CD326 EVALUATION IN HEPATOCELLULAR CARCINOMA PATIENTS WITH AND WITHOUT PORTAL VEIN TUMOR THROMBOSIS

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Key Words: cluster of differentiation 326, circulating tumor cell, patient management, hepatocellular carcinoma, flow cytometry

ABSTRACT.

Aim: to evaluate the presence and clinical relevance of a cluster of differentiation (CD)326+subset of circulating tumor cells (CTCs) in blood samples of hepatocellular carcinoma (HCC) patients with portal vein tumor thrombus and without portal vein tumor thrombosis, who had undergone curative or palliative intervention, in order to find a novel prognostic factor for patient management and follow-up.

Method: In total, 47 HCC patients, along with 21 with PVTT and 26 without PVTT were included. The easily transferable methodology of flow cytometry, along with multiparametric antibody staining were used to selectively evaluate CD326+CTCs in the peripheral blood samples of HCC patients. The multiparametric selection allowed any enrichment methods to be avoided thus rendering the whole procedure suitable for clinical routine.

Result: The presence of CD326+ cells was strongly correlated with poor survival and high metastasis rates in this novel patient population. as well as PVTT probability for CD 326 > or < 0.45 was 88.0%. Several factors independently correlated with CD326 using univariate multiple logistic regression of CD 326 with laboratory parameters. Correlation was found between EPCAM activity and AST, ALT, AFP at P Value less than 0.01

In conclusion: CD326+ CTCs are an independent prognostic factor for tumor aggressiveness rate in multivariate analysis, suggesting that their evaluation could be an additional factor for liver cancer progression risk evaluation in patient management.
INTRODUCTION

Hepatocellular carcinoma (HCC) is responsible for significant morbidity and mortality in cirrhosis and also accounts for between 85% and 90% of primary liver cancer\textsuperscript{[1-2]}. Most of HCCs in the world occur in the setting of cirrhosis and over half-million of people develop liver cancer every year and an almost equal number die of it\textsuperscript{[3]}. The most important causes leading to HCC are the HBV and HCV infections, heavy alcohol consumption, aflatoxin B1, age and gender (males are more susceptible than females), tobacco consumption, obesity associated with nonalcoholic fatty liver disease, and the increase of the Diabetes II mellitus (that rises the risk factor between 2 and 3), genetic hemochromatosis, primary biliary cirrhosis, and alpha1-antitrypsin deficiency and autoimmune hepatitis\textsuperscript{[4]}. Usually HCC develop during long process of inflammation and fibrosis eventually leading to cirrhosis\textsuperscript{[5]}. Portal vein tumour thrombosis is common complications of HCC that occur in approximately 10-40% of HCC patient at time of diagnosis, although great improvement have been made in diagnosis of HCC with PVTT and treatment during the past few decades the long term survival of these patients remain unfavourable, due to high mortality. Conventional prognostic marker for HCC alpha feto protein and tumor node metastasis, but their value varies between patients with this diseases\textsuperscript{[6]}, therefore new method are needed to provide predictive information about existing metastasis and probability of early recurrence.

In the field of biology of tumors, some expressions have been coined for the different types of circulating cellular elements. The term circulating tumor cells (CTC) defines a small population of cancer cells that have escaped from the primary tumor into the body’s circulatory system, and establish metastases in distant organs, The presence of CTC reflects the aggressiveness nature of a solid tumor. Many attempts have been made to develop assays that reliably detect and enumerate these cells. The clinical results obtained with such assays suggest that in some tumor types, CTC detection and identification can be used to estimate prognosis and may serve as an early marker to assess antitumor activity of treatment.

