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Review Article

Chronic Hypoxia Emerging as One of the Driving Forces behind Gene Expression and Prognosis of Hepatocellular Carcinoma

Jos F. van Pelt, Hannah van Malenstein, Frederik Nevens, and Chris Verslype

Liver Research Facility and Lab of Hepatology, University Hospital Gasthuisberg-Leuven, Herestraat 49, bus 703, 3000 Leuven, Belgium

Correspondence should be addressed to Jos F. van Pelt, jos.vanpelt@med.kuleuven.be

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Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors and an important cause of death. It has become evident that the tumor microenvironment, including hypoxia, plays a major role in the development of HCC. This paper focuses on the role of chronic hypoxia in HCC. Recently, we have shown the importance of chronic hypoxia on gene expression and behavior of liver cells. Using a cell culture model, we identified a distinct gene expression pattern and demonstrated clinical relevance for a 7-gene subset that can predict survival and early recurrence in patients. Recently, it was also shown that chronic hypoxia is associated with the upregulation of β-catenin and Hif1α and that suppression of β-catenin reduced the metastatic potential of the tumor. Both studies demonstrate the importance of chronic hypoxia for the prognosis of HCC. Identifying the molecular pathways can help us to understand the mechanisms responsible for tumor aggressiveness.

1. Introduction—Hepatocellular Carcinoma: The Clinical Problem

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors and an important cause of cancer death. Worldwide, it ranks fifth among the solid tumors and is the third cause of cancer-related mortality in males with ~600.000 deaths each year [1]. It is most prevalent in Asia (the annual incidence in China equals 100 per 100,000) and Africa. In Africa, HCC is mostly related to chronic hepatitis B virus (HBV) infection in Asia to hepatitis B or C. The incidence in Europe and the USA is considerably lower with an annual incidence between 1 and 10 per 100.000, but it is rising mainly due to hepatitis C (HCV) virus infection with an expected fivefold increase peaking around 2015 [2]. In more than 80% of cases, HCC is associated with cirrhosis or with advanced fibrosis [3, 4] Cirrhosis is mostly due to chronic infection with the HBV or HCV, alcohol abuse, or metabolic disorders like hemochromatosis. More recently, nonalcoholic steatohepatitis (NASH) resulting from the insulin resistance syndrome is emerging as another important risk factor for cirrhosis and HCC [5, 6].

Hepatocarcinogenesis is a multistep process in which a number of mutational genetic alterations accumulate in a cell. It involves the transition of a normal cell via the so-called initiated cell to a preneoplastic lesion that develops into malignant tumor and to clinical liver cancer [7].

Symptomatic HCC frequently runs a rapidly progressive clinical course and has a low rate of resectability and generally a poor response to nonoperative treatment and therefore a bad prognosis [8, 9]. HCC in general develops over a long period (20–30 years) and is usually asymptomatic in the early stages. Therefore, a large group of patients have already an incurable disease at the time of diagnosis with the present available therapies. Identifying an HCC in a nodular (cirrhotic) liver represents a real challenge, especially if the lesion is smaller than two centimeters, which is an increasing problem due to the implementation of surveillance programs of patients at risk for HCC. HCC shows a variety of imaging features that reflect the variable pathological characteristics of this tumor. The cirrhotic liver harbors large regenerative nodules and dysplastic nodules (DNs), which may be very challenging to distinguish from small HCCs. The vascular supply to the lesion represents the key pathologic factor for differential diagnosis that is also reflected in imaging characteristics.

Through the progression from low-grade DN, to high-grade DN and finally early HCC, there is a development

of arteries which become the dominant blood supply [7]. This neoangiogenesis allows the imaging diagnosis of HCC. Imaging techniques may establish the diagnosis of HCC in nodules larger than two centimeters showing characteristic early arterial hypervascularisation on a dynamic imaging technique, even with a normal serum alpha-fetoprotein (AFP) value. In lesions ranging from one to two centimeters, two different dynamic imaging techniques should demonstrate the typical vascular features. If not, special workup for these lesions may consist of either followup or preferably obtaining a pathological proof using a safe approach. A lesion that is increasing in diameter, the presence of a tumor capsule, internal mosaic architecture, or invasion of portal vein branches are strongly suggestive of HCC [10].

