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Combined Use of B7-H4 and Dickkopf-1 for Accurate Diagnosis of Hepatitis C-Related Hepatocellular Carcinoma

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Abstract: Despite the advent of newer imaging modalities for accurate and earlier hepatocellular carcinoma (HCC) detection, no method is sensitive enough. Here, we aimed to evaluate the accuracy of B7-H4 and dickkopf-1 (DKK1) combination for early HCC differentiating from premalignant liver cirrhosis or fibrosis. A total of 210 chronic hepatitis C (CHC) patients (110 with HCC and 100 were non-HCC patients (45 cirrhosis and 55 fibrosis) were included. Both B7-H4 and DKK1 were screened for all cases. HCC patients were distinctly ($P<0.05$) associated with high B7-H4 (44.8±5.3) and DKK1 (5.4±2.1) levels (ng/mL) compared to cirrhotic (38.9±4.6, 2.6±0.8, respectively) and fibrotic (33.5±6.6, 2.2±0.6, respectively) controls. For separating HCC, both B7-H4 (AUC=0.863) and DKK1 (AUC=0.852) had a good diagnostic power superior to AFP (AUC=0.802). Multiplying B7-H4 with DKK1 exhibited values that significantly ($P=0.0001$) increased in HCC patients (192.3 (120.2-380.1)) versus patients with hepatic cirrhosis (99.9 (85.6-111.2)) and fibrosis (77.2 (63.1-100.1)). This index improves HCC diagnostic performances [AUC=0.892; sensitivity 80.9%, specificity 77%, PPV 79.5%, NPV 78.6% and efficiency 79.1%]. Elevated B7-H4×DKK1 values were strongly correlated with aggressive features including multiple lesions, large size, Child-Pugh and BCLC late stages. In conclusion, B7-H4×DKK1 is reliable, feasible and simple HCC diagnostic blood based method that could improve diagnostic sensitivity and accuracy and could represent an addition in follow up and management of cases with premalignant liver disorders.

Keywords: Hepatocellular carcinoma; Hepatitis C virus; Diagnosis; B7-H4; DKK1.

Introduction

Worldwide, HCC is one of the most serious health problems, it is globally the sixth most frequent tumor and the fourth reason of tumor-related death [1]. Beside non-alcoholic steatohepatitis, hepatitis C (HCV) and B (HBV) viruses are remains the most noted HCC risk factors [2] as both increase HCC risk by 20-fold [3]. In Egypt, the most critical HCC risk factor is HCV [4].

Although the survival rate is about 5 years, if HCC still untreated this rate is assessed to be 18%, and the median survival is only seven months [1, 5]. HCC cases are usually diagnosed at advanced stages when treatment options are very limited and treatment is restricted to multi-kinase inhibitors and the systemic therapy which are related to adverse side effects [1, 6]. Owing to relatively low sensitivity of most HCC diagnostic approaches including imaging modalities, α-fetoprotein (AFP) and other tumor markers, there were only small percentage (about 12%) of cases could be detected via current surveillance guidelines [6, 7]. Therefore for early HCC diagnosis, there is a great clinical urgent for identification of potential new efficient markers [8].

B7 homolog 4 (B7-H4), also known as B7S1, B7x or VTCN1, is a serious member of B7 family co-regulatory ligands [9]. By controlling T cells activation, cytotoxicity development and cytokine secretion, B7-H4 could negatively modulate T cell-mediated

immunity [10]. In many normal human somatic tissues, expression of B7-H4 cell surface protein is greatly absent, whereas overexpressed in several tumors including HCC [11], esophageal, pancreatic, gastric, renal, prostate, uterus, breast, ovarian, and lung tumors [10, 12, 13]. By binding to an unknown receptor, previously studies have reported that B7-H4 could be important driver in human carcinomas development and progression [13]. Although the potential mechanism remains elusive, there is only some evidence suggesting that blood B7-H4 appears to be a candidate therapeutic target and a potential biomarker for HCC [11, 14, 15].

