Original Article
The value of tumor angiogenesis activity for stratification of HCC patients

Włodzimierz Otto¹, Janusz Sierdziński², Maria Król³, Ewa Wolińska⁴, Magdalena Feliksbrot-Bratosiewicz⁵, Urszula Wilkowojska⁵

¹Departments of General, Transplant & Liver Surgery, Central Teaching Hospital, Medical University of Warsaw, Poland; ²Departments of Medical, Informatics & Telemedicine, Central Teaching Hospital, Medical University of Warsaw, Poland; ³Departments of Oncology, Hematology & Internal Medicine, Central Teaching Hospital, Medical University of Warsaw, Poland; ⁴Departments of Pathology, Central Teaching Hospital, Medical University of Warsaw, Poland

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Abstract: The study aimed to evaluate whether the identification of angiogenesis factors improves the system of clinical classification and allows for choice of more appropriate candidates for surgery. The cohort study covered 180 patients. Ninety two of them underwent radical treatment: 48 liver resection, 44 liver transplantation. Eighty eight patients were selected for palliative treatment. Endothelial progenitor cells in the blood were assessed with a flow cytometer, serum VEGF concentration was measured by ELISA. HCC samples were examined for morphologic characteristics and stained for VEGF and VEGFR concentration within tumor tissue. Patients were followed up to 3 years after the surgery. With the use of the multivariate analysis model we identified the angiogenesis factors that are significant for the appropriate stratification of candidate for the radical treatment (P-value < 0.05). Patients considered as eligible for the radical surgical treatment had lower levels of angiogenesis factors than those chosen for the palliative care (P < 0.001). The optimal cutoff identified for the rate of circulating EPCs, serum VEGF level, and tumor VEGF concentration was 47.08%, 327.2 pg/mL and 83.16 morphometric units, respectively. In 30% of patients undergoing surgery HCC recurred within 3 years. The preoperative level of angiogenesis factors exceeded the value of the cutoff in all of them (P < 0.001). The multivariate analysis showed an increased EPCs level as the strongest independent factor indicating tumor unfavorable biology and the possibility of relapse after surgery (Odds Ratio = 15.5, P < 0.001). We conclude that the assessment of angiogenesis factors improves the clinical evaluation and allows for more accurate stratification of patients for surgery.

Keywords: Hepatocellular carcinoma, tumor angiogenesis, vascular endothelial growth factor, endothelial progenitor cells, HCC patients stratification

Introduction

Crucial factors enabling medical doctors to stratify and discriminate patients with hepatocellular carcinoma (HCC) have been gathered in a number of commonly applied systems of clinical assessment. They also allow to assess the risk of tumor recurrence after radical surgery [1-3]. However, even with the best selection of criteria and restrictive qualification of patients, the number of early tumor recurrences exceeds 25%, thus testifying to the low viability of the applied qualification systems [4-6]. This creates a clear incentive to seeking new, additional assessment criteria which would base, to a much larger extent than at present, on the pathologic characteristics of the tumor and its properties [7-9]. Research on mechanisms of angiogenesis that support tumor development constitutes one of the directions in this area. Many reports point to the existence of mutual relation between the level of expression of angiogenesis factors, tumor pathologic properties, and the disease recurrence after radical surgery [10-15].

The report analyses the levels of the vascular endothelial growth factor and its receptor-2 (VEGF/VEGFR-2) in the peripheral blood and in the tumor tissue, as well as the number of...
Endothelial progenitor cells (EPCs) circulating in the peripheral blood in patients with HCC undergoing surgical treatment. The assumption was that the eradication of the tumor is conditional upon the intensity of angiogenesis. If so, the assessment of angiogenesis factors should allow to distinguish patients with high probability of tumor recurrence already at the stage of qualification.

**Material and methods**

**Study design & setting**

The cohort study covered 180 patients with hepatocellular carcinoma treated at the Department in the years 2009-2012 in the research project supported by the National Science Center (Grant No. NN 403 177940) and approved by the Bioethics Committee of Medical University of Warsaw (Approval No. 235/209).

**Patients**

The HCC patients were classified for either radical or palliative therapy group on the basis of the clinical evaluation of their health status, liver function and tumor stage in accordance with Child-Pugh classification and TNM as well as BCLC and the Milan Criteria [1-3, 16].

