RESEARCH ARTICLE

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The Combined Use of Dickkopf-1 and Soluble Axl Improves Hepatocellular Carcinoma Diagnostic Efficacy in Hepatitis C Patients

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Abstract

Background: Standard tools are not sensitive enough for hepatocellular carcinoma (HCC) early detection. This study aimed to evaluate the accuracy of dickkopf-1 (DKK1) and soluble Axl (sAxl) and their combined for early differentiating of HCC from premalignant benign liver diseases. **Methods:** A total of 210 chronic hepatitis C (CHC) patients (55 fibrotic, 45 cirrhotic and 110 HCC) were enrolled. Both DKK1 and sAxl were tested using ELISA for all participants. **Results:** HCC patients were accompanied by a significant increase (P<0.05) in DKK1 (5.38±2.05 ng/mL) and sAxl (178.02±49.39 ng/mL) compared to patients with fibrosis (2.16±0.6, 97.63±19.71 ng/mL, respectively) and cirrhosis (2.62±0.8, 121.84±34.66 ng/mL, respectively). Both DKK1 (AUC=0.852) and sAxl (AUC=0.882) had a good diagnostic accuracy in separating HCC from all non-HCC patients. Multiplying DKK1 with sAXL yielded values that significantly (P=0.0001) increased in patients who developed HCC (674.3 (434.2-1413.9)) versus fibrotic (204.9 (161.7-262)) and cirrhotic (254.4 (205.4-343.7)) patients. This model improves HCC diagnostic performances [AUC=0.921; sensitivity 90.9%, specificity 87%, PPV 88.5%, NPV 89.7% and efficiency 89.1%]. Elevated DKK1×sAxl values were associated with aggressive tumor features including multiple nodules, large size, Child-Pugh and BCLC late stages. **Conclusions:** combined use of DKK1×sAxl is simple and feasible HCC diagnostic model that could enhance HCC diagnostic accuracy and could replace AFP in follow up of patients with premalignant diseases.

Keywords: HCC- HCV- early detection- DKK1- soluble Axl

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Introduction

Liver cancer incidence is rising worldwide and is estimated to reach more than one million cases by 2025 [1]. HCC is the most frequent primary liver tumor and accounts for ~90% of liver cancer patients and is related to a high mortality and morbidity rates and healthcare expenditure [2, 1]. Although HCC incidence displays strong male preponderance and progressively elevates with advancing age [1], viral infections hepatitis C (HCV) and B (HBV) beside non-alcoholic steatohepatitis (NASH) are remains the most prominent HCC risk factors which can resulted in hepatic fibrosis and cirrhosis [3].

Despite achievements in disease treatment, HCC is still tackled with high recurrence and low remission and survival rates [4]. Moreover, at times of diagnosis, 40-50% of HCC patients are at advanced stages and their treatment options are very limited [5]. Liver cirrhosis is the most common premalignant event and the main HCC risk factor [6]. Compared to non-cirrhotic patients (0.5-1.0%), HCC incidence reaches 3-6% in cirrhosis patients [5]. Despite many available treatment options, in such patients HCC prognosis remains generally poor and is largely related to late diagnosis [7]. Owing to relatively low sensitivity of imaging tools and tumor markers including α-fetoprotein (AFP), there were only up to 12% of HCC patients could be diagnosed through current surveillance recommendation [5, 8]. Thus, there is great clinical urgency in identification of potential new efficient approach for early HCC diagnosis [9].

Dickkopf-1 (DKK1) is typicaly secretory antagonist of the Wnt signaling pathway [10]. Regarding tumorigenesis, there are conflicting reports on DKK1 effects in tumor suppressive or oncogenic activities; these opposing effects may be owing to underlying genetic components and the cell type [11]. Indeed, DKK1 acts as proto-oncogene in HCC and it is highly expressed in this tumor and involved in HCC aggressiveness [11, 12]. From another hand, one of the TAM receptors family is tyrosine kinase Axl receptor which is comprised of Mer, tyro3 and Axl

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[13]. Axl is stimulated by binding of ligand growtharrest specific protein six causing downstream signaling and dimerization, which enhanced tumor invasion, survival, proliferation and metastasis [14]. Moreover, proteolytic processing of Axl results in the release of soluble protein (sAxl) of 80-kDa that can be detected in serum [15]. Compared to AFP, some studies demonstrated that serum levels of sAxl had superior accuracy than for HCC early diagnosis [14, 16].