In addition, CTC can be used to predict progression-free survival and overall survival. CTC are an interesting source of biological information in order to understand dissemination, drug resistance and treatment-induced cell death\textsuperscript{[7-8]}. In HCC animal models showed that 10 to 10 000 CTC are capable to initiate new metastasis. Even after curative resection, the tumor recurrence rate remains high.\textsuperscript{[9]}

Although CTC detection has been applied and well documented in different types of cancer, CTC detection is not routinely performed in HCC follow-up and remains in the experimental field. However, HCC
CTC detection might bring new interesting information of metastatic process might be used as diagnostic tool of early recurrence and may allow a better patient selection for liver transplantation.\textsuperscript{[10]} but there are a number of problems affect CTC identification and enumeration; each method used for their detection has several drawbacks\textsuperscript{[11]} . The scarcity of CTCs in peripheral blood samples means that an enrichment step is often required prior to the analysis\textsuperscript{[12]} , however, all methods used to enrich CTCs from blood samples (i.e., filtration, density gradient separation and magnetic isolation) exhibit a low purity grade\textsuperscript{[13]}.

Also, major techniques used to identify CTCs (i.e., quantitative polymerase chain reaction and CellSearch system) are too expensive and time consuming to be predominantly used in the clinical routine\textsuperscript{[14]} . In addition, nucleic acid-based methods are markedly affected by the presence of a huge number of contaminant cells inside peripheral blood samples\textsuperscript{[15]} while intact cell analysis by the use of specific markers is defective due to the lack of unambiguous specificities\textsuperscript{[16]} ; in fact several of the markers expressed by cancer cells are shared by leucocytes. Flow cytometry is a suitable technique to analyze CTCs, as it allows single cell analysis and permits the researcher to include or exclude doubtful origin populations and suspect objects from the analysis at any time following sample acquisition. The universally recognized markers of CTCs are the epithelial specificities, CD326 (EpCAM) and the cytokeratins\textsuperscript{[17]} , however, recent findings have highlighted the complex nature of cancer cell dissemination, which involves deep cell changes, including the epithelial-to-mesenchymal (EMT) transition. The types of modifications that occur in the cell in such transitions are not univocally clarified; it has been demonstrated that cells reduce the epithelial characteristics as mesenchymal features appear, this transition appears to promote cancer cell dissemination\textsuperscript{[18]}.

In addition, it has been reported that intermediate phenotypes are observable in CTCs with the presence of epithelial and mesenchymal markers\textsuperscript{[19]} . The assessment of biologically significant markers is likely to provide more clinically relevant information than simple enumeration\textsuperscript{[20]} , so Recent work suggest using Flow cytometry to identify expression of CD326 as tumor marker in CTCs of hepatocellular carcinoma with PVTT compared to patient without PVTT.
for improvement standard cancer staging criteria and assessment risk at early stage.

**Recruitment of individuals**

Patients collected between January 2020 and March 2020 from HCC CLINIC, NATIONAL LIVER INSTITUTE, Menoufia university. Patients with diabetes were excluded from these studies. In total, 47 patients, consisting of 26 without portal vein tumor thrombus and 21 with portal vein tumor thrombus patients, were included. According to Magnetic resonance imaging and computerized tomography scan 9 HCC patients with patent portal vein and 17 HCC patients with distal portal vein. Furthermore, 21 patients, including 16 patients with partial portal vein tumor thrombus in the remaining five patients with complete portal vein tumor thrombus.

The following clinicopathological parameters were recorded: Age, gender, blood transfusion, surgery, focal lesion number, ALT, AST, AFP.

**The following was done for each patient:**

1. **Flowcytometric technique**

10 ml were withdrawn from 48 HCC patient to measure the percentage of CD326+ cells using flowcytometric technique. These blood samples were separated by RBCs lysing buffer. Then, the mononuclear cells were extracted. Using phosphate buffer solution (PBS), a process of washing the mononuclear cells is done twice by incubation for 7 minutes at room temperature and Centrifugation at 250 x g for 7 minutes. After that, the staining process is done by adding (FITC which was conjugated with anti-CD 45 antibody, PE which was conjugated with antiCD326 antibody) into appropriate amount of sediment. This system was placed in a dark place for 15 minutes at 37°C; after which the cells were washed with PBS. Finally, the CD326+ cells are measured by flowcytometry (Miltenyi Biotec, Germany).

2. **Liver enzyme** were represented by measuring the level of AST, ALT, AFP by automated biochemistry Huma Star 300 SR German.