The radical therapy group (RT Group) consisted of 92 patients and included:

48 patients (29 M, 19 F; Mean age: 51 years) with Stage I tumor (0-A1 Stage acc. to BCLC) in whom a liver resection was performed. In 19 of them the tumor developed in a healthy liver, in 29 in a cirrhotic liver (but with normal synthetic function and without features of portal hypertension) after viral hepatitis (HBV/HCV). The blood serum alpha-fetoprotein level averaged 28.5 ng/ml (SD +/− 1457), the number of blood platelets 158,000 mcl (SD +/− 74,000), INR 1.06 (SD +/− 0.15).

In the group of 44 patients (23 M, 21 F; Mean age: 47 years) with Stage I/II tumor (A2-A4 Stage acc. to BCLC), satisfying the Milan Criteria, in whom a liver transplantation was performed. Liver cirrhosis after viral HBV or HCV infection was found in all the patients, with A/B liver efficiency acc. to Child-Pugh. Their alpha-fetoprotein level averaged 53.35 ng/ml (SD +/− 3444), the number of blood platelets...
Angiogenesis activity of HCC

The post-operative approach was based on the overall condition, liver functioning, and a nutritional status in all patients after extended surgical intervention for HCC. In the immediate postoperative period after hepatic resection or liver transplantation all patients were held in the intensive care unit for 2-14 days (mean 9 days) depending on their cardiovascular and respiratory conditions as well as on other metabolic and functional disorders. Maintenance of an adequate fluid balance and a normal renal function were of primary importance. All patients underwent broad-spectrum antibiotic therapy. The patients transplanted for HCC received immunosuppressive therapy with calcineurin inhibitors, antimetabolites and corticosteroids. Patients were followed up continuously for 3 years undergoing routine clinical examination and USG examination every 6 months. Their alpha-fetoprotein level was also determined every 6 months. Its presence, suggestive in any case of cancer relapse, was verified with a CT scan.

The palliative therapy group (PT Group) included 88 patients (48 M, 40 F; Mean age 63 years) with an advanced neoplastic process (III/IV Clinical Stage, B-C acc. to BCLC). In 16 of the patients the tumor developed in a healthy liver, in 72 in a cirrhotic liver in the course of HBV/HCV infection. Their alpha-protein level averaged 65.25 ng/ml (SD +/- 1.798), the number of blood platelets 116,000 mcl (SD +/- 82,000), INR 1.12 (SD +/- 0.11).

Demographic data are presented in Tables 1 and 2. The flow of the patients is presented in Figure 1.

Determination of circulating EPCs

Prior to the treatment (1-3 days before the operation or upon a palliative therapy decision) blood samples were collected from the patients. Two mL of venous blood were collected for K3 EDTA and analyzed for the number of EPCs in peripheral blood with a flow cytometer. The endothelial progenitor cells were defined by the phenotype of CD309+ within a subpopulation of CD34+CD45+ cells and CD34+CD45- cells. The standardization was based on the use of state-of-the-art bright fluorochrome conjugates and the combination of the HSC marker CD34 and CD133 with CD45 counterstaining. The gating strategy was established to separate the CD34+CD45+ and CD34+CD45- cell fractions from irrelevant cell populations, as recommended by the International Society of Hematology and Graft Engineering (ISHAGE). Following many reports indicating the human EPCs as the primitive progenitors within the hematopoietic line of the stem cells, we added the surface marker CD133 to the original ISHAGE protocol, as the presence of CD133 positivity indicated both the stemness and the hematopoietic lineage of the cells. Assuming that the cells positive to CD34, CD133 and CD309 produce the lowest counts in the circulation we also increased the total number of acquired events in the flow cytometer analysis to at least 2,000,000. The immunofluorescence of the cells for CD309 was assessed after identification of CD34 cells within the fraction of cells positive and negative to the CD45 marker. Their counts were added up and expressed as a percentage of EPCS in CD34+ cells population. Flow cytometry pictures indicating the low and high expression of endothel-
Angiogenesis activity of HCC

Angiogenesis activity of HCC

Figure 2. The rating of CD309+ cells in the population of CD34+CD45- and CD34+CD45+ cells of WBC in HCC patients. The cells of CD309+ were identified in both populations in relation to the isothypic control. Pictures indicate the small number of the CD309+ cells.

libraries progenitor cells in patients who remained asymptomatic and in patients with tumor recurrence are presented in Figures 2 and 3.