Despite the promising reported association between these proteins and HCC, an opposite results may be obtained by other studies [17]. However, there is an agreement

between most of these studies that combining these tumor markers with each others could enhance the sensitivity of HCC diagnosis [8]. Thus, in this study, we aimed to evaluate the role of serum DKK1 and sAxl in diagnosis of HCV-related HCC in Egyptian patients with CHC infection. Moreover, we aimed to evaluate a new HCC diagnostic model based mainly on combination of these proteins to improve the diagnostic sensitivity of HCC.

Materials and Methods

Patients

This is a retrospective hospital-based study included a total of 210 CHC collected from Gastrointestinal Surgery Center, Mansoura University Hospitals, Egypt. The study protocol was conformed to 1975 Helsinki ethical guidelines and approved by Mansoura University Hospitals Ethics and Scientifc Committees. Patients were pathologically classified into 55 with liver fibrosis, 45 with liver cirrhosis and 110 HCC patients. HCC was diagnosed based on imaging tests such as computed tomography and magnetic resonance imaging. If available and acceptable, HCC was histopathology confirmed with liver biopsy. Data about number of nodules, tumor size, Child-Pugh [18, 19] and Barcelona clinic liver cancer (BCLC) [20] staging systems were collected and evaluated from patients' reports. Patient with any other malignant tumors were excluded. None of the patients underwent surgical interference or radiofrequency ablation, or had received chemotherapy or transarterial embolization. From all participants, informed consents were obtained concerning the disease nature and the involved diagnostic procedures.

Sample size

Using MedCalc (Belgium) software, sample size was calculated based on former demonstrated area under receiver operating characteristics (ROC) curve (AUC) for both DKK1 [21] and sAxl [14] in HCC diagnosis. Null hypothesis for AUC value was 0.5. Significance (α) level of 5% and a statistical power (1-β) level of 80% were used. The sample size of 30 (15 disease and 15 controls) was required to achieve confidence range, so the sample size of our study (n=210) was very sufficient to perform statistical analysis.

Biochemical measurements

Venous blood (5mL) was withdrawn from all patients and serum was freshly obtained. On an automated biochemistry analyzer (A15, Biosystem, Spain), fresh serum samples were tested for liver enzymes [alanine (ALT) and aspartate transaminases (AST) and alkaline phosphatase (ALP)], bilirubin, albumin and creatinine. Blood portion treated with sodium citrate solution was used for prothrombin international normalized ratio (INR) measurment. Another part of blood samples treated with KEDTA were used for complete blood count analysis (Sysmes, Japan). Serum AFP was measured using chimiluminescence immunoassay (Siemens, Germany). Both DKK1 (Cat E0630Hu, Bioassay Technology Laboratory, Shanghai, China) and sAxl (Cat E4707Hu, Bioassay Technology Laboratory, Shanghai, China) were measured using commercial human ELISA kits according to the manufacturer's instructions.

Statistical analysis

Both SPSS v.20.0 (SPSS, Chicago) and GraphPad Prism v.8.0 (GraphPad, San Diego) were used for all statistical analyses. Categorical variables were expressed as absolute numbers. Based on variable normality distribution, results were expressed as mean±standard deviation (SD) or median (interquartile range (IQR)). Differences between independent groups were compared using ANOVA and Kruskal-Wallis tests, appropriately follwed by LSD as post-hoc test. Stepwise multivariate discriminant analysis and ROC curves were performed to assess the independent HCC discriminative power of each blood parameter. Cutoff points were obtained based on the point on ROC curve closest to the (0, 1) point [22]. A logistic regression function (model) including the most HCC discriminatory independent factors was constructed. For simplification, very small non-significant constants and coefficients were removed and the model diagnostic ability was not affected. Diagnostic performances were derived from a 2×2 contingency table.