3. **To assess infection with hepatitis B virus and hepatitis C virus**

were measured using a (Stat Fax-4200 USA) device, qualitatively and quantitatively, depending on the technique of immune absorption linked to the enzyme (ELISA).

4. **Statistical analyses** were performed by IBM® SPSS® Statistics software version All data are expressed as mean ± standard deviation (X±SD). Comparisons were analyzed by ANOVA one-way analysis A value of P <0.05: significant, P < 0.001: more significant. that was considered statically significant. ROC curve was done to determine the cutoff point, area under curve (AUC), sensitivity (Sn), specificity (Sp),
positive predictive value (PPV) and negative predictive value (NPV) of presences of portal vein thrombosis. Measure the diagnostic power of indirect scores which came in modern scientific journals according to the following equations:

1- **AST: platelets ratio index (APRI)** was calculated using Wais formula: (AST (upper limit of normal)/ALT (IU/L) \*100)/platelet count (platelets x 109/L) X 100.

2- **Fibrosis index (FI)** was calculated using this formula as:
   
   8.0-0.01 x platelet count (x109/L) – serum albumin (g/dl).

**RESULT**

Total of 50 patient were included in this study 3 patient were omitted because of missing data leaving 47 patients in final analysis 26 patient were HCC without PVTT. median age in all studied group (patent, dilated, partial and complete PVTT) (table 1)

(60.0 ± 5.38, 58.53 ± 5.68, 60.19 ± 6.66, 65.40 ± 6.34)

Majority were male n = 39, and 44.6 % had HCC with portal vein tumor thrombus , etiology of underlying liver cancer included ( HCV n =46, HBV n = 1).

**Table 1: Comparison between studied groups as regard Age and Gender**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>Group1 N=9</th>
<th>Group2 N=17</th>
<th>Group3 N=16</th>
<th>Group4 N=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>9 (100%)</td>
<td>13 (76.5%)</td>
<td>14 (87.5%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0 (0.00%)</td>
<td>4 (23.5%)</td>
<td>2 (12.5%)</td>
<td>2 (40.0%)</td>
</tr>
<tr>
<td>Age (mean ± sd)</td>
<td>60.00±5.38</td>
<td>58.53±5.68</td>
<td>60.19±6.66</td>
<td>65.40±6.34</td>
<td></td>
</tr>
</tbody>
</table>

Mean blood level of CD326 in 4 studied group was (0.49± 1300 , 3.06±1.39 , 3.18 +3.18 , 6.62 +5.99 )(table 2), the enzyme activities of ALT ,AST in partial portal vein tumor thrombus [94.63 ± 3.12 , 160.06 ± 8.06] and complete PVTT patients [ 99.40 ± 0.89 , 181.40 ± 5.77] were significant higher than in patient without PVTT. In contrast, significant differences were found in AFP between (patent, dilated) versus (partial, complete) (P = 0.000).

**Table 1: Comparison between studied groups as regard Laboratory data**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group1 N=9 Mean ± SD</th>
<th>Group2(N=17) Mean ± SD</th>
<th>Group3(N=16) Mean ± SD</th>
<th>Group4(N=5) Mean ± SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>53.89 ± 5.07</td>
<td>63.75 ± 6.63</td>
<td>94.63 ± 3.12</td>
<td>99.40 ± 0.89</td>
<td>0.000</td>
</tr>
<tr>
<td>AST(U/L)</td>
<td>104.67 ± 3.71</td>
<td>127.59 ± 8.65</td>
<td>160.06 ± 8.06</td>
<td>181.40 ± 5.77</td>
<td>0.000</td>
</tr>
<tr>
<td>AFP(ug/ml)</td>
<td>11.89 ± 3.09</td>
<td>99.53 ± 123.38</td>
<td>503.69 ± 103.97</td>
<td>964.80 ± 145.52</td>
<td>0.000</td>
</tr>
<tr>
<td>Cd326</td>
<td>0.49 ± 0.77</td>
<td>1.39 ± 3.06</td>
<td>2.68 ± 3.18</td>
<td>6.62 ± 5.99</td>
<td>0.008</td>
</tr>
</tbody>
</table>
Correlation between CD326 and different variables are illustrated in Table 3. A highly significant association was observed between EPCAM CD326 and the AFP. Furthermore, liver enzymes ALT and AST were significant associated P< 0.01 with CD326 in all HCC patient included in this study.

