoto K, Ogawa K, Tohyama T, et al. Serosal invasion is a strong prognostic factor for hepatocellular carcinoma after hepatectomy. *Hepatol Res*. 2019;49(4):419-431.
9. Alawyia B, Constantinou C. Hepatocellular carcinoma: a narrative review on current knowledge and future prospects. *Curr Treat Options Oncol*. 2023;24(7):711-724.
10. Ding J, Wen Z. Survival improvement and prognosis for hepatocellular carcinoma: analysis of the SEER database. *BMC Cancer*. 2021;21(1):1157.
11. Sarveazad A, Agah S, Babahajian A, Amini N, Bahardoust M. Predictors of 5 year survival rate in hepatocellular carcinoma patients. *J Res Med Sci*. 2019;24:86.
12. Luk JM, Lam CT, Siu AF, et al. Proteomic profiling of hepatocellular carcinoma in Chinese cohort reveals heat-shock proteins (Hsp27, Hsp70, GRP78) up-regulation and their associated prognostic values. *Proteomics*. 2006;6(3):1049-1057.

13. Gehrmann M, Cervello M, Montalto G, et al. Heat shock protein 70 serum levels differ significantly in patients with chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. *Front Immunol*. 2014;5:307.
14. Diehl JA, Yang W, Rimerman RA, Xiao H, Emili A. Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase. *Mol Cell Biol*. 2003;23(5):1764-1774.
15. Calderwood SK, Khaleque MA, Sawyer DB, Ciocca D. Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem Sci*. 2006;31(3):164-172.
16. Joo M, Chi JG, Lee H. Expressions of HSP70 and HSP27 in hepatocellular carcinoma. *J Korean Med Sci*. 2005;20(5):829-834.
17. Lim SO, Park SG, Yoo JH, et al. Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. *World J Gastroenterol*. 2005;11(14):2072-2079.
18. Kang GH, Lee BS, Lee ES, Kim SH, Lee HY, Kang DY. Prognostic significance of p53, mTOR, c-Met, IGF-1R, and HSP70 overexpression after the resection of hepatocellular carcinoma. *Gut Liver*. 2014;8(1):79-87.
19. Dai W, Zong WX. Glutamine synthetase: a tumor suppressor in hepatocellular carcinoma? *J Mol Cell Biol*. 2023;15(1):mjad007.
20. Dai W, Shen J, Yan J, et al. Glutamine synthetase limits beta-catenin-mutated liver cancer growth by maintaining nitrogen homeostasis and suppressing mTORC1. *J Clin Invest*. 2022;132(24):e161408.
21. Uthamalingam P, Das A, Behra A, Kalra N, Chawla Y. Diagnostic value of glypican3, heat shock protein 70 and glutamine synthetase in hepatocellular carcinoma arising in cirrhotic and non-cirrhotic livers. *J Clin Exp Hepatol*. 2018;8(2):173-180.
22. Morita M, Nishida N, Sakai K, et al. Immunological microenvironment predicts the survival of the patients with hepatocellular carcinoma treated with anti-PD-1 antibody. *Liver Cancer*. 2021;10(4):380-393.
23. Fu J, Li M, Wu DC, Liu LL, Chen SL, Yun JP. Increased expression of CAP2 indicates poor prognosis in hepatocellular carcinoma. *Translat Oncol*. 2015;8(5):400-406.
24. Mohammed MA, Moustafa Omar N, Mohammed SA, Galal Deiab A. Identification of cyclase-associated protein-2 as a novel biomarker for early-stage hepatocellular carcinoma. *J Clin Gastroenterol Hepatol*. 2017;1(3):26.
25. Steele JC, Torr EE, Noakes KL, et al. The polycomb group proteins, BMI-1 and EZH2, are tumour-associated antigens. *Br J Cancer*. 2006;95(9):1202-1211.
26. Liu Y, Yang Q. The roles of EZH2 in cancer and its inhibitors. *Med Oncol*. 2023;40(6):167.
27. Li X, Yang Z, Song W, et al. Overexpression of Bmi-1 contributes to the invasion and metastasis of hepatocellular carcinoma by increasing the expression of matrix metalloproteinase (MMP)-2, MMP-9 and vascular endothelial growth factor via the PTEN/PI3K/Akt pathway. *Int J Oncol*. 2013;43(3):793-802.
28. Lee RM, Russell MC. Is screening for hepatocellular carcinoma effective? *Adv Surg*. 2023;57(1):73-86.
29. Zhang L, Li HT, Shereda R, et al. DNMT and EZH2 inhibitors synergize to activate therapeutic targets in hepatocellular carcinoma. *Cancer Lett*. 2022;548:215899.
30. Fu WM, Zhu X, Wang WM, et al. Hotair mediates hepatocarcinogenesis through suppressing miRNA-218 expression and activating P14 and P16 signaling. *J Hepatol*. 2015;63(4):886-895.