On the other hand, Dickkopf-1 (DKK1) is soluble low molecular weight secreted protein that typicaly acts as Wnt/β-catenin pathway inhibitor [16, 17]. This plasma protein was reported to be implicated in management of several pathological and physiological processes, including osteoclastogenesis, embryonic development, head formation and tumor cell related processes (migration, survival, proliferation and invasion) [18]. Indeed, DKK1 acts as proto-oncogene in HCC and it is highly expressed in this tumor and involved in HCC aggressiveness [19, 20]. In HCC, DKK-1 was reported to be related to poor prognosis, recurrence, metastasis and carcinogenesis [21].

Combining varied tumor markers with each others could enhance the sensitivity of HCC diagnosis [7]. Thus in Egyptian patients with CHC, the aim of this study was to evaluate the value of combined serum B7-H4 with DKK1 in diagnosis of HCV-related HCC in a trial to improve the diagnostic sensitivity of HCC.

Subjects and methods Patients

Ethical consideration

The study protocol was approved by Institutional Research Board, Mansoura University Hospitals, Egypt and conformed to 1975 Helsinki ethical guidelines. From all cases, informed consents were assigned regarding the involved research procedures.

Patients

Before any intervention, a total of 210 Egyptian CHC patients were included in this retrospective hospital-based study. Pathologically, they were classified into 110 HCC patients and 100 non-HCC patients (45 with cirrhosis and 55 with liver fibrosis). All cases were collected from Gastrointestinal Surgery Center, Mansoura University Hospitals, Egypt. Diagnosis of HCC was mainly via imaging approaches like computed tomography and magnetic resonance imaging and, if acceptable, liver biopsy was used for HCC histopathology confirmation. None of the included cases underwent any therapy intervention [radiofrequency, surgical interference, chemotherapy, transarterial embolization]. Some tumor features such as number of nodules and tumor size were collected from patients' reports. Also, two common HCC staging systems: Child-Pugh [22, 23] and Barcelona clinic liver cancer (BCLC) [24] were assessed. Patient with any other chronic diseases or malignant tumors were excluded.

Sample size

Using MedCalc (Belgium) software, sample size was assessed according to former demonstrated difference of mean serum level of B7-H4 between HCC and controls [15] and according to previously reported area under receiver operating characteristics (ROC) curve (AUC) for DKK1 [25] in HCC diagnosis. Significance (α) level of 5% and a statistical power (1-β) level of 80% were used. To achieve confidence range, the highest reported sample size was 30 (15 HCC and 15 controls), so the sample size of our study (n=210) was very sufficient to perform statistical analysis.

Biochemical measurements

From all cases, blood (5mL) was withdrawn and one part was used to obtain fresh serum, another part was treated with KEDTA for complete blood count analysis (Sysmes, Japan) and the final part was sodium citrate treated and used for measuring prothrombin international normalized ratio (INR). Fresh sera and automated biochemistry analyzer (A15, Biosystem, Spain), were used for liver enzymes [alanine (ALT) and aspartate transaminases (AST) and alkaline phosphatase (ALP)], bilirubin, albumin and creatinine measurments. According to the manufacturer's instructions, human commercial ELISA kits (Bioassay Technology Laboratory, Shanghai, China) were

used to detect serum B7-H4 (Cat E0630Hu) and DKK1 (Cat E0630Hu). AFP was measured using chimiluminescence immunoassay (Siemens, Germany).

Statistical analysis

Both SPSS v.20.0 (SPSS, Chicago) and GraphPad Prism v.8.0 (GraphPad, San Diego) were used for all statistical analyses. Results were expressed as absolute numbers, median (interquartile range (IQR)) or mean±standard deviation (SD), appropriately. Differences were compared using *ANOVA* and *Kruskal-Wallis* tests, appropriately follwed by LSD as post-hoc test. The diagnostic power of each biomarker and their combination was assessed by and cutoff points were determined based on the point closest to the (0, 1) point [26]. Diagnostic performances were derived from a 2×2 contingency table. Logistic regression index including independent HCC discriminatory factors was constructed. Very small non-significant coefficients and constants were removed for simplification, and the diagnostic ability was not affected.

