

ORIGINAL PAPER

THE PROGNOSTIC RELATIONSHIP BETWEEN HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL FEATURES OF HEPATOCELLULAR CARCINOMA, INTRAHEPATIC CHOLANGIOCARCINOMA AND MIXED TYPE

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Hepatocellular carcinoma, intrahepatic cholangiocarcinoma, and combined hepatocellular and cholangiocarcinoma are the most common cancers of the liver. In this study, our first aim is to evaluate the relationship between prognosis and clinicopathological parameters. The second aim involves investigating the need for immunohistochemical staining and patterns of tumours to differentiate between them.

Sixty-one cases were included in this study. For IHC, we used Hep par-1, CK7, CK19, CD56 and p53 staining, and the patterns of tumours were evaluated in haematoxylin-eosin sections.

No significant differences were found in Kaplan-Meier life analysis between the tumour types and OS and DFS values, but these values were greater in HCC than in ICC. There were no relationships between clinicopathologic parameters and OS and DFS. Although the multifocality, stage and grade of tumour were higher in HCC than in ICC, the perineural invasion and lymph node metastasis were more common in ICC than in HCC.

The diagnosis was changed in 4 cases, from HCC to ICC in one case and to combined type in 3 cases after IHC. Pathologist should be alert to mixed patterns in terms of diagnosis and IHC, because it helps differential diagnosis in these cases.

Key words: hepatocellular carcinoma, liver neoplasms, cholangiocarcinoma, risk factors.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide [1]. Intrahepatic cholangiocarcinoma (ICC) is the second most common cause of primary liver malignancies after HCC and constitutes approximately 10-15% of all primary liver cancers [2, 3, 4].

Combined hepatocellular-cholangiocarcinoma (cHCC-ICC) is a rare tumour, with variations reported between 1.0 and 4.7% of all primary hepatic tumours in a series of patients undergoing hepatic resection [5]. HCC is derived from hepatocytes, ICC from intrahepatic bile duct, and cHCC-ICC both from hepatocytes and biliary duct epithelial cells [6].

The most common aetiological factor for HCC is hepatitis B or hepatitis C or both infections [1].

Causes for liver damage, such as alcoholic liver disease, HCV, HDV, HIV, diabetes, nonspecific cirrhosis, and parasitic infections, are considered common risk factors in the development of ICC [7]. The risk factors for cHCC-ICC are similar to those for pure HCC [8]. Although the pathogenesis is uncertain, there is evidence that progenitor or stem cells play a role in the cause of these lesions [2, 8].

Trabecular pattern is the most common in well and moderately differentiated HCCs [2]. HCCs could show a gland-like pattern (pseudo acinar), usually admixed with the trabecular pattern [2]. The most common pattern in poorly differentiated HCCs is solid or compact pattern [2]. In classical morphology, ICCs appear in small or wide glandular patterns, with very few micropapillaries, HCC-like trabecular pattern, and all mixed-mixed types. Most ICCs have a tubular pattern of growth with variable size lumina, although micropapillary, acinar or cord like patterns also occur [2, 4, 9]. The most typical form of classic cHCC-ICC demonstrates morphological and histochemical evidence of a mixture of hepatocellular and ductular (or glandular) elements throughout the tumour [2, 8]. Also, a new type of cHCC-ICC, showing stem cell properties, has been described [2].

There is no need for immunohistochemical markers in the diagnosis of classical HCC. However, in the presence of different patterns, it may sometimes be necessary to distinguish between different types. Confirmation of hepatocellular differentiation is easily provided by immunohistochemical staining with Hep Par (granular cytoplasmic staining) [2]. ICC is negative with Hep Par. CK 19 can be positive both in HCC and ICC [2, 8]. CK7 is mainly positive in ICC [8]. Combined HCC-ICC with stem cells has features of mature-appearing hepatocytes with peripheral clusters of small cells positive for stem cell markers such as CK7, CK19, and CD133, epithelial cell adhesion molecule (EpCAM), and CD56/ neural cell adhesion molecule (NCAM) [2, 7, 8, 10, 11]. A classical combined type is CD56 negative. In the literature, CD56, CK19, and p53 are found to be significant in terms of prognostic parameters [12, 13].