Examination of the blood serum VEGF concentration

Eight ml of blood were collected for the serological examination of the blood serum VEGF concentration. After centrifugation at 3000 rotations/minute for 10 minutes, the serum sample was stored at -80°C. The VEGF concentration was determined with the help of the immunoenzymatic assay Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN, USA. The sensitivity of the method was 9 pg/ml, and the precision coefficient was 4.4-6.7% and between examinations 6.2-8.8%.

Pathologic analysis

Pathologic analysis was performed on material from 92 tumors excised during surgery and 88 specimens collected during a core needle biopsy in patients under palliative therapy. The preparations were fixed in 10% formalin and immersed in paraffin. Tissue blocks were sliced into 4 µm-thick sections and stained with hematoxylin/eosin. The degree of tumor cells differentiation acc. to the Edmondson-Steiner scale, the structural variant and the presence of microvascular invasion by the tumor were evaluated.

Morphometric determination of VEGF/VEGFR tissue concentration

The tissues were stained for the presence of VEGF and VEGFR with the immunohistochemistry technique, using anti-VEGF and anti-VEGFR antibodies (Dako-Cytomation) and a set of secondary antibodies combined with horseradish peroxidase particles (ImmPress anti-mouse-HRP Detection Kit, Vector Laboratories). The staining was conducted in the Leica Bond-Max autostainer. The immune reactivity level of
Angiogenesis activity of HCC

VEGF proteins was determined by morphometry with the use of an automatic image analyzer in 5 representative fields of vision (Media Cybernetics). The analysis covered the immunological reaction surface field and the intensity of the reaction. The product of the mean surface field and the mean reaction intensity expressed in morphometric units (m.u.) was adopted as the mean level of VEGF protein immunoreactivity in the tissue. Pictures showing the results of the immunohistochemical staining for the expression of VEGF and VEGFR in the samples of HCC are presented in Figures 4 and 5, respectively.

Statistical analysis

The dataset was recorded on a disc and analyzed in the SAS 9.4 statistical software (SAS Institute Inc. 100 SAS Campus Drive Cary, NC 27513-2414, USA). Continuous variables were expressed as a mean +/- SD, with a sample representativeness of 95% CI, discrete variables were presented as numbers or letters, and categorical variables were adequately labeled. The statistical analysis of mutual relations between the variables studied was carried out with the use of Student t-Test and the U Mann-Whitney Test, while correlations between the variables were evaluated with Spearman Test. Factors that improve effectiveness of the stratification of candidates for radical surgical treatment were established by the model of multivariate analysis of clinical-pathologic features by Okuda and BCLC classification alone and together with factors indicating the activity of angiogenesis (the logistic regression model Wald test). The clinical and pathologic data presented in Tables 1 and 2, and the angiogenesis features presented in Tables 3 and 4 were used as dependent variables to describe relations between tumor propriety and tumor recurrence. Patients who stayed asymptomatic for over 3 years after a radical surgical treatment were the reference group. Results of P < 0.05 were adopted as statistically significant.
Angiogenesis activity of HCC

Figure 4. Results of immunohistochemical staining for expression of VEGF in the HCC samples. A: Immunoreactivity for VEGF in normal liver tissue; B: Weak immunoreactivity for VEGF in HCC cells; C: Mild intensity of VEGF expression in HCC cells; D: Strong immunoreactivity for VEGF in HCC cells. Images A-C: Magnification of 10x, image D: Magnification of 20x.

