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

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) remain the most prominent HCC risk factors which can result 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 typically 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.
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 Scientific 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) measurement. 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 followed 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
Patienst’s 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 in an 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

<table>
<thead>
<tr>
<th>Variables</th>
<th>Fibrosis</th>
<th>Cirrhosis</th>
<th>HCC</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female)</td>
<td>20/35</td>
<td>17/28</td>
<td>30/80</td>
<td>0.096</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.0±6.9</td>
<td>51.5±7.0</td>
<td>54.2±9.7</td>
<td>0.001</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>58.6 (51-70)</td>
<td>55.5 (41-73)</td>
<td>65 (43-79)</td>
<td>0.027</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>48.5 (38-61)</td>
<td>69 (58-93)</td>
<td>81 (62-103)</td>
<td>0.001</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>86 (56-105)</td>
<td>105 (85-127)</td>
<td>168.5 (116-278)</td>
<td>0.001</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.2±0.55</td>
<td>3.6±0.63</td>
<td>3.2±0.62</td>
<td>0.001</td>
</tr>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.8±0.29</td>
<td>1.7±0.31</td>
<td>2.4±0.61</td>
<td>0.001</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.80±0.16</td>
<td>0.81±0.14</td>
<td>0.94±0.22</td>
<td>0.059</td>
</tr>
<tr>
<td>INR</td>
<td>1.14±0.07</td>
<td>1.36±0.14</td>
<td>1.39±0.20</td>
<td>0.001</td>
</tr>
<tr>
<td>α-fetoprotein (ng/mL)</td>
<td>4.0 (2-122)</td>
<td>139.1 (79-407)</td>
<td>266 (225-486)</td>
<td>0.005</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>14.18±1.35</td>
<td>11.56±2.08</td>
<td>11.55±2.49</td>
<td>0.001</td>
</tr>
<tr>
<td>Red blood cells (x10^12/L)</td>
<td>5.1 (4.6-5.3)</td>
<td>5.5 (3.8-5.8)</td>
<td>4.1 (3.5-5.2)</td>
<td>0.038</td>
</tr>
<tr>
<td>White blood cells (x10^12/L)</td>
<td>6.1 (5-7.3)</td>
<td>3.9 (3.5-4.2)</td>
<td>4.3 (3.5-4.7)</td>
<td>0.644</td>
</tr>
<tr>
<td>Platelets (x10^9/L)</td>
<td>181 (148-215)</td>
<td>116 (68-165)</td>
<td>75 (62-112)</td>
<td>0.001</td>
</tr>
<tr>
<td>Size small (&lt;2.5 cm)/large (&gt;2.5 cm)</td>
<td>—</td>
<td>—</td>
<td>30/80</td>
<td>—</td>
</tr>
<tr>
<td>Lesion(s) single/multiple</td>
<td>—</td>
<td>—</td>
<td>60/50</td>
<td>—</td>
</tr>
<tr>
<td>Child-Turcotte-Pugh (A/B/C)</td>
<td>—</td>
<td>—</td>
<td>49/31/30</td>
<td>—</td>
</tr>
<tr>
<td>BCLC (A/B/C/D)</td>
<td>—</td>
<td>—</td>
<td>28/26/23/33</td>
<td>—</td>
</tr>
</tbody>
</table>

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.
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.
Table 2. Diagnostic Performances for HCC Detection

<table>
<thead>
<tr>
<th>Categories</th>
<th>AUC (95% CI)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DKK1 ≥ 2.6 ng/mL</td>
<td>0.852 (0.80-0.90)</td>
<td>78.2</td>
<td>75</td>
<td>77.5</td>
<td>75.8</td>
<td>76.7</td>
</tr>
<tr>
<td>sAXL ≥ 116 ng/mL</td>
<td>0.882 (0.83-0.93)</td>
<td>83.6</td>
<td>80</td>
<td>82.1</td>
<td>81.6</td>
<td>81.9</td>
</tr>
<tr>
<td>DKK1×sAXL ≥ 340 ng/mL</td>
<td>0.921 (0.88-0.96)</td>
<td>90.9</td>
<td>87</td>
<td>88.5</td>
<td>89.7</td>
<td>89.05</td>
</tr>
<tr>
<td>AFP ≥ 240 U/L</td>
<td>0.802 (0.76-0.88)</td>
<td>72.7</td>
<td>75</td>
<td>76.2</td>
<td>71.4</td>
<td>73.8</td>
</tr>
</tbody>
</table>

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

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 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.

References