In all three tumours, the histological grade, tumour diameter, lymphovascular invasion, perineural invasion, portal venous or hepatic venous invasion, regional lymph node metastasis, tumour grade, and tumour size should be indicated as prognostic parameters in the report, according to the 8th edition of the TNM, AJCC Staging Manual classification [14]. In terms of prognosis, classical HCC is generally very poor, particularly for cases in which partial or complete portal vein thrombosis and ICC are associated with a high rate of fatality because of early invasion and widespread metastasis. The prognosis for patients with combined HCC-ICC without stem cell fea-

tures is thought to be worse than for pure HCC [2]. The prognosis of combined HCC-ICC with stem cell features is unknown [2].

In this study, the contribution of immunohistochemical markers and histological patterns in differential diagnosis of HCC, ICC, and cHCC-ICC types and the relationship between clinicopathologic features and prognostic parameters in all three tumour types, were investigated.

Material and methods

A total of 61 cases, with clinical follow-up of 50 cases with HCC diagnosis, 9 cases with ICC diagnosis, and 2 cases with cHCC-ICC diagnosis, between 2009 and 2013 were included in the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. The clinicopathologic features of these cases were determined. Haematoxylin-eosin preparations were re-evaluated by two pathologists in terms of histopathologic features. Then, formalin-fixed paraffin-embedded tissue samples, cut at 3 μ m thickness, were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by boiling at 98°C for 40 minutes in 0.01 mol/l sodium citrate buffer (pH 6.0), or in Tris/EDTA buffer (pH 9.0). Immunohistochemistry was performed using a Leica Bond Max Automated IHC/ISH Stainer. Primary antibody CK7 (Leica Novocastra, OV-TL, Mouse monoclonal; 1/50), CK19 (Leica Novocastra, b170, Mouse monoclonal; 1/50), CD56 (Leica Novocastra, CD56), Mouse monoclonal; 1/100), *Hep par-1* (Leica Novocastra, 0CH1E5, Mouse monoclonal; 1/50), and ve P53 (Leica Novocastra, DO-7, Mouse monoclonal; 1/100). As controls, gastric mucosa for CK7, skin for CK19, colon mucosa for CD56, liver tissue for Hep par-1, and hepatocellular carcinoma for p53 were used. The cases were evaluated as negative, with focal staining \leq 50% and strong staining $>$ 50%, according to staining patterns. Changes in the diagnosis with IHC were evaluated.

The differences in prognostic parameters between HCC and other types, after diagnosis with IHC, were evaluated. For evaluation of prognostic parameters, the tumours were divided into two groups, HCC and ICC. Because of the low number of cHCC-ICC, they were excluded from further analysis. The significant difference between overall survival (OS) and disease free survival (DFS) for two types of tumours were also evaluated. The effect of prognostic parameters on OS and DFS were evaluated independently of tumour type.

The SPSS 21 (Armonk, New York, USA) software was used for statistical calculations. OS and DFS

were compared using the Kaplan-Meier method, and the survival differences between the two groups were compared using the log-rank test.

The distribution was analysed with the help of the Kolmogorov-Smirnov test. The categorical variables were assessed using the χ^2 test. The quantitative variables were analysed by means of the independent simple T test. The OS and DFS rates among the patients were calculated by the Kaplan-Meier method and compared with the log rank test. The statistical significance was indicated by p values less than 0.05.