Results

Patients' characteristics

Clinicopathological characteristics of the studied patients are summarized in Table 1. HCC patients were associated with older age and elevated liver enzymes, total bilirubin, AFP levels. Serum albumin levels were lower in HCC patients in comparison to cirrhosis and fibrosis groups. Haemoglobin and red cell count were also lower in HCC group in comparison to cirrhosis and fibrosis groups. Data about number of nodules, tumor size, Child-Pugh and BCLC staging systems were also shown in Table 1.

HCC development was associated with DKK1 and sAxl elevated levels

Patients who had HCC were accompanied by a significant increase ($P<0.05$) in the concentration of DKK1 (5.38±2.05 ng/mL; Figure 1A) and sAxl (178.02±49.39 ng/mL; Figure 1B) when compared to patients with fibrosis (2.16±0.6, 97.63±19.71 ng/mL, respectively) and cirrhosis (2.62±0.8, 121.84±34.66 ng/mL, respectively). As revealed by ROC analysis, both DKK1 (AUC=0.852; Figure 1C) and sAxl (AUC=0.882; Figure 1D) had a good

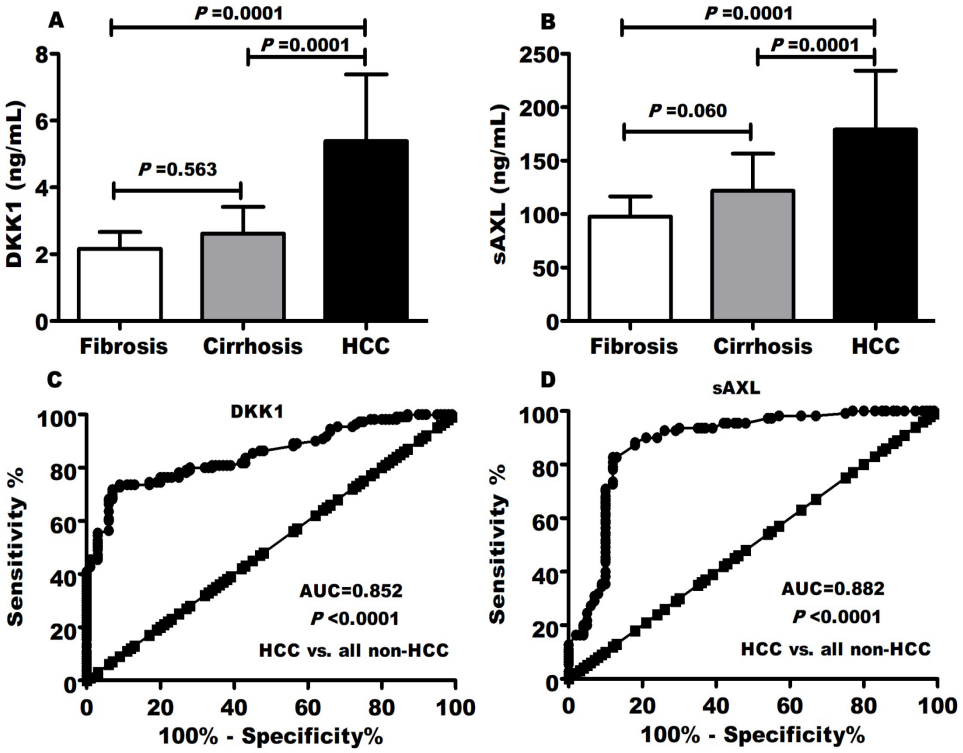

Figure 1. (A) DKK1 and (B) sAxl was associated with HCC development and their levels were increase with disease progression. The area under receiver-operating characteristic curve of (C) DKK1 and (D) sAxl revealed that both were had a good ability to discriminate patients with HCC from non-HCC patients (liver fibrosis and cirrhosis combined). P<0.50 is considered significant.

diagnostic accuracy in HCC diagnosis for separating HCC from all non-HCC patients (Fibrosis and cirrhosis combined) that was superior to AFP (AUC=0.802).