Results

Patients' clinical characteristics

Patients' characteristics and clinicopathological data were summarized in Table 1. HCC cases were older than cirrhotic and fibrotic controls. Also, they were related to elevated enzymes activities and bilirubin and AFP concentrations. HCC patients had lower serum albumin, haemoglobin levels and red cell counts in comparison to liver cirrhosis and fibrosis. Tumor features including nodules number, tumor size and Child-Pugh and BCLC stages were shown in Table 1.

Serum B7-H4 and DKK1 levels and HCC development

HCC cases had distinctly (P<0.05) higher serum B7-H4 (44.8±5.3 ng/mL; Figure 1A) and DKK1 (5.4±2.1 ng/mL; Figure 1B) levels compared to cirrhotic (38.9±4.6, 2.6±0.8 ng/mL, respectively) and fibrotic (33.5±6.6, 2.2±0.6 ng/mL, respectively) controls. Compared to AFP (AUC=0.802), both B7-H4 (AUC=0.863; Figure 1C) and DKK1 (AUC=0.852; Figure 1D) had a good superior diagnostic ability for separating HCC from all non-HCC patients (Fibrosis and cirrhosis combined) as revealed by ROC analysis.

Diagnosis accuracy was improved with model development

The best mathematical score was developed based on linear logistic regression analysis: [B7-H4×DKK1]. Multiplying B7-H4 with DKK1 yielded values that significantly (P<0.05) elevated in HCC cases (192.3 (120.2-380.1)) versus patients with hepatic cirrhosis (99.9 (85.6-111.2)) and fibrosis (77.2 (63.1-100.1)) (Figure 2A).

Compared to each marker alone, B7-H4×DKK1 had an AUC equal to 0.892 for separating HCC from non-HCC patients (Figure 2B). As shown in Table 2, the index had sensitivity, specificity, PPV, NPV and efficiency for HCC detection of 80.9, 77, 79.5, 78.6 and 79.1%, respectively. Interestingly, elevated B7-H4×DKK1 values were associated with aggressive tumor features including multiple nodules (Figure 3A), large tumor size (Figure 3B) and Child-Pugh (Figure 3C) and BCLC (Figure 3D) late stages.