Results

The diagnosis changed in four cases after immunohistochemical (IHC) examinations. After IHC staining, the diagnosis was changed from HCC to ICC in one case and to cHCC-ICC in three cases. In 46 cases (75.4%), the HCC diagnosis remained unchanged. The histological features were trabecular in 36 cases (78%) and mixed (trabecular, tubular/pseudoacinar, solid) in 10 cases (22%) among the cases with

unchanged diagnosis of HCC (Fig. 1). The staining characteristics of HCC cases were all positive with Hep par-1, all negative with CK19 and CD56, and focal positively with CK7 and p53 in a few cases (Fig. 2). A mixed histological pattern was observed where the diagnosis changed to ICC. The histologic features of the 10 cases with ICC were 5 small glandular (50%), 3 large glandular (30%), and 2 mixed (large glandular, trabecular) type (20%). The staining properties of ICC were all negative with Hep par-1, all positive with CK7 and CK19, and positive with CD56 and p53 in some cases. A total of five cases, with three cases diagnosed after IHC, and two cases previously diagnosed with and before IHC, were accepted as cHCC-ICC. All cHCC-ICC cases were in mixed pattern histologically. All of the cases with Hep par-1, CK7, and CK 19 were positive; however, positive staining with p53 were seen in some of them. When the CD56 staining characteristics were examined, it was determined that staining was negative in two cases of classical cHCC-ICC and focal positive in 3 cases of cHCC-ICC with stem cell properties; however, due to their small number, they were collected