Results

The differences in patients clinical-pathologic presentation

Although candidates for liver resection differed from candidates for liver transplantation in terms of HCC staging (0-A1 for L.r. and A2-A4 for L.t. group), underlying liver disease and the health status, they were analyzed as one radical treatment group (RT Group). The reason was that the development of an early cancer (stage 0-A4 according to BCLC classification) was recognized in all of them in the assessment by the clinical classification systems (Child-Pugh, Okuda and BCLC). Candidates for liver resection also had a higher number of blood platelets, a lower INR level and a lower blood serum AFP level. Post-HBV/HCV inflammation cirrhosis was found only in 61% of them. The pathologic examination showed prevalence of tumors of trabecular and acinar structure (85.8%), well or medium differentiated (90.2%). Relevant data are presented in Tables 1 and 2.

There were statistically significant differences neither in the mean values of the VEGF/VEGFR protein expression in the blood serum and tumor tissue nor in the mean number of EPCs in circulation between patients treated with liver resection (L.r.) and with liver transplantation (L.t.), as determined in preoperative settings (see Table 3).

The patients qualified for palliative therapy (PT Group) were characterized, apart from differences in the number and stage of neoplastic changes, by a higher mean blood serum AFP level. The pathologic examination showed prevalence of tumors of trabecular and acinar structure (73.9%) but 36.3% of them were poorly diversifed, as presented in Tables 1 and 2.

This group differed in a statistically significant way from patients qualified for radical surgical
Angiogenesis activity of HCC

Figure 5. Results of immunohistochemical staining for expression of VEGFR in the HCC samples. A: Strong immunoreactivity for VEGFR in HCC cells.; B: Absence of immunoreactivity for VEGF in HCC; C: Mild intensity of VEGFR expression in HCC cells; D: Marking of HCC cells on the wedge of cancer. Images A-D: Magnification of 10x.

treatment (RT Group) by higher levels of angiogenesis factors (P < 0.001), except for the mean level of the VEGF blood serum concentration, as shown in Table 3.

ROC curve and cutoff values for angiogenesis factors

The mean value of each of the angiogenesis factors determined on the basis of measurements performed in all the 180 HCC patients presented as follows: 47.8% (SD +/- 17.5, 44.5-49.6 95% CI) for the percentage of EPCs, 327.2 pg/ml (SD +/- 284.0, 285.1-368.9 95% CI) for the VEGF concentration in blood serum and 83.1 m.u. (SD +/- 12.8, 81.2-85.4 95% CI) and 139.3 m.u. (SD +/- 58.1, 30.7-140.8 95% CI) for VEGF and VEGFR in tumor tissues. Details are given in Table 3. We adopted them as the cutoffs for the further analysis instead of values established by the receiver operating characteristic (ROC curve) because they constituted a better point of reference in the analysis of factors favoring cancer recurrence in the 92 patients after radical surgical treatment (RT Group). The ROC values were as follows: 46.75% for the percentage of EPCs, 221.9 pg/ml for the VEGF concentration in the blood serum, and 65.8 m.u. and 197.03 m.u. for VEGF and VEGFR in tumor tissues. The cutoff value for the percentage of EPCs was established at the same level in both cases (mean value 47.8 % versus ROC value 46.75%). The ROC analysis for EPCs is presented in Figure 6.

Clinical-pathologic predisposition for recurrence

In the group of 92 patients after radical surgical treatment (RT), tumor relapse occurred within 3 years from the procedure in 28 patients (35%): 15 after liver resection and 13 after liver transplantation. All patients with tumor relapse showed an elevated blood serum level of AFP in
Angiogenesis activity of HCC

Table 3. Expression of angiogenesis factors in individual groups of 180 HCC patients