Table 3: correlation between CD326 and liver function test

<table>
<thead>
<tr>
<th>Variables</th>
<th>patent PV (N=9)</th>
<th>dilated PV (N=17)</th>
<th>partial PVTT (N=16)</th>
<th>complete PVTT (N=5)</th>
<th>Total (N =47)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>0.670*</td>
<td>0.657*</td>
<td>0.761**</td>
<td>0.936*</td>
<td>0.531**</td>
</tr>
<tr>
<td>AST(U/L)</td>
<td>0.779*</td>
<td>0.577*</td>
<td>0.926**</td>
<td>0.920*</td>
<td>0.606**</td>
</tr>
<tr>
<td>AFP(ng/ml)</td>
<td>0.770*</td>
<td>0.733**</td>
<td>0.880**</td>
<td>0.944*</td>
<td>0.687**</td>
</tr>
</tbody>
</table>

*: significant at p= 0.05  **: significant at p= 0.01

The diagnostic abilities of CD326 versus those of APRI and FIB4 are illustrated in Table 4. Unfortunately, there are not significant correlation between CD326 and (APRI or FIB4) among 4 studied group.

Cytometric analysis for CD326

A multiparametric cytometric analysis was used to evaluate the CD326+ CTCs present in the peripheral blood samples of the PVTT HCC patients and without PVTT HCC. The cells were first gated on physical parameters in a dot plot [forward scatter versus side scatter (SSC)] to exclude debris. Subsequently, in a CD45 versus SSC dot plot, the CD45-cell population position was identified by discarding all hematopoietic contaminants. Finally, a CD326 dot plot, + CTCs were identified. Finally, we calculated the cut point using the partial and complete PVTT groups together as positive HCC group, patient without PVTT(n= 26) served as negative group. ROC analysis showed the best cut point at a sensitivity of about 81%, 88% for CD26 and CD 326. The specificity and sensitivity values are as follows in fig 1.

Table 4: correlation between CD326 and (APRI, FIB 4)

<table>
<thead>
<tr>
<th>Variables</th>
<th>partial pv (N=16)</th>
<th>dilated pv (N=17)</th>
<th>patent pv (N=9)</th>
<th>complete pv (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APRI SCORE</td>
<td>-0.41102</td>
<td>-0.15308</td>
<td>-0.18365</td>
<td>-0.25981</td>
</tr>
<tr>
<td>FIB 4 SCORE</td>
<td>-0.20775</td>
<td>-0.12181</td>
<td>-0.15877</td>
<td>-0.45762</td>
</tr>
</tbody>
</table>

DISCUSSION

In the past few decades, many promising candidate biomarkers for HCC had been found, but most of them were not applied to clinical diagnosis due to their limited practicability and high cost. Nevertheless, these new markers have potential to be applied in clinical diagnosis for their higher sensitivity and specificity. So far, α-fetoprotein (AFP) and imaging technology (e.g., ultrasound or computed tomography) are two primary methods to diagnose HCC in hospitals.
AFP has been used as a serum marker for HCC for many years, but its sensitivity was only about 39%–65% \(^{[22]}\).

Recently, we have identified epithelial cell adhesion molecule (EpCAM), to be an early biomarker of HCC because its expression is highly elevated in premalignant hepatic tissues \(^{[23]}\). EpCAM (also known as CO17-1A, EGP, EGP40, GA733-2, KSA, Ly74, M1S2, M4S1, MIC18, MK-1, TROP1, and hEGP-2) is highly expressed in many human cancers with an epithelial origin.