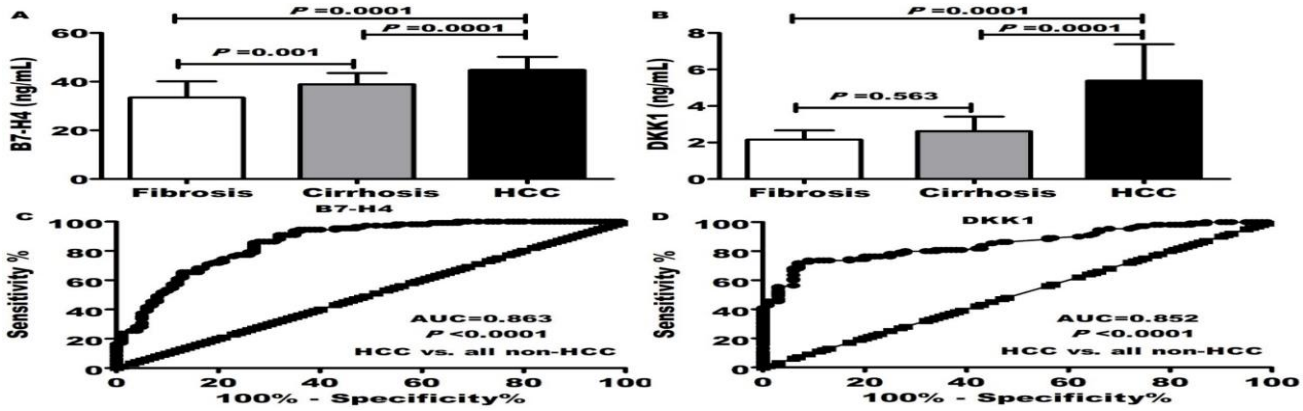

Figure 1. (A) B7-H4 and (B) DKK1 were related to HCC. Receiver-operating characteristic curve revealed that both (C) B7-H4 and (D) DKK1 had a good ability to discriminate HCC from non-HCC patients (fibrosis and cirrhosis combined). P<0.50 is considered significant.

Table 1. Patient's clinical characteristics

Variables	Fibrosis	Cirrhosis	HCC	*P* value
Gender (male/female)	21/34	18/27	31/79	0.092
Age (years)	45.1±7.0	51.6±7.1	54.3±9.8	0.001
ALT (U/L)	58.7 (52-71)	55.45 (40-74)	66 (43-78)	0.027
AST (U/L)	48.6 (39-62)	70 (59-94)	80 (61-102)	0.001
ALP (U/L)	86 (57-104)	105 (86-128)	168.4 (116-277)	0.001
Albumin (g/dL)	4.21±0.56	3.6±0.63	3.21±0.61	0.001
Bilirubin (mg/dL)	0.8±0.29	1.7±0.31	2.4±0.61	0.001
Creatinine (mg/dL)	0.80±0.16	0.81±0.14	0.94±0.22	0.059
INR	1.14±0.07	1.36±0.14	1.39±0.20	0.001
α-fetoprotein (ng/mL)	4.0 (2-122)	139.1 (79-407)	266 (225-486)	0.005
Hemoglobin (g/dl)	14.18±1.35	11.56±2.08	11.55±2.49	0.001
Red blood cells ($x10^{12}$/L)	5.1 (4.6-5.3)	5.5 (3.8-5.8)	4.1 (3.5-5.2)	0.038
White blood cells ($x10^{9}$/L)	6.1 (5-7.3)	3.9 (3.5-4.2)	4.3 (3.5-4.7)	0.644
Platelets ($x10^{9}$/L)	182 (148-215)	117 (68-165)	76 (62-112)	0.001
Size small (<2.5 cm)/large (>2.5 cm)	—	—	30/80	—
Lesion(s) single/multiple	—	—	60/50	—
Child-Turcotte-Pugh (A/B/C)	—	—	49/31/30	—
BCLC (A/B/C/D)	—	—	28/26/23/33	—

Normally and non-normally distributed variables were expressed as mean±SD and median (interquartile range), respectively. Significant differences were determined using Chi-squared (X^2) *ANOVA* and *Kruskal-Wallis* test, appropriately. $P<0.05$ was significant. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; BCLC: Barcelona clinic liver cancer staging system.

Table 2. Diagnostic performances for HCC detection

Categories	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
B7-H4≥ 40.1ng/mL	0.863(0.81-0.91)	79.1	74	77	76.3	76.7
DKK1≥ 2.6 ng/mL	0.852(0.80-0.90)	78.2	75	77.5	75.8	76.7
B7-H4×DKK1≥ 110 ng/mL	0.892(0.85-0.93)	80.9	77	79.5	78.6	79.05
AFP≥ 240 U/L	0.802(0.76-0.88)	72.7	75	76.2	71.4	73.8

Cutoff values were obtained from ROC analysis. HCC patients were compared to all non-HCC patients. PPV: Positive predictive value; NPV: Negative predictive value.