Model development and diagnosis improvement

Linear logistic regression analysis revealed the best mathematical scoring formula combining DKK1 and sAxl was [DKK1 × sAxl]. Multiplying DKK1

Table 1. Patient's Characteristics

Variables	Fibrosis	Cirrhosis	HCC	P value
Gender (male/female)	20/35	17/28	30/80	0.096
Age (years)	45.05±6.9	51.56±7.0	54.27±9.7	0.001
ALT (U/L)	58.6 (51-70)	55.5 (41-73)	65 (43-79)	0.027
AST (U/L)	48.5 (38-61)	69 (58-93)	81 (62-103)	0.001
ALP (U/L)	86 (56-105)	105 (85-127)	168.5 (116-278)	0.001
Albumin (g/dL)	4.2±0.55	3.6±0.63	3.2±0.62	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)	181 (148-215)	116 (68-165)	75 (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 (X2) 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.

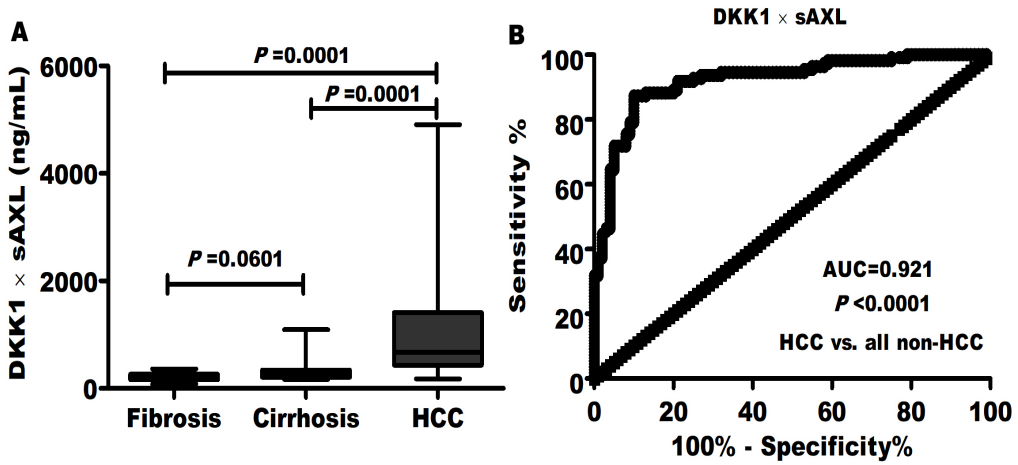

Figure 2. (A) Multiplying DKK1 with sAxl values was more associated with HCC compared to each marker alone. (B) As reported form the area under receiver-operating characteristic curve this model enhances the diagnostic power for HCC detection. P<0.50 is considered significant.

with sAXL yielded values that significantly (P<0.05) increased in patients who developed HCC (674.3 (434.2-1413.9)) versus patients who have hepatic fibrosis (204.9 (161.7-262)) and cirrhosis (254.4 (205.4-343.7)) (Figure 2A).

Compared to each protein alone, the model per se gave an AUC equal to 0.921 for separating patients with HCC from non-HCC patients (Figure 2B). It had diagnostic performances superior to DKK1 and sAxl separately in HCC prediction (Table 2). Sensitivity, specificity, PPV, NPV and efficiency for HCC detection were 90.9, 87, 88.5, 89.7 and 89.1%, respectively. Interestingly, elevated DKK1×sAxl 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.