Alpha-fetoprotein is the only serological marker commonly used in diagnosis and has a poor sensitivity ranging from 39% to 65% and a specificity ranging from 76% to 97%. This high variability relates to the different cutoffs used in various (retrospective) studies. At values over 200 IU/mL, AFP is reliable as tumor marker but the percentage of patients with such high levels is very small and false positives are quite frequently seen especially in viral cirrhosis. The availability of other suitable serological early diagnostic markers would be very useful. Some new tumor markers are emerging, such as human cervical cancer oncogene and human telomerase reverse transcriptase mRNA, and these have higher accuracies than AFP.

Furthermore, some other tumor markers, such as glypican-3 (GPC-3), Golgi Protein 73, vascular endothelial growth factor (VEGF), des-gamma-carboxyprothrombin (DCP), and transforming growth factor-beta1, combined with AFP seem to have a greater sensitivity than AFP alone.

The treatment of patients with HCC is based on staging, which includes assessment of tumor extent, liver function, portal pressure, and clinical performance status. The widely used Barcelona-Clinic-Liver-Cancer (BCLC) staging system links staging of HCC in cirrhosis with treatment modalities [8, 9]. The system identifies those patients with early HCC (stage 0 and A) who may benefit from curative therapies (resection, radiofrequency ablation, and liver transplantation), those at intermediate or advanced stage (stage B, C) who may benefit from palliative treatments (such as transarterial chemo- or radioembolisation), and the patients with a very poor life expectancy (stage D) where best supportive care is the only option [9, 11] (see Figure 1).

Until recently, patients with advanced hepatocellular carcinoma (BCLC-stage C) could only be offered best supportive care or systemic chemotherapy with an unfavorable risk-benefit ratio. The advent of targeted drugs has offered hope to our patients. Sorafenib is a multikinase inhibitor targeting the Raf serine/threonine kinases and the VEGFR1-3, PDGFR-β, c-Kit, Flt3, and p38 tyrosine kinases, that block the VEGF and PDGF-dependent angiogenesis. The drug is now considered the standard of care in patients with advanced hepatocellular carcinoma and preserved liver function, based upon two phase III trials that showed an improved overall survival and progression-free survival compared to placebo. Median survival increased from 7.9 to 10.7 months (hazard ratio 0.69, 95% CI 0.55–0.87) [12, 13]. Most drug-related and manageable adverse events include fatigue, anorexia, diarrhea, and hand-foot skin reaction. Unfortunately, all patients with advanced HCC still die from the disease and there is an unmet need for other drugs, either as a single agent or in combination with sorafenib. A phase III clinical trial with sunitinib, a drug with a similar mode of action as sorafenib, has recently been stopped because of higher toxicity and no superior efficacy compared to sorafenib. Other clinical trials explore the safety and efficacy of everolimus that targets mTOR, which is a central regulator of cell growth and angiogenesis.

2. Tumor Microenvironment

Supported by a growing number of scientific reports, it has become clear over recent years that the tumor environment also plays an important role in tumorigenesis and tumor progression [14]. Cancer cells are not as autonomous as once thought; they depend on angiogenesis, inflammatory cells, and fibroblasts [15, 16]. Obvious, in the cirrhotic liver there is an abundance of fibroblasts and there is emerging evidence that some are cancer-associated fibroblasts (CAFs) [17]. In HCC, the progression of malignant hepatocytes frequently depends on transforming growth factor (TGF)-β provided by the stromal cells (fibroblasts, macrophages, etc.). This TGF-β is one of the factors that can induce an epithelial-to-mesenchymal transition (EMT) [18]. For different types of tumors, it has been demonstrated that an EMT switch is associated with a worse prognosis as seen in esophageal squamous cell carcinoma [19], gastric cancer cells [20], bladder cancer [21], nonsmall cell lung cancer [22], and pancreatic ductal adenocarcinoma [23]. For HCC, this correlation has also been found with an independent effect of Twist and Snail in promoting metastasis of hepatocellular carcinoma [24–27].

Characteristics for EMT are a shift in gene expression with an upregulation of Twist [23, 28], Snail [29], VE-cadherin [30], Vimentin [31], downregulation of E-cadherin [32], hepatocyte transcriptional factor HNF4α, and changes in the cytoskeleton. Important molecular pathways involved in EMT are Wnt/β-catenin signaling [27, 33] and through growth factor signaling like PDGF [34].

In EMT, there is a destabilization of adherent junctions [35] and the cells develop more invasive properties [31]. In mouse models, both *in vitro* and *in vivo*, epithelial-to-mesenchymal transition has been shown to promote invasion and metastasis [26, 36].

Two major risk factors associated with HCC (hepatitis B virus and hepatitis C virus) have been shown to contribute to EMT in HCC tumor progression [37, 38].