<table>
<thead>
<tr>
<th>Rated groups</th>
<th>No. pts.</th>
<th>% EPCs</th>
<th>VEGF (pg/ml) Mean +/− SD</th>
<th>Tissue VEGF (units)</th>
<th>Tissue VEGFR (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver resection (L.r. group)</td>
<td>48</td>
<td>36.4, +/- 12.9</td>
<td>356.3, +/- 208</td>
<td>77.9, +/- 14.2</td>
<td>110.1, +/- 58.5</td>
</tr>
<tr>
<td>Liver transplantation (L.t. group)</td>
<td>44</td>
<td>40.5, +/- 13.1</td>
<td>309.7, +/- 245</td>
<td>80.9, +/- 13.1</td>
<td>126.4, +/- 50.1</td>
</tr>
<tr>
<td>Radical treatment (L.r. + L.t. = R.T. group)</td>
<td>92</td>
<td>35.5, +/- 13.1</td>
<td>334.8, +/- 226</td>
<td>79.4, +/- 13.7</td>
<td>117.9, +/- 55.1</td>
</tr>
<tr>
<td>Palliative treatment (P.T. group)</td>
<td>88</td>
<td>56.1, +/- 17.6</td>
<td>319.6, +/- 336</td>
<td>87.8, +/- 10.4</td>
<td>161.6, +/- 52.8</td>
</tr>
<tr>
<td>All HCC patients</td>
<td>180</td>
<td>47.8, +/- 17.5</td>
<td>327.2, +/- 284</td>
<td>83.1, +/- 12.8</td>
<td>139.3, +/- 58.1</td>
</tr>
<tr>
<td>Student t-Test L.r. versus L.t. group</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Student t-Test R.T. versus P.T. group</td>
<td>P &lt; 0.001</td>
<td>NS</td>
<td>t = -6.88, P &lt; 0.001</td>
<td>t = -3.41, P &lt; 0.001</td>
<td>t = -5.51, P &lt; 0.001</td>
</tr>
</tbody>
</table>

Table 4. Expression of angiogenesis factors in patients with and without tumor recurrence at the time of 3 years of follow-up after radical surgical treatment

<table>
<thead>
<tr>
<th>Rated groups</th>
<th>No. pts. (%)</th>
<th>% EPCs</th>
<th>VEGF (pg/ml) Mean +/− SD</th>
<th>Tissue VEGF (units)</th>
<th>Tissue VEGFR (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pts. free of tumor</td>
<td>64 (65%)</td>
<td>31.8, +/- 9.26</td>
<td>325.9, +/- 245</td>
<td>77.5, +/- 11.9</td>
<td>113.5, +/- 54.2</td>
</tr>
<tr>
<td>Pts. with recurrence</td>
<td>28 (35%)</td>
<td>53.3, +/- 7.0</td>
<td>352.7, +/- 178</td>
<td>85.2, +/- 16.6</td>
<td>128.1, +/- 56.3</td>
</tr>
<tr>
<td>Cutoff value</td>
<td>47.8</td>
<td></td>
<td>327.2</td>
<td>83.1</td>
<td>139.3</td>
</tr>
<tr>
<td>Student t-Test Pts. free of tumor vs. Pts. with recurrence</td>
<td>t = -10.95, P &lt; 0.001</td>
<td>NS</td>
<td>t = -1.94, P &lt; 0.05</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

The preoperative clinical settings (t = -1.7, P < 0.01). However, clinical evaluation systems revealed no significant differences between patients with and without cancer relapse in terms of the general health status and tumor staging (BCLC Staging = early cancer, Okuda score = NS). There were also no significant differences in tumor tissue differentiation, its structure variants and frequency of microvascular invasion found on the autopsy examination of HCC preparations collected during surgery from patients with or without a tumor relapse.

**High angiogenesis activity predisposes to tumor recurrence**

All patients with tumor relapse had an elevated level of VEGF/VEGFR-2 protein expression in peripheral blood and in HCC tissue as well as an increased number of EPCs in blood circulation, exceeding the cutoff value in each of them. The data are given in Table 4.

Factors indicating unfavorable tumor biology predisposing to early recurrence

The multivariate logistic regression model (Wald test, Software SAS 9.4) was used to describe factors of unfavorable tumor biology predisposing to early tumor recurrence after radical surgery. When only the clinical and pathologic features of the tumor were used as dependent variables, the analysis indicated an increased INR (OR = 8.15), a low PLT count (OR = 3.12), tumor microvascular invasion (OR = 3.35) and an increased AFP level (OR = 2.74, P < 0.01) as significant independent predisposing factors (see Table 5). Unfortunately, patients with an abnormal INR and a low platelets count are not qualified for surgical treatment in principle and the knowledge about the tumor microvascular invasion is available only after operation. However, when angiogenesis features of the tumor were used together with clinical/pathological features in the model, the analysis indicated an increased level of endothelial progenitor cells in the blood circulation as the
Angiogenesis activity of HCC

Figure 6. The ROC curve analysis indicating cutoff value for the percentage of EPCs in the blood circulation in patients with HCC.