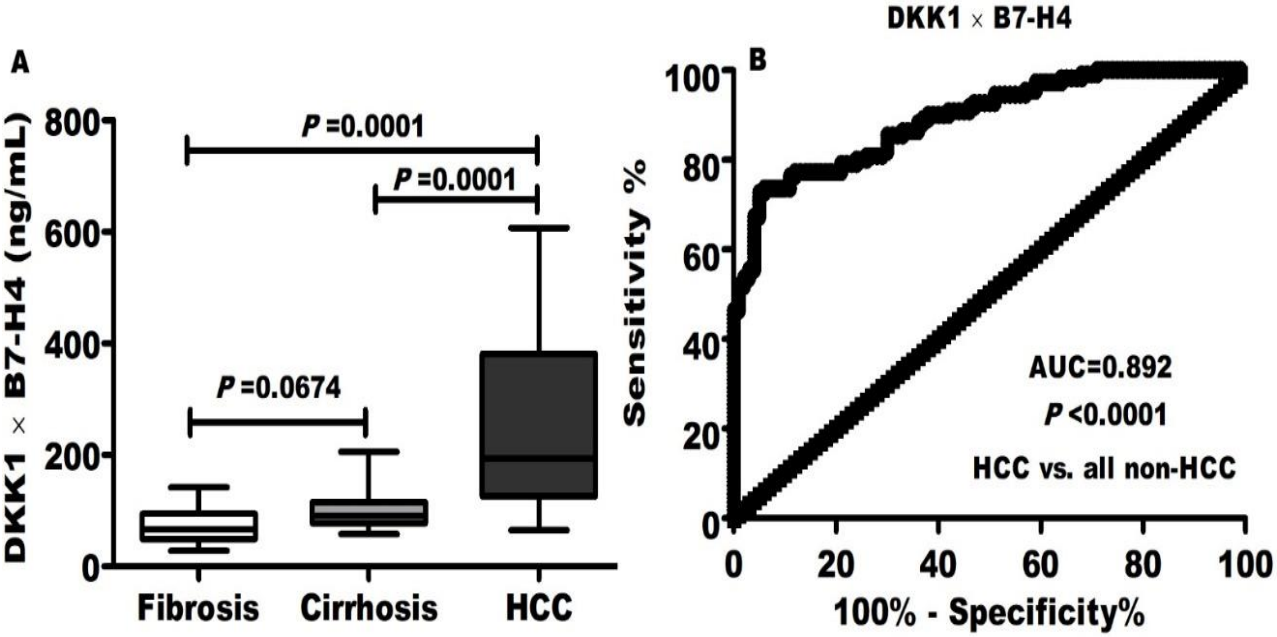

Figure 2. Despite each marker alone, (A) multiplying B7-H4 with DKK1 levels was more correlated with HCC. (B) This index improves the diagnostic power for HCC detection. P<0.50 is considered significant.