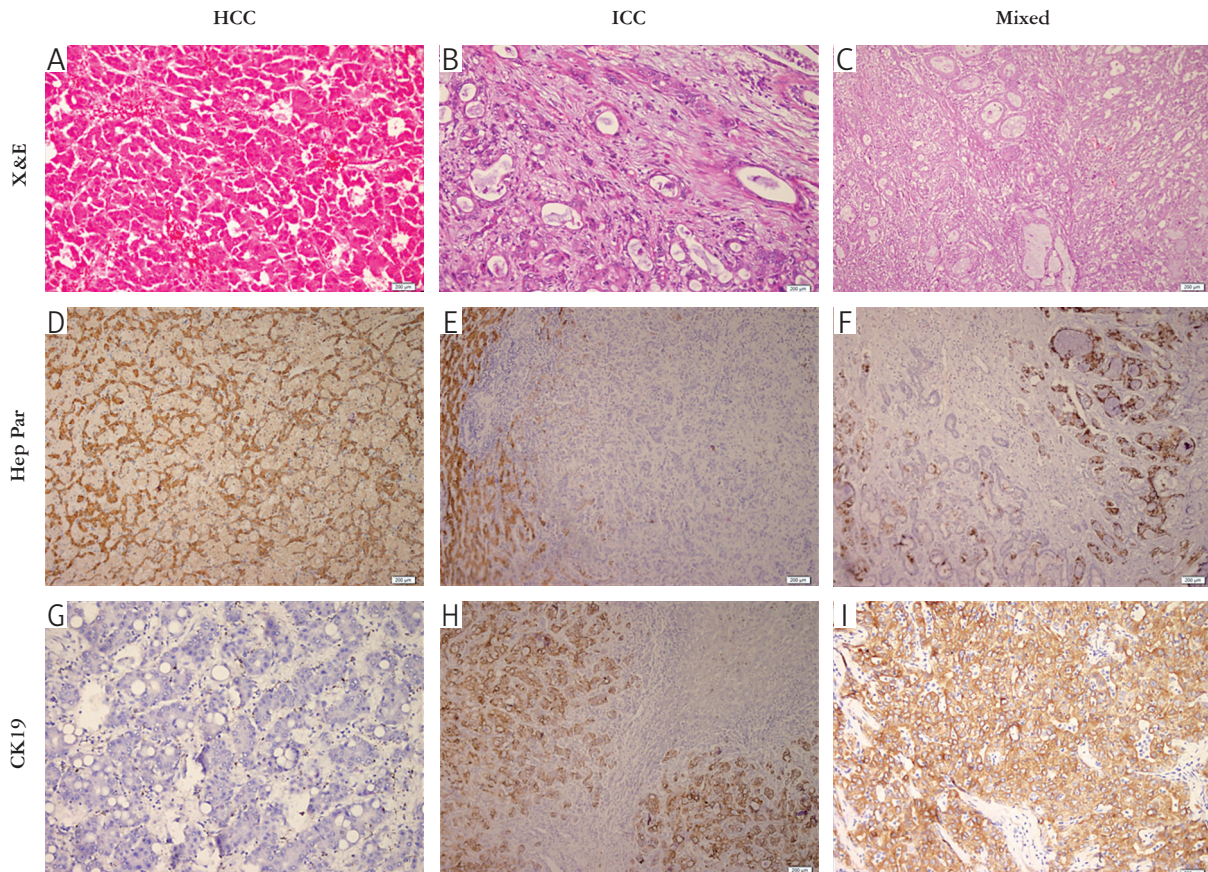


Fig. 1. The histologic features were: A) trabecular pattern, HE, 200 \times ; B) small glandular areas in ICC, HE, 100 \times ; C) and mixed (trabecular, tubular/pseudoacinar, solid) type, HE, 100 \times ; Hep par-1 stain: D) diffuse positivity in HCC, Hep par-1, 200 \times ; E) negative stain of Hep par-1 in ICC Hep par-1, 200 \times ; F) focal positivity in tumor cells in mixed type with Hep par-1 stain Hep par-1, 200 \times ; CK19 stain was: G) negative in HCC and positive in H) ICC and I) mixed type CK19, 200 \times