The number of HCC recurrences after surgical treatment indicates that patients qualified for surgery are those with a prognosis of an unlikely or impossible recovery due to the aggressive biological activity of the tumor. Unfortunately, none of the algorithms based on the system of the clinical assessment takes into account the tumor potential to disturb the homeostasis of the host organism and determine the invasiveness of the neoplasm [4, 5, 17]. The current study presents results of a multifactor analysis of clinical variables commonly used to determine the HCC stage as well as variables testifying to the biological activity of the neoplasm. The dominant factor predicting the unsuccessful outcome due to likely occurrence of the recurrence of HCC (OR = 15.5, P < 0.001, Table 6). Notably, the angiogenesis activity of the tumor can be readily ascertainable at the time of preoperative settings.

Discussion

Accurate forecasting of the outcome of treatment and proper stratification of patients with an early HCC seem of essential importance [28-30]. Early relapse of the disease is not only an unfortunate development for the patient but jeopardizes the sense of the procedure to be undertaken. The situation can be improved with more far-reaching and adequate determination of the tumor angiogenesis parameters at the time of the patient’s qualification for a research was carried out on a selected, homogenous group of 92 patients with an early-stage hepatocellular carcinoma in whom we determined the factors of tumor-associated angiogenesis. We also examined 88 patients with an advanced-stage HCC to show changes in the biological activity of the tumor. As the distribution of results in the study groups corresponded to the Gauss curve distribution, we used a parametric test to analyze the data. The analysis pointed to an unequivocal difference in angiogenesis activity between tumors promising a successful cure by radical surgical treatment and those eligible just for palliative treatment. The difference is most clearly expressed in a significant increase in the circulating endothelial progenitor cells population discriminating advanced tumors [13, 14, 18-21]. Surprisingly, the study revealed that the level of blood/tissue VEGF/VEGFR protein expression and the count of endothelial progenitor cells (EPCs) in the population of CD34+ stem cells in patients with the tumor recurrence significantly exceeded the mean values of angiogenesis factors that were established in the preoperative settings for the group eligible for radical surgical management (P < 0.001). Our findings indicate that the determination of angiogenesis factors can prove helpful in the proper discrimination and stratification of patients in clinically ambiguous cases [9, 22-27]. And this is the main contribution of the present study.
angiogenesis from the 1996 findings of Mise.
Numerous authors draw attention to the fact that the level of VEGF protein concentration in the blood serum of HCC patients differs from its level in the blood serum of healthy people or patients with liver cirrhosis but without a neoplasm [9, 18, 20, 31, 32]. The prognostic importance of VEGF protein appears mostly in patients with advanced HCC and a level of blood serum VEGF concentration exceeding 400 pg/mL [27, 31]. In such cases, the level of the VEGF factor may prove a convenient indicator of the effectiveness of a bridging or palliative treatment with TACE [27, 34]. In our study, the level of VEGF concentration in the blood serum of patients with HCC recurrence exceeded the level of 400 pg/mL just in a few cases. Though the mean level of VEGF serum concentration was higher than the determined cutoff value, the difference was not significant. The level of the VEGF serum concentration correlated with increased levels of the VEGF and VEGFR protein in the tumor tissue of patients with HCC recurrence (t = -1.94, p < 0.05 for VEGFR). The findings correspond to the experience reported by others [9, 31, 32, 35]. What proved to be the best indicator was, however, the number of endothelial progenitor cells of CD34+, CD309+ phenotype circulating in the blood. The experience of many research centers indicates that the level of endothelial progenitor cells (EPCs) is essential for tumor growth [10, 12, 14, 15, 19, 31]. Recently, Zhu et al. revealed that EPCs are mobilized and incorporated into tumor vessels throughout the whole process of HCC growth and Sun et al. demonstrated the role of EPCs in HCC neovascularization [11, 13, 36]. EPCs constitute a relatively small, re-aching less than 3%, subpopulation of hematopoietic CD45+ stem cells and a larger, amounting to 35%, subpopulation of vascular endothelium CD45- cells [21]. The study showed that in patients with an early HCC the number of endothelial progenitor cells (EPCs) in the population of CD34+ stem cells is significantly lower than in patients with an advanced HCC (35.5% versus 56.1%; t = -6.88, P < 0.001). It also revealed that in all early HCC patients with a relapse within 3 years from radical surgery, the number of EPCs determined at the time of the patients’ qualification for treatment was significantly higher (31.8%, SD +/-.10.95, p < 0.001). Also, it exceeded significantly the cutoff value set at 47.8% (t = -10.95, p < 0.001). These results indicate that the activity of angiogenesis is proportional to how advanced the tumor is. Consequently, the determination of the EPCs in circulation provides an effective way of identifying patients