Discussion

Usually, HCC is detected during the disease advanced stages as this malignancy is often asymptomatic and, thus, early detection is important for improving patient survival

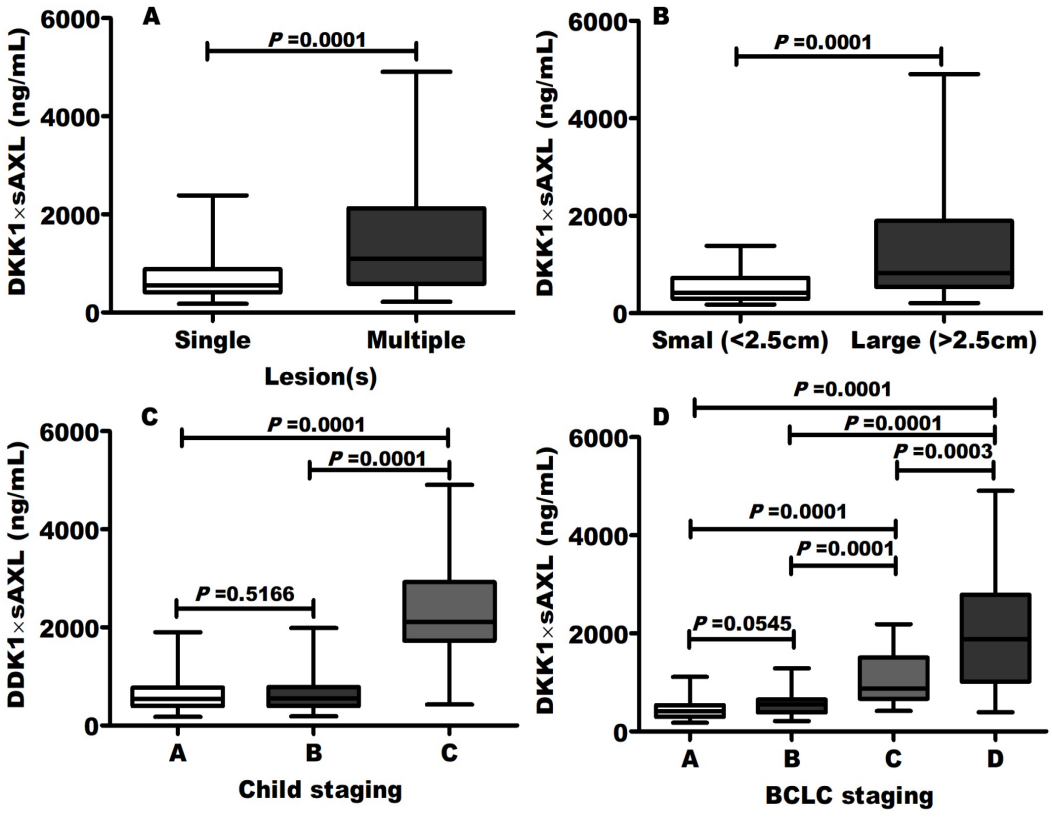

Figure 3. Elevated DKK1×sAxl Values were Associated with HCC Aggressiveness Including (A) multiple nodules, (B) large size and (C) Child and (D) BCLC late stages. P<0.50 is considered significant.

Table 2. Diagnostic Performances for HCC Detection

Categories	AUC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
DKK1≥ 2.6 ng/mL	0.852 (0.80-0.90)	78.2	75	77.5	75.8	76.7
sAXL≥ 116 ng/mL	0.882 (0.83-0.93)	83.6	80	82.1	81.6	81.9
DKK×sAXL ≥ 340 ng/mL	0.921 (0.88-0.96)	90.9	87	88.5	89.7	89.05
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.

[23]. In patients with HCC, evaluation and validation of reliable novel markers for HCC can enhance early diagnosis and ultimately survival [24]. In this study, we evaluated the performance of DKK1 and sAxl for the early detection of HCC in a cohort of Egyptian patients with CHC infection. Also, this study aimed to develop a model combining the two proteins in a trial to improve the diagnostic accuracy.