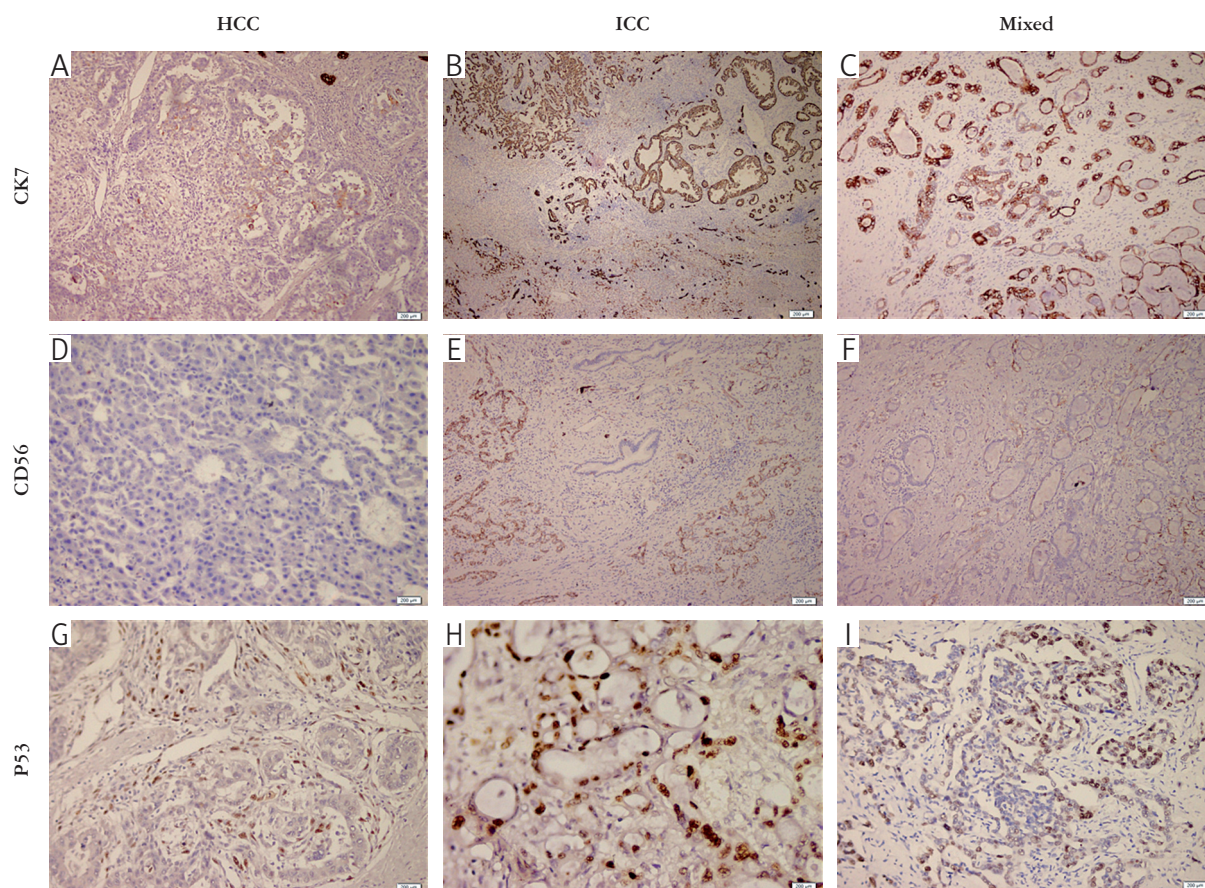


Fig. 2. CK7 stain was A) focal positive in HCC; B) diffuse positive in ICC and C) mixed type CK7, 200 \times ; CD56 stain was D) negative in HCC, 200 \times ; E) focal positive in ICC and F) in mixed type CD56, 100 \times ; P53 stain was focal positive in G) HCC, H) ICC and I) in mixed type, 200 \times

Table I. Distribution of cases before and after IHC

N	HCC	ICC	Mikst HCC-ICC	TOTAL N BEFORE IHC
HCC	46	1	3	50
ICC	–	9	–	9
Mikst HCC-ICC	–	–	2	2
Total n after IHC	46	10	5	61

within a group of cHCC-ICC. The cases with changed diagnosis and staining characteristics of all tumours are shown in Tables I and II, respectively.

In terms of their clinicopathological features, the analysis was conducted by dividing them into three groups: HCC, ICC and cHCC-ICC types, at the final diagnosis. All HCC cases were hepatectomized for the purpose of transplantation due to cirrhosis. Four cases of ICC and mixed types were developed due to cirrhosis. While most of the ICCs underwent partial hepatectomy, most of the mixed types underwent total hepatectomy. Other clinicopathological and histopathological features are summarised in Tables III and IV.

The OS of patients was 35.3 months (0 to 87, ± 27.176) and DFS was 33 months (0 to 87, ± 25.880). Patients who died and recurred during this period are identified in Table IV. HCC and ICC types were evaluated for statistical significance in recurrence, death, grade, angiolymphatic invasion (ALI), perineural invasion (PNI), lymph node metastasis, necrosis, grade, tumour diameter, and OS and DFS values. The grade and stage were significantly higher in HCC patients ($p = 0.000$ and $p = 0.003$, respectively). Lymph node metastasis and perineural invasion was common in tumour types other than HCC ($p = 0.032$ and $p = 0.000$, respectively). There were no significant differences between tumour types in terms of diameter ($p = 0.064$). The OS and DFS

Table II. Immunohistochemistry features of HCC, ICC and mixed type carcinomas

	HCC (N)	ICC (N)	MIXED (N)
Hep par-1			
Negative	0	10	0
≤ 50%	2	0	4
> 50%	44	0	1
CK 7			
Negative	41	0	0
≤ 50%	5	1	5
> 50%	0	9	0
CK 19			
Negative	46	0	0
≤ 50%	0	0	3
> 50%	0	10	2
CD 56			
Negative	46	6	2
≤ 50%	0	2	3
> 50%	0	2	0
P53			
Negative	43	0	1
≤ 50%	1	5	2
> 50%	2	5	2

values were significantly higher in HCC than in others ($p = 0.008$ and $p = 0.011$, respectively; Table V). However, no significant differences were found in the Kaplan-Meier life analysis between the tumour types and OS and DFS values (0.705 and 0.200 respectively; Figs. 3 and 4).

In the Kaplan-Meier survival analysis of OS and DFS with independent prognostic parameters, such as angiolymphatic invasion independent of tumour type, PNI, hepatoportal venous invasion (HPVI), lymph node metastasis, necrosis, tumour diameter, tumour foci, stage and grade, no significant results could be obtained. Only the relationship between angiolymphatic invasion with OS trended towards significance ($p = 0.052$).