### Table 5. Analysis of maximum likelihood of unfavorable tumor biology predisposing to tumor recurrence

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wald Chi-Square</th>
<th>Pr &gt; ChiSq</th>
<th>Odds ratio estimates</th>
<th>95% Wald Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased INR</td>
<td>18.36</td>
<td>&lt; 0.001</td>
<td>8.15</td>
<td>3.12</td>
</tr>
<tr>
<td>Low platelet count</td>
<td>7.74</td>
<td>&lt; 0.005</td>
<td>3.12</td>
<td>1.40</td>
</tr>
<tr>
<td>Increased AFP level</td>
<td>6.50</td>
<td>&lt; 0.01</td>
<td>2.74</td>
<td>1.26</td>
</tr>
</tbody>
</table>

Clinical features of BCLC classification were used as the dependent variables. Patients who stayed asymptomatic over 3 years after radical surgical treatment consisted of the reference group.

### Table 6. Analysis of maximum likelihood estimates of unfavorable tumor biology predisposing to tumor recurrence

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wald Chi-Square</th>
<th>Pr &gt; ChiSq</th>
<th>Odds ratio estimates</th>
<th>95% Wald Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased rate of EPCs</td>
<td>30.04</td>
<td>&lt; 0.001</td>
<td>15.5</td>
<td>5.45</td>
</tr>
<tr>
<td>Poor tumor differentiation</td>
<td>15.00</td>
<td>&lt; 0.001</td>
<td>0.31</td>
<td>0.079</td>
</tr>
<tr>
<td>Increased INR</td>
<td>4.40</td>
<td>&lt; 0.035</td>
<td>6.75</td>
<td>2.23</td>
</tr>
<tr>
<td>Low platelet count</td>
<td>4.96</td>
<td>&lt; 0.025</td>
<td>3.3</td>
<td>1.29</td>
</tr>
<tr>
<td>Increased AFP level</td>
<td>5.84</td>
<td>&lt; 0.015</td>
<td>3.46</td>
<td>1.37</td>
</tr>
<tr>
<td>Okuda score</td>
<td>5.28</td>
<td>&lt; 0.025</td>
<td>2.89</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Clinical features of BCLC classification and the pathologic tumor features were used together with the indicators of angiogenesis activity as the dependent variables. Patients who stayed asymptomatic over 3 years after radical surgical treatment consisted of the reference group.
with an advanced tumor. Yet, most importantly, a high number of circulating EPCs indicates the presence, among early HCC patients determined on the basis of the clinical classification system, of patients with a tumor of exceptionally high biological activity characteristic of more malignant, more advanced tumors with poor prospects for successful surgical treatment. Determination of the number of circulating EPCs at the time of the qualification of candidates for radical surgical treatment may thus constitute a valuable clue in the proper stratification of patients.

The study indicates that it is not enough to base the algorithm of clinical assessment on physical parameters, such as the size and number of neoplastic foci, pathological data informing on the microvascular invasion and clinical liver function parameters which constitute the foundation of BCLC, Child-Pugh, Milan Criteria and other, similar classification systems. The single end point of our study used to assess the performance of the different factors was tumor recurrence at the 3 year follow-up after a radical surgery. The multifactorial analysis with the use of clinical factors, determined according to the standards of the qualification of patients for surgical treatment referred to above, showed that the risk of recurrence does not depend so much on the extent of the tumor impact as on disturbances resulting from a liver dysfunction. Significantly, the same analysis made with the use of standard clinical factors and angiogenesis factors revealed a major role of the tumor angiogenesis potential and the degree of tumor cell maturity in the appearance of HCC recurrences. It is therefore possible to ‘tighten’ the systems of clinical classification and improve the stratification of patients for radical surgical treatment. Determination of the EPCs in circulation is a non-invasive, simple and inexpensive method and the obtained results seem to be much easier to interpret than the ‘capricious’ VEGFR factor.