Our results revealed that both DKK1 (AUC=0.852) and sAxl (AUC=0.882) outperformed AFP (AUC=0.802) for HCC early detection. HCC patients were accompanied by a significant increase (P<0.05) in DKK1 and sAxl levels compared to patients with liver fibrosis and cirrhosis. DKK1 expression dysregulation has been demonstrated in a variety of human tumors [25]. In HCC, it was previously reported that DKK1 was upregulated [25, 26]. Using immunohistochemistry, DKK1 was upregulated in HCC tissues but was weakly expressed in cirrhotic tissues and its expression was associated with tumor number and size [25]. In HCC cells (HUH-7and HepG2), DKK1 genetic depletion impaired tumor formation, invasion, colonyforming ability and the proliferation [25]. DKK1-mediated tumorigenicity and proliferation of HCC cells may be dependent on the Wnt/β-catenin signaling pathway [25]. Other studies confirmed that DKK1 exerts its oncogenic effect in HCC through upregulating the expression of oncogenes, downregulating that of tumor suppressor genes and promotes inflammation, migration and tumor invasion [27]. DKK1 may facilitate cancer invasion and migration through TGF-β1 by inducing inflammation and tumor microenvironment remodelling [27].

Similar to our results, Song et al. study indicated that sAxl has differentiating power to HCC diagnosis in HBV patients, especially for AFP-negative HCC and disease early stages [14]. Reichl et al. [28] had reported that sAxl expression was higher in HCC than other tumors including colorectal, ovarian and breast tumors. During fibrosis progression, it was suggested that sAxl was predominantly produced from myofibroblasts supported (significantly released in 6 out of 7 of the tested liver cell lines) [16]. This releasing was associated with cirrhosis and advanced fibrosis compared to healthy controls [16]. There is a great deal between our study and other studies indicated that Axl is relevant with tumorigenesis such as metastasis, invasion, survival and proliferation [29-31]. To overcome limitation in HCC diagnosis and improve the diagnostic ability, some studies recommended the combined use of sAxl with other HCC markers [14].

In this study, linear logistic regression analysis revealed that multiplying DKK1 with sAXL [DKK1 × sAxl] is the best mathematical scoring formula for HCC detection. Model values were significantly (P=0.0001) higher in HCC (674.3 (434.2-1413.9)) patients compared to non-HCC fibrotic (204.9 (161.7-262)) and cirrhotic (254.4 (205.4-343.7)) patients with the highest diagnostic ability (AUC=0.921) compared to each protein alone. Its sensitivity, specificity, PPV, NPV and efficiency for HCC detection were 90.9, 87, 88.5, 89.7 and 89.1%, respectively. Interestingly, elevated DKK1×sAxl values were related to aggressive tumor including multiple nodules, large size and Child-Pugh and BCLC late stages indicating it's a valuable opportunity in disease surveillance.

In the line of our results, some studies reported that serum DKK-1 levels were correlated with HCC progression according to TNM and BCLC staging as DKK1 was higher in advanced compared to early stages [21]. Also, Song et al., and Reichl et al., reported that the increased in median sAxl level was found with the increase in BCLC stages [14, 28]. The promising diagnostic performances of DKK1×sAxl were comparable to other models combining DKK1 [32] and sAxl [14] with other established HCC tumor markers.

This study has a few limitations. Firstly, HCV is the dominant HCC etiology in this study; this feature is similar to most of our country studies where HCV infection is more commonly found. Secondly, the model was developed in a single centre and thus required confirmation from other centres of different geographic regions.

In conclusion, data of this study demonstrated a significant diagnostic accuracy of DKK1 and sAxl for early HCC detection. In addition, multiplying DKK1 with sAxl values developed a novel HCC diagnostic model that improves the diagnostic accuracy and may prove to be useful in the screening and early detection of HCC. This preliminary study should be proved in a multicentre larger study involved more cases from each studied group.

Author Contribution Statement

All authors contributed equally in this research.

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Helsinki ethical guidelines and approved by Mansoura University Hospitals Ethics and Scientific Committees.

Competing interests

All authors declare that there they have no conflicts of interest.

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