P53 and CD 56 staining rates were evaluated in terms of prognostic parameters. In all tumour types, no significant relationships were found between CD 56 and death ($p = 0.433$), recurrence ($p = 0.314$), ALI ($p = 0.576$), PNI ($p = 0.703$), necrosis ($p = 0.637$), HPVI ($p = 0.791$), and lymph node metastasis ($p = 0.791$). While analysing the relation between p53 and prognostic parameters, only necrosis ($p = 0.027$) and lymph node metastasis ($p = 0.001$) were significant. No relationship was found between p53 and death ($p = 0.955$),

Table III. Clinical parameters of tumours

	HCC, N (%)	ICC, N (%)	MIXED, N (%)
Sex			
Female	6 (13)	5 (50)	0 (0)
Male	40 (87)	5 (50)	5 (100)
Age ±SD	56.63 ±8.46	52.5 ±11.12	54.20 ±9.98
Recurrence			
Yes	9 (20)	3 (30)	1 (20)
No	37 (80)	7 (70)	4 (80)
Exitus			
Yes	10 (22)	2 (20)	1 (20)
No	36 (78)	8 (80)	4 (80)
Stage			
Stage 2	18 (39.14)	2 (20)	3 (60)
Stage 3	10 (21.73)	4 (40)	0 (0)
Stage 4	15 (32.61)	0	0 (0)
Etiologic factor			
HBV	32 (69)	1 (10)	4 (75)
HCV	0 (0)	2 (20)	0 (0)
HBV + HCV	7 (15)	0 (0)	0 (0)
HBV + HDV	3 (6)	0 (0)	0 (0)
Alcohol	2 (4)	1 (10)	0 (0)
Unknown	2 (4)	6 (60)	1 (25)

recurrence ($p = 0.340$), ALI ($p = 0.381$), PNI ($p = 0.550$), and HPVI ($p = 0.550$). Also, no significant relationship was observed in the Kaplan-Meier life analysis between p53 and OS ($p = 0.419$) and DFS ($p = 0.348$).

Discussion

The three most common malign tumour types in the liver are HCC, ICC, and cHCC-ICC. Because the cHCC-ICC type is less common, the prognosis is not clear. ICC is known to have a poor prognosis with early invasion and metastasis. It has been reported that ICC and cHCC-ICC have worse prognosis than HCC [8, 15, 16, 17, 18]. In our study, HCC and ICC types were compared in terms of prognostic parameters. Statistically significant results were obtained between grade, lymph node metastasis, focality, perineural invasion, stage, overall survival, and disease-free survival. In our study, lymph node metastasis in HCC-diagnosed cases was as low as mentioned in the literature [15, 19]. The presence of lymph node metastasis and perineural invasion suggests poor prognosis in the non-HCC group [10]. Grade,

Table IV. Pathologic parameters of tumours

	HCC, N (%)	ICC, N (%)	MIXED, N (%)
Focality			
Single	19 (41.30)	9 (90)	3 (60)
Multiple	27 (58.70)	1 (10)	2 (40)
Tumor Diameter			
≤ 2 cm	2 (4.35)	2 (20)	0 (0)
2-3 cm	5 (10.87)	2 (20)	3 (60)
≥ 3 cm	39 (84.78)	6 (60)	2 (40)
Grade			
Grade 1	6 (13.04)	9 (90)	1 (20)
Grade 2	35 (76.09)	1 (10)	3 (60)
Grade 3	5 (10.87)	0(0)	1 (20)
ALI			
Yes	7 (15.22)	1 (10)	0
No	39 (84.78)	9 (90)	5 (100)
PNI			
Yes	0	4 (40)	0
No	46 (100)	6 (60)	5 (100)
Lymph node metastasis			
Yes	0	1 (10)	1 (20)
No	46 (100)	9 (90)	4 (80)
HPVI			
Yes	2 (4.35)	0 (0)	0 (0)
No	44 (95.65)	10 (100)	5 (100)
Necrosis			
Yes	13 (27.66)	5 (50)	3 (60)
No	33 (72.34)	5 (50)	2 (40)
Histology			
Trabecular	36 (78)	0 (0)	0 (0)
Mixed trabecular	10 (22)	0 (0)	0 (0)
Big glandular	0 (0)	3 (30)	0 (0)
Small glandular	0 (0)	5 (50)	0 (0)
Mixed glandular	0 (0)	2 (20)	5 (100)