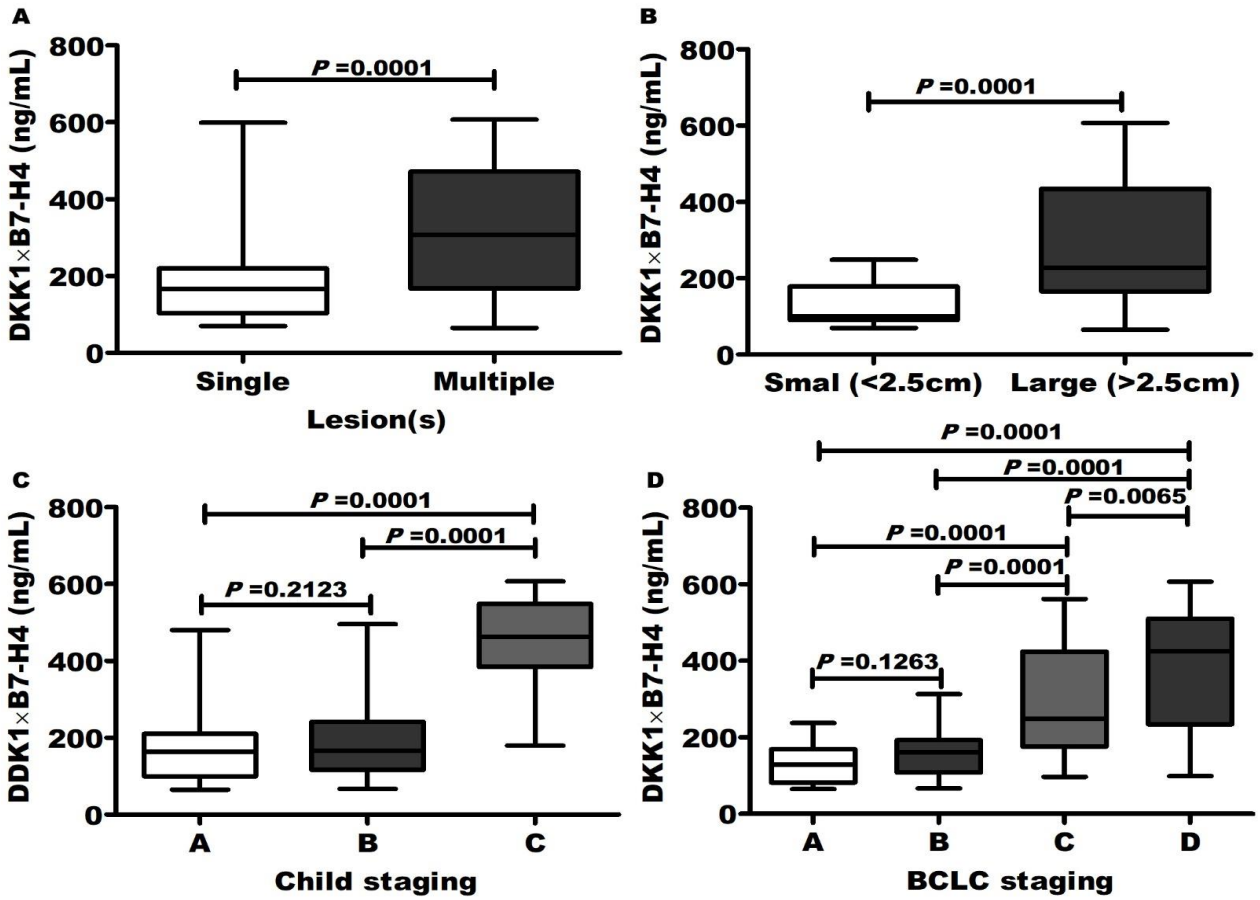

Figure 3. Elevated B7-H4×DKK1 was associated with tumor aggressiveness including (A) multiple lesions, (B) large size and (C) Child and (D) BCLC late stages**.** *P*<0.50 is considered significant.

Discussion

Among hepatitis high risk patients, AFP is recommended for HCV cases surveillance even alone or combined with liver radiological modalities [27]. For early HCC detection and owing to AFP low sensitivity for accurate HCC detection, many trials were applied to improve AFP sensitivity [27]. In patients with HCC, evaluation and validation of reliable novel markers for HCC can enhance early diagnosis and ultimately survival [28]. In this research, we determined the performance of the combination between B7-H4 and DKK1 for early HCC detection in a cohort of Egyptian CHC patients in a trial to improve the diagnostic accuracy.

Our findings revealed that both serum B7-H4 and DKK1 were distinctly (P<0.05) elevated in HCC cases compared to cirrhotic and fibrotic controls. As revealed by ROC analysis, both B7-H4 (AUC=0.863) and DKK1 (AUC=0.852) outperformed AFP (AUC=0.802) for HCC diagnosis.