Kim et al. has shown that epithelial-to-mesenchymal transition gene signature can predict clinical outcome of hepatocellular carcinoma. They used RT-PCR to determine expression of EMT-related genes and could predict survival of patients using only four genes (E-cadherin (CDH1), inhibitor of DNA binding 2 (ID2), matrix metalloproteinase 9 (MMP9), and transcription factor 3 (TCF3) [39].

The role of hypoxia in initiation and progression of HCC is only partly understood. Hypoxia is involved in

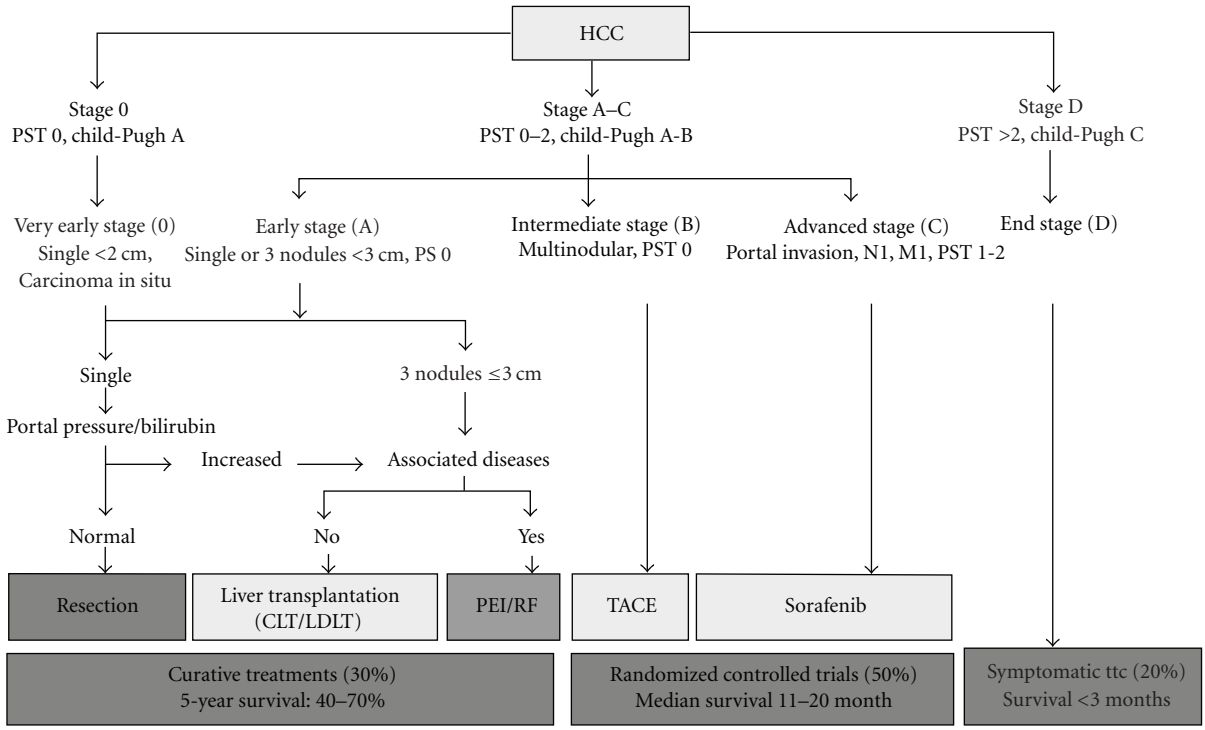

Figure 1: Barcelona Clinic Liver Cancer (BCLC) staging classification and treatment schedule (Adapted from [9]). PST: primary systemic therapy, CLT: cadaveric liver transplantation, LDLT: living donor liver transplantation, PEI: percutaneous ethanol injection, RF: radio frequency ablation, and TACE: transcatheter arterial chemoembolization.

neoangiogenesis and the formation of new blood vessels and it induces gene expression through the transcription factor Hif1α that regulates more than 50 targets genes. Hypoxia through Hif1α [40] stimulates EMT [41, 42] involving PI3 kinase/AKT signaling [43–45] and redox mechanisms [46]. Hypoxia further influences the tumor microenvironment by stimulation of stellate cells, and it promotes fibrosis [42].