ALI – angiolymphatic invasion; PNI – perineural invasion; HPVI – hepato-portal vein invasion

tumour foci, and stage were higher in HCC than for other tumour types. In accordance with the literature, in our study, most of the cases with HCC and cHCC-ICC were grade 2 [3, 15, 17]. Unlike other studies in the literature, multifocality of HCC type was found more frequently in our study [3]. In the literature, HCC is generally reported at early stages (stage I/II), whereas in our study the patients were at more advanced stages (stage III-IV) [3, 10]. Patients in cHCC-ICC were diagnosed mainly at

Table V. The difference of prognostic factors between HCC and ICC

PROGNOSTIC PARAMETERS	HCC (N)	ICC (N)	P
ALI	7	1	0.672
PNI	0	4	0.000
HPVI	2	0	0.506
Lymph node metastasis	0	1	0.032
Necrosis	13	5	0.186
Focality	27	1	0.006
Stage (III-IV)	25	4	0.003
Grade (2-3)	40	1	0.000
Recurrence	9	3	0.470
Exitus	10	2	0.904
Age	56.6	52.5	0.192
DFS	38.7	15.8	0.011
OS	40.7	17.6	0.008

ALI – angiolymphatic invasion; PNI – perineural invasion; HPVI – hepato-portal vein invasion; DFS – disease free survival; OS – overall survival

stage II instead of stage I [17]. Important parameters affecting stage in HCC were tumour diameter and multifocality. Patients with one HCC of 5 cm or smaller, or up to three nodules of 3 cm or smaller, without vascular invasion or extrahepatic spread, had a 4-year survival rate of 75% with a recurrence rate of below 15% [20]. Multifocality is associated with high recurrence and impaired survival [20]. The mean HCC diameter was higher in our study compared to the other tumour types. In our study, the average tumour diameter in HCC was found to be 7.7 cm. In the literature, this size was between 3.1 and 6.4 cm on average [6, 15, 19, 21]. In ICC and cHCC-ICC types, our average tumour diameter was smaller in magnitude than the tumour diameters in the literature [18, 21]; the stage was lower in these tumours. We found no significant correlation between diameter and tumour type. OS and DFS were statistically significant in two tumour groups. In our study, OS in HCC was 40.7 months, OS in cHCC-ICC was 20 months, and OS in ICC was 17.6 months. These findings support a higher OS in HCC than ICC [6, 15, 16, 17, 18]. OS was longer in HCC patients, but not significant in the Kaplan-Meier life analysis. This may be because HCC cases were at more advanced stages than the cases in other studies in the literature.

When we examined the clinicopathological parameters, the male sex was more pronounced in all tumour types, which is consistent with the incidence in Asian countries in terms of mean age and sex [2, 6, 19]. HBV was detected most frequently in HCC as well, which is consistent with the fact that HBV