The function of EpCAM and the regulation of its expression are largely unknown \(^{[24]}\). In the adult liver, hepatocytes are negative and bile duct epithelium is positive for EpCAM expression \(^{[25]}\). However, in the embryonic liver, the majority of hepatocytes express EpCAM. In the cirrhotic liver, EpCAM is expressed in proliferating bile ductus that are thought to be derived from HPC \(^{[26]}\) but its distribution pattern is altered in hepatocellular carcinoma according to previous study published by Gi Hong Choi who examined mRNA levels for K19, EpCAM, and CD44 in peripheral blood and HCC tissues before and after operation using real-time RT-PCR. Study participants were divided into high and low ratio groups, according to the ratio of postoperative to preoperative mRNA levels for each marker.. Their findings revealed that Preoperative peripheral levels of K19 and EpCAM mRNA were higher in liver transplantation patients than in resection patients, and they were also significantly higher in cirrhotic patients of Child–Pugh Class B or C than those of Child–Pugh Class A (p<0.05 for all). Preoperative peripheral levels of K19 and EpCAM mRNA were influenced by background liver status and HCC. Additionally, the ratio of postoperative to preoperative mRNA levels for CSC markers, especially K19 and EPCAM, was shown to be important to predict HCC recurrence \(^{[27]}\).

Another study by Taro Yamashita who identified EpCAM-positive HCCs by cDNA microarray in 40 HCC cases and validated by oligonucleotide microarray analysis of 238 independent HCC cases, which was further confirmed by immunohistochemical analysis of an additional 101 HCC cases. EpCAM-positive HCC displayed a distinct molecular signature with features of hepatic progenitor cells including the presence of known stem/progenitor markers such as cytokeratin 19, c-Kit, EpCAM, and activated Wnt-B-catenin signaling, whereas EpCAM-negative HCC displayed genes with features of mature hepatocytes. Moreover, EpCAM-positive and EpCAM-negative HCC could be further subclassified into four groups with prognostic implication by determining the level of A-fetoprotein (AFP). These four subtypes displayed distinct gene expression patterns with features resembling certain stages of hepatic lineages. Taken together, they proposed an easy classification system defined by EpCAM and AFP to reveal HCC subtypes similar to hepatic cell maturation lineages, which may enable prognostic stratification and assessment of HCC patients with adjuvant therapy and provide new insights into the potential cellular origin of HCC and its activated molecular pathways \(^{[28]}\).

These results once more underline the pathological role of CD326 in HCC. Therefore, the current study selected EPCAM to explore the CTC populations in the
peripheral blood of HCC patients by flow cytometry. Our goal was to validate the usefulness of the CD326 as diagnostic marker in progression liver cancer.

Our cohort revealed that Portal vein tumor thrombosis patients of HCC had the highest number of CD326+ CTCs and Blood levels of CD326+CTCs correctly predicted tumor prognosis in 85.7% of the cases, by contrast, negative patient (patient, dilated) expressed decrease number of cd 326+ CTC in 84.4% of cases, with a test specificity of 88%. (Fig 1).

Our second goal was to examine possible correlation between CD 326 activity and other laboratory parameters of liver function. We focused on 3 parameters that were associated with prognosis in HCC patients. Included were markers of liver damage such as AST, ALT, also included were AFP that were correlated with tumor aggressiveness.

Consistent with previous published data by UC Okonkwo et al who analyzed bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and albumin in 64 patients with HCC and 120 patients without HCC they observed that AST and ALT were elevated in 30(46.9%) and 31(48.4%) patients, respectively, while ALP was elevated in 33(52%). Hyper bilirubinaemia was present in 34(53%) and hypo albuminaemia in 54(84.3%) of the patients. Except for bilirubin, liver function test was more frequently abnormal in HCC than in non-HCC cases. This seems logical and in agreement with our study which revealed highly significant difference among studied groups in liver enzyme (AST, ALT) at P<0.001 and highly significant correlation with CD326 at P <0.001.