3. Molecular Classification for HCC

Several studies have tried to classify HCCs based on their gene expression or on their chromosomal alteration. Obtaining a molecular classification of hepatocellular carcinoma, however, remains a striking challenge because of the overwhelming genomic complexity of HCC [47]. Hitherto, there are several large studies published that classify HCC based on gene expression. Two of them could identify subgroups with common affected pathways such as β-catenin and proliferation signals [48, 49] and one group [50, 51] was able to correlate the subgroups with survival. Other studies tried to find common affected genes that are associated with prognostic factors such as vascular invasion and metastasis, but the overlap between these studies is very poor. Three groups studied the surrounding liver tissue, instead of the tumor tissue itself [52–54]. Based on their findings, late recurrence can be predicted in cirrhotic patients but these recurrences are probably "second primary tumors in an at-risk liver."

Combining all these studies led to the transcriptome classification as developed by Zucman-Rossi and Boyault [48, 55]. A subgroup of patients have activation of pathways related to cell proliferation (IGF signaling, RAS/MAPK, and mTOR signaling) and differentiation (Wnt-β-catenin and Hedgehog). Overall, we can classify as follows: (1) a proliferation subgroup with Akt/mTOR activation, (2) the closely related DNA-repair/cell-cycle subgroup, (3) the β-catenin subgroup, (4) the immune-related subgroup, (5) the MAPK-/c-jun-/c-Myc-related genes and Jak/STAT-related genes, and (6) the metastasis-related genes group.

Molecular analysis also helped to identify important cellular pathways and with them possible therapeutic targets. The first molecule that in this way has shown clinical application for liver cancer is the multikinase inhibitor sorafenib, others are currently in different stages of clinical studies. The prognostic implication of this clustering based on pathways remains unclear.

4. Hypoxia and HCC

One of the factors that appears to affect cancer cell behavior and patient prognosis is hypoxia [56]. Hypoxia is associated with poor prognosis in several malignancies, such as cervix and breast carcinoma, and with the development of resistance to chemotherapeutic agents and radiation [57, 58]. And although HCC is a hypervascular malignancy, still there are regions with reduced oxygen supply [59]. Hypoxic regions are already present in the early stage

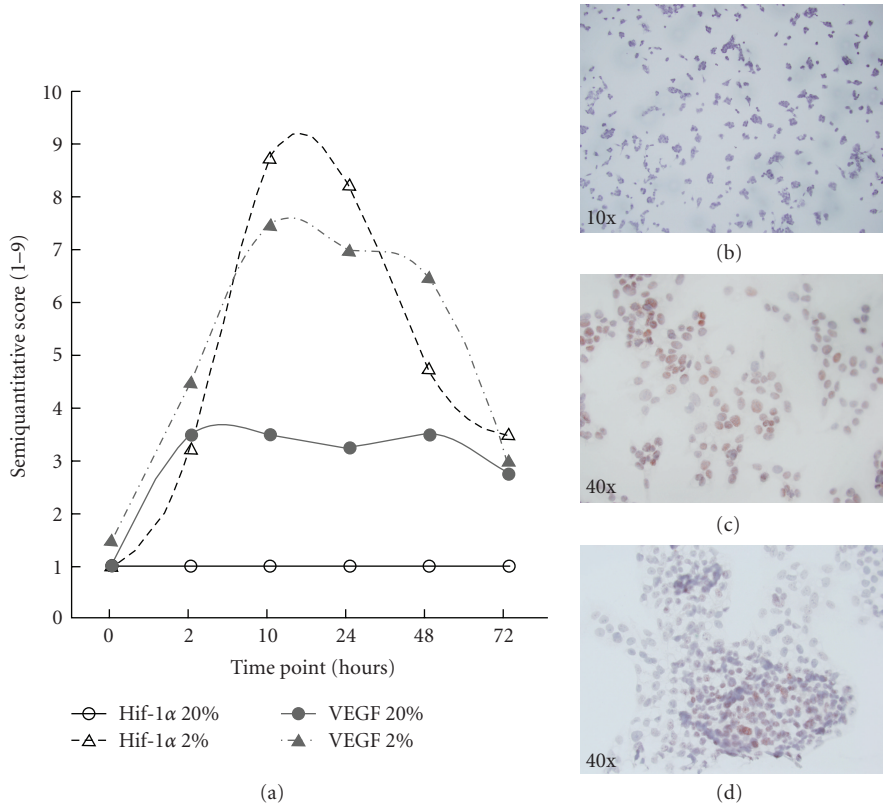

Figure 2: Immunohistochemical staining for Hif-1α and VEGF after exposure to normal (20%) or impaired (2%) oxygen at several time points. (a) To evaluate the staining, a semiquantitative quickscore (1–9) was used which combines positivity (P) with a range from 1 to 6 and intensity (I) with a range from 0 to 3. There is a strong induction of both proteins in the acute phase (0–24 hours), but after prolonged hypoxia a new balance occurs. Hif-1α is not expressed under normal oxygen (20%) conditions, whereas VEGF has a low constitutional expression. (b–d) *Immunohistochemical staining under hypoxic conditions*: (b) Hif-1α staining at 0 hrs—there is no Hif-1α present; (c) Hif-1α staining after 24 hrs—almost all cells are positive; (d) Hif-1α staining after 72 hrs—some cells are positive.