In agreement with our results, some previous studies demonstrated that serum B7-H4 detection might serve as a clinical predictor in HCC diagnosis [11, 29]. In HCC patients, Zhang et al. found that B7-H4 blood levels were significantly greater than healthy controls and its high levels were markedly related to higher recurrence probability and poor survival [29]. Also in contrast to normal human liver tissues, Yuan et al. observed that B7-H4 was expressed in HCC patient tissues and HCC cell lines [30]. In the same line, Kang et al. found that B7-H4 was highly overexpressed in HCC cells and tissues [11]. By inducing apoptosis *in vitro*, they found that B7-H4 downregulation notably inhibited HCC cell stemness, invasion and growth [11]. By Western blotting and Flow cytometry, B7-H4 expression was detected in several HCC cell lines compared to normal LO2 cell line [31]. They found that B7-H4 expression Knockdown induced HCC cells apoptosis and elevated several apoptosis-related proteins, including Bax, cleaved caspase-7, cleaved caspase-3 and surviving [31]. Moreover in nude mice, siRNA intra-tumor *in*

vivo injection targeting B7-H4 can markedly suppressed HepG2 cells growth [31]. Similarly, other study demonstrated that blocking B7-H4 channel promotes HCC autophagy and cell apoptosis via PI3K signaling pathway and thus might be a potential HCC therapeutic strategy [32].

On the other hand, in a variety of human solid cancers, DKK1 expression dysregulation has been suggested [33]. Despite weakly expression in cirrhotic tissues, Zhang et al. reported by using immunohistochemistry that DKK1 was upregulated in HCC tissues and its expression was related to lesions number and size [33]. They found in HCC cells that DKK1 genetic depletion impaired cancer formation, invasion, colony forming ability and the proliferation [33]. Other similar previously study reported that DKK1 was upregulated in HCC [34]. DKK1-mediated HCC cells tumorigenicity and proliferation may be related to Wnt/β-catenin signaling pathway [33]. Other reports demonstrated that DKK1 oncogenic effect in HCC may be via upregulating oncogenes expression, downregulating tumor suppressor genes and promotes inflammation, migration and tumor invasion [35]. DKK1 may enhance tumor invasion and migration via TGF-β1 by inducing inflammation and tumor microenvironment remodelling [35].

To overcome limitation in HCC diagnosis and improve the diagnostic ability, combining varied tumor markers with each others could enhance the sensitivity of HCC diagnosis [7]. In this study, logistic regression analysis showed that multiplying B7-H4 with DKK1 [B7-H4×DKK1] is the best mathematical index for diagnosing HCC. Index values were distinctly (P=0.0001) higher in HCC (192.3 (120.2-380.1)) patients compared to non-HCC fibrotic (77.2 (63.1-100.1)) and cirrhotic (99.9 (85.6-111.2)) patients with the highest diagnostic ability (AUC=0.892) compared to each protein alone. Its sensitivity, specificity, PPV, NPV and efficiency for HCC detection were 80.9, 77, 79.5, 78.6 and 79.1%, respectively. Interestingly, increased values of B7-H4×DKK1 were related to aggressive tumor including multiple lesions, large size and Child-Pugh and BCLC late stages.

In the line of our results, Kang et al. found that B7-H4 overexpression strongly associated with HCC TNM stage, early recurrence and overall survival [11]. Similarly in HCC patients, Yuan et al. reported that B7-H4 expression level was positively associated with differentiation degree, lymph node metastasis and TNM stage [30]. Serum DKK-1 levels also were reported to be correlated with HCC progression, TNM stage and BCLC staging as DKK1 was higher in late compared to early stages [25]. B7-H4×DKK1 promising diagnostic performances were comparable to other models combining them with other established HCC tumor markers [36].

Conclusion and recommendations

In conclusion, our findings suggested a significant diagnostic accuracy in HCC early diagnosis of B7-H4 and DKK1. In addition, multiplying serum B7-H4 with DKK1 levels developed a novel index for HCC diagnosis that improves the diagnostic sensitivity and may prove to be useful in early HCC detection and screening. Further confirmation studies in a multicentre larger cohort are needed.

Conflict of interest none

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