**Expectations and limits of the study**

There are at least several patient and tumor-related factors responsible for the ultimate outcome of the patient after surgical treatment. Coexisting HCV infection, tumor number and size, as well as tumor histologic grade and microvascular invasion are the most frequently reported. Factors indicative of tumor angiogenesis activity may be considered, to a certain extent, just an addendum to the wide range of the known prognostic factors. They certainly are but, unlike the currently used factors reflecting the clinical-pathological features of the disease, the enhanced angiogenesis activity of the tumor is not only indicative of poor prognosis but also points to a possible complementary treatment, i.e. an angiogenesis-suppressing therapy. Antiangiogenic therapy has already entered clinical trials in HCC patients and holds the promise of providing an effective novel treatment for HCC. It is not possible to answer, basing on this study results, whether such a therapy applied as a neo-adjuvant or adjuvant treatment to the surgical management of early HCC could improve the recurrence-free period of time in ambiguous surgical cases. On the other hand, it seems to be obvious that enhanced angiogenesis cannot be the criterion that would exclude patients with HCC clinical stage 0-A from curative treatment by liver resection or liver transplantation. First of all, such issues were not considered in the study assumptions, secondly, the study has several limitations. It was conducted in a single center and concerned a limited cohort of patients. However, the sample of 180 patients evaluated in the study corresponds to the number of cases presented in many other reports concerning biomarkers and factors predicting the angiogenesis propriety of HCC. As presented in our previous reports the levels of endothelial progenitor cells were found to correlate significantly with the manifestation of clinical and biochemical symptoms of the disease [21, 37]. Thus, it was necessary to confirm whether the count of the endothelial progenitor cells might be considered an additional factor contributing to better evaluation of HCC patients in pre-treatment settings and a helpful determinant of the optimal management. The answer seems to be positive. Our study should also be considered in terms of the limitations of the ISHAGE methodology that was used for the cell count. The ISHAGE protocol was adopted as a standardized and sensitive flow cytometric method to quantify the progenitor cells that bear the CD34 antigen in peripheral blood. The immunofluorescence of the cells for CD309 was achieved by adding the CD309 surface marker to the original ISHAGE protocol. Being
Angiogenesis activity of HCC

aware that the identification of the EPCs by flow cytometry is a “rare event”, depending exclusively on the number of positive events (n), we increased the total number of acquired events to at least 2,000,000. Despite the above mentioned limitations we consider flow cytometry a method sensitive enough when the count of peripheral blood EPCs is conceived as a biomarker for the stratification of early HCCs.

We conclude that an increased number of EPCs in the blood is a reliable prognostic indicator. Radical surgery with an intention to cure the disease in patients with an over 47% higher rate of circulating EPCs should be proposed with full awareness of failure without an adjunctive therapy.

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Disclosure of conflict of interest

None.

Abbreviations

HCC, hepatocellular carcinoma; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2; EPCs, endothelial progenitor cells; BCLC, Barcelona Clinic Liver Cancer classification; TNM, tumor nodules metastases classification; AFP, alpha fetoprotein; ALP, alkaline phosphatase; PLT, platelets count; CD (34, 133, 45, 3019), cluster of differentiation antigens; K3 EDTA, ethylenediaminetetraacetic acid; INR, International Normalized Ratio of prothrombin time of blood coagulation; ISHAGE, International Society of Hematology and Graft Engineering; HBV/HCV, hepatitis B virus/hepatitis C virus.

Address correspondence to: Dr. Janusz Sierdziński, Department of Medical, Informatics & Telemedicine, Central Teaching Hospital, Medical University of Warsaw, Banacha 1a, Warsaw 02097, Poland. Tel: (+48) 22 658 29 97; E-mail: jsierdzinski@wum.edu.pl

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Angiogenesis activity of HCC


Angiogenesis activity of HCC