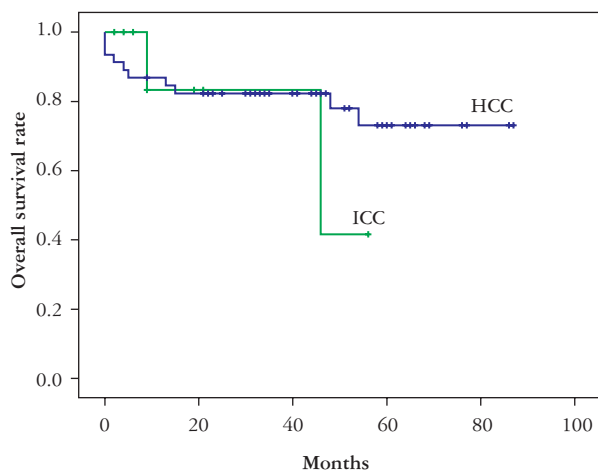


Fig. 3. Overall survival rate of HCC and ICC tumours

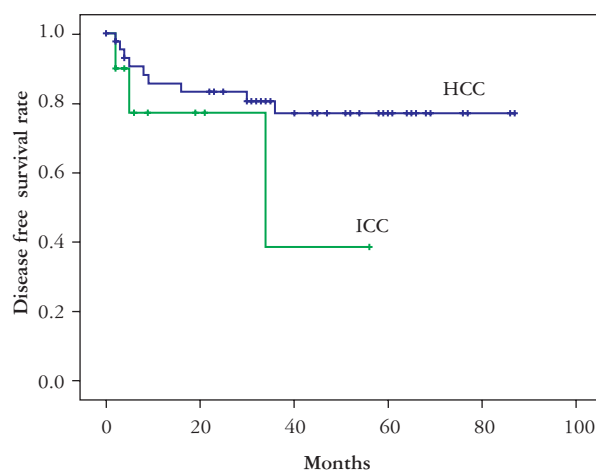


Fig. 4. Disease free survival rate of HCC and ICC tumours

is the most common cause in developing and Asian countries [8, 19].

When we evaluated the histological pattern with IHC, Hep par-1, which stains hepatocyte-derived cells, was positive in the classic trabecular and mixed pattern in HCC [22]. With CK19 and CK7 staining, which are for biliary epithelial cells, only two cases of focal positivity with CK7 was observed. These two cases demonstrated a mixed pattern. In typical trabecular pattern, CK7 and CK19 was negative. However, there are also publications in the literature that find CK19 positive in HCC [21]. In our study, when the IHC was re-evaluated in the previously diagnosed tumours, the diagnosis was changed only in four patients, who underwent HCC diagnosis and showed were more mixed type areas. The IHC study should be conducted as required after pattern analysis. In particular, tumours of mixed pattern should receive support from IHC.

Studies have shown that CK 19 positivity is associated with a poor prognosis in HCC [2, 23]. There are studies that associate CK19 positivity with a grade of HCC, but they did not show a significant relationship [20, 23]. All HCC cases were negatively stained with CK19. Because CD56 was positive in stem cell, it can be used for diagnosis of stem cells, subtype of cHCC-ICC [5, 10]. In our study, prognostic parameters were also compared with CD56, but no significant results were obtained. This could be due to the lower number of cases both in our study and in the literature [13, 21]. The literature suggested that p53 is a prognostic marker between stage, metastasis, and OS [24]. In our study, we obtained significant results only with tumour necrosis and lymph node metastasis with p53.

The vascular pattern is important in the prognosis of HCC [25, 26]. In particular, sinusoidal neovascularisation pattern has worse prognosis than capillary pattern in terms of DFS and OS [25]. For evalua-

tion of vascular pattern CD34 and CD31 could be used [25, 26]. Also, Glypican-3 (GPC-3) which plays an important role in regulating cells growth, could also be used for prognosis [27, 28]. It is postulated that GPC-3 is an important molecule for differentiation of dysplastic nodules from HCC and determination of poor prognosis but more studies are needed to confirm these results [27, 28].

In our study, although the sample size was small, the most common types of liver primary carcinomas were examined in a wide spectrum of clinicopathological and prognostic parameters. When HCC and other tumour types were compared in terms of prognostic parameters, perineural invasion and lymph node metastasis were higher in the non-HCC group, whereas the stage, grade, and multifocality were higher in the HCC group. There was no significant difference in tumour types between DSF and OS. The HCC patients in our study were considered to have worse OS than expected because they were in an advanced stage compared to many studies in the literature. When tumour types were diagnosed, we suggested that IHC should be studied especially in morphologically mixed cases. As a prognostic marker, p53 was more significant than CK 19 and CD 56.

The authors declare no conflict of interest.

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