when the vasculature is not sufficiently extended and in more advanced stages when rapid cell proliferation induces hypoxia [60]. Moreover, liver cancer usually develops in a cirrhotic environment where the blood flow is already impaired and, more importantly, during the expansion of the tumor, the neovascularization is unorganized with leaky blood vessels, arteriovenous shunting, large diffusion distances, and coiled vessels. These structural and functional defects lead to both acute hypoxia due to fluctuating flow and to chronic hypoxia due to diffusion distances of more than 150 μm [61, 62].

Hypoxia induces a transcription response that is mainly initiated by hypoxia inducible factor-1 alpha (Hif-1α). In normoxic conditions, Hif-1α is rapidly broken down in the cytoplasm through ubiquitination by the cooperation between Von Hippel Lindau protein and the oxygen sensors prolyl hydroxylase (PHD) and factor inhibiting Hif (FIH). When oxygen is lacking, Hif-1α accumulates and can translocate to the nucleus and form the transcriptionally active complex Hif-1 by coupling to Hif-1β (also ARNT). Hif-1 is a master control gene with over fifty target genes and alters different pathways, such as angiogenesis (VEGF), glycolysis (GLUT1), apoptosis (BNIP), and cell proliferation (IGF2) among others [57]. Hitherto, studies evaluated only the early changes in gene expression of cells exposed to maximum 24 hours of hypoxia [63–65]. From *in vitro* studies, it has come forward that there is a relationship between mTOR inhibition and hypoxia [66]. Hypoxia can also elicit multidrug resistance to chemotherapeutics [67] and protect cells from drug-induced apoptosis [68–70]. Several studies have found a link between hypoxia and oxidative stress, one of the factors strongly associated with the development of cirrhosis and HCC and therefore an attractive candidate for antitumor therapy [71–73].

Many studies using either overexpression or gene silencing by siRNA have shown possible interactions in cell lines under hypoxia. The relevance of the separate observations and the interplay with other mechanisms need to be validated in a clinical relevant setting.

5. Chronic Hypoxia: The Additional Dimension

We hypothesized that, in HCC, there are regions with sustained hypoxia which induces a characteristic gene expression pattern and, further, that the extend of hypoxic gene expression determines the aggressiveness or, more in general, the prognosis.

In our approach, we addressed the problem by starting from an *in vitro* situation in which the results are not

clouded by the heterogeneous nature of HCC from patients with different etiologies that would otherwise obscure the underlying processes.

To test our hypothesis, we determined the gene expression in human HCC cells HepG2 under different oxygen conditions (20, 5, 2, and 1%) and different incubation periods (24 and 72 hours). We tested 6 genes (CCNG2, EGLN3, ERO1L, MAT1A, RCL1, and WDR45L), and a score was determined by RT-PCR based on the relative expression to cells at 20% O_2. For 5% O_2, we did not observe big changes in expression. The most pronounced effect was observed at 72 hours and 2% O_2. Cells cultured in 1% O_2 for 24 hours had a hypoxia score almost equal to 2% O_2 at 72 hours, but this was dominated by only 3 genes (CCNG2, EGLN3, and ERO1L). When HepG2 cells were cultured with 2% O_2 continuously, no extensive cell death was noted, the cell doubling time increased, and the cultures could be passaged by trypsinization for at least 2 months.

The difference in gene expression between acute (<24 hours) and prolonged or chronic hypoxia was further demonstrated by immunological staining (Figure 2, semi-quantitative quickscore [74]). At 2% O_2, both Hif-1α and VEGF are upregulated from normoxic conditions (20% O_2) at 72 hours; this was below the peak between 24 and 48 hours that represents the early (acute) reaction to hypoxia.