In present study we showed highly significant correlation between Cd326 and AFP, at p value< 0.01 in total 47 patient, similar to our result Leonardo do Prado Lima, et al, (2018) concluded that, EpCAM+ expression was associated with AFP+ (OR = 12.5, 95% CI, 1.9-84.1, p<0.001) in child Pugh A HCC patient undergoing curative surgical resection.

As predicted in our study, HCC patients showed a positivity for CD326+ even when the primary cancer had a low pathological grade, suggesting that CTCs can identify high-risk HCC patients at early stages. Notably, the spread of CD326+ cancer cells into the blood circulation is a common event and 81% of all analyzed patients exhibited these cells in their peripheral blood (figure 1) we have also proven to be useful in detecting minor subgroups of cells present in the primary tissue which might eventually be the cause of treatment resistance or relapse of the disease.

Fig 1: CD326
ROC curve of CD326 between Patients group

<table>
<thead>
<tr>
<th>Cut off</th>
<th>Area under the curve</th>
<th>Sensitivity</th>
<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD326 &gt;0.45</td>
<td>0.831</td>
<td>88.0</td>
<td>81.0</td>
<td>84.6</td>
<td>85.7</td>
<td>0.01</td>
</tr>
</tbody>
</table>

REFERENCES


shifts the state of cadherin-mediated adhesions from strong to weak. Experimental cell research, 285(1): 50-58.


29. تقييم CD326 لمرضى سرطان الكبد مع أو بدون الوريد البابي المتخثر

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بعد سرطان الكبد مسؤول عن الوفاة بنسبة 85% حتى 90% و معظم الحالات بسبب تليف الكبد و نصف مليون شحص حول العالم يموت بسبب ذلك و من اهم اسباب سرطان الكبد فيروس الكبد ب ، فيروس الكبد س ، افراط في تناول الكحول و السمنة المفرطة و مرضي السكري النوع الثاني عادة سرطان الكبد يتطور خلال عملية طويلة من الالتهاب و التليف، مما يؤدي في النهاية إلى تليف الكبد ثم السرطان.

في مجال بيوجينا الآلر وجدت العديد من التعريبات المسئولة عن الكشف عن الخلايا السرطانية و في هذا السياق يمكن تعريف الخلايا السرطانية بأنها خلايا هريت من ورم ايداني و انتقلت في الدم.
لكي تستوطن في عضو آخر، وكونت مكيسي بالانبثاث، وهذه الخلايا مسؤولة عن تطور المرض، وهذا فان هناك العديد من المحاولات لفحص هذه الخلايا وعدها، وبالتالي تمكن اهميتها في معرفة مدى تطور المرض.

هناك العديد من التقنيات التي استخدمت لفحص الخلايا السرطانية مثل جهاز Cell PCR و Cell search system. ولكن تكلفتها عالية ومهيدة للوقت، ولكن الأبحاث الجديدة أثبتت فعالية جهاز التعرف الخلويا. فهو يقوم بعد خليهة خليهة ويقوم باستئصال الخليهة المشكوك في فيه CD326، ولذا فان بحثنا يقوم بعد الخلايا السرطانية الموجودة في سرطان الكبد عن طريق عد CD326 الموجود على سطح الخلايا أو EPCAM.

المرضى الذين استخدموا في الدراسة جمعتهم بين كانون الثاني / يناير 2020 أذار / مارس عام 2020 من معهد الكبد، جامعة المنوفية. المرضى الذين يعانون من مرض السكري استبعدوا من هذه الدراسة. في المجموع 47 المرضى، تتكون 26 بدون وريد بابي متخثر، و 21 مع الوريد البابي المتخثر.

تم اخذ جميع بيانات المريض، وقام بالانسحاب لكل مريض استهداف الكبد AST, ALT, AFP، وقياس نسبة CD326 الموجود على الخلايا السرطانية APRI، وقياس APRI، وقياس FIBRO index.

قد وجدنا أن 326 من مرضى الحالات المبكرة من سرطان الكبد و استطاع تشخيص المرض بنسبة 80%، كما أنه أظهر علاقة إيجابية مع AFP و انزيمات الكبد و لم يظهر أي علاقة مع APRI, FIBRO INDEX.