Using these conditions, we determined the differentially expressed genes in HepG2 cells that were cultured for 72 hours at either 20% oxygen or in hypoxic conditions at 2% oxygen by microarray. A total of 37,707 spots showed a representative signal of which 3,592 (8%) with a fold change above 2 and a P value < .05 (1,879 upregulated and 1,713 downregulated). A correction for multiple testing was used, and probes with a P value below .01 and a fold change of >2 were selected. This resulted in a list of 265 highly significant genes designated as the hypoxic gene set. Next, we used all presently available published datasets [48–50, 75] to investigate the prognostic correlation with our *in vitro* derived hypoxia gene set. The first three training datasets contained HCCs of 247 patients and the validation dataset 91 HCCs. To test whether the overall expression pattern of these 265 hypoxia genes is significantly related to the prognostic factor considered for each of the three training datasets, the global test of Goeman et al. was used [76]. This resulted in a significant enrichment of the hypoxia gene set for all three training sets (P value .03595 for Boyault, P value < .00001 for Lee, and P value .0064 for Wurmbach), keeping only the significant genes with a z-score above 1,130 genes remained for the dataset of Lee et al., 43 genes for Boyault et al., and 58 genes for Wurmbach et al. Finally, genes for which the direction of altered expression did not correspond to the direction observed *in vivo* were removed. With this approach, we were able to downsize our hypoxia gene set to seven genes, the hypoxia signature, found to overlap between the three training datasets (see Figure 3).

These 7 genes were used to define the hypoxia score based on the mean (expression ratio UPregulated genes in log base 2)—mean (expression ratio DOWNregulated genes in log base 2). This hypoxia score was then used to classify the patients in the 3 training sets and to calculate the area under the ROC curve (AUC), to assess the predictive performance in all data sets. The hypoxia score based on these seven genes could significantly divide patients with and without vascular invasion (Wurmbach, AUC 88.9%), with a FAL-index >0.128 and ≤0.128 (Boyault, AUC 72.8%), and with cluster A and cluster B gene expression (Lee et al., AUC 84.9%). The performance of our hypoxia score was confirmed in the Chiang dataset with the BCLC classification as prognostic characteristic. The seven genes significantly separate the BCLC group 0/A/B from C (AUC 91%) as well as the group 0/A from B/C (AUC 71.5%).

Table 1: List of the 7 hypoxia-related prognostic genes in HCC included in the hypoxia score. CCNG2, EGLN3, ERO1L, and WDR45L will be upregulated under hypoxia in HCC and FGF21, MAT1A, and RCL1 will be downregulated under hypoxia in HCC.

Genes in the hypoxia signature		
Gene	Full name	Function or Pathway
CCNG2	Cyclin G2	Regulation of the cell cycle
EGLN3	Egl nine homolog 1 (*C. elegans*)	Hif1α-transcription factor network
ERO1L	Endoplasmic reticulum oxidoreductin-1 L	Protein processing and oxidoreductase activity
WDR45L	WDR45-like	Autophagy and response to starvation
FGF21	Fibroblast growth factor 21	Hepatic lipid metabolism
MAT1A	Methionine adenosyltransferase I alpha	Biological oxidation and metabolism
RCL1	RNA terminal phosphate cyclase-like 1	Ribosome biogenesis

The hypoxia score was tested for correlation with survival and recurrence. In a retrospective survival analysis on the 135 patients of the study by Lee et al., if we use a cut-off value of 0.35 for the hypoxia score (log rank test P = .0018), we will be able to demonstrate significant differences in survival in 135 patients with a Kaplan-Meier survival curve (Figure 4(a)). The median survival for patients with a hypoxia score >0.35 (n = 42) was 307 days, whereas the median survival for patients with a hypoxia score ≤0.35 (n = 93) was 1602 days (P = .002). We should make a differentiation between early recurrence (<2 yrs) and late recurrence (>2 yrs) [77, 78]. Recurrence after 2 years is usually a second primary tumor that arises in a cirrhotic liver and has no relation with the first tumor. When early recurrence is the result of dissemination of the primary tumor and tumor characteristics determine the risk of recurrence, our hypoxia score determined on the tumor tissue itself should be able to predict early recurrence. Again, when we use a cutoff of 0.35 for the hypoxia score, the Kaplan-Meier curve shows a significant difference in early recurrence (P = .005) (Figure 4(b)).

In multivariate statistical analysis on 135 patients from the study by Lee et al., using variables such as AFP level (≤300 versus >300), tumor size (≤5 cm versus >5 cm), age (≤65 y versus >65 y), and differentiation grade (1-2 versus 3-4), the hypoxia score was a significant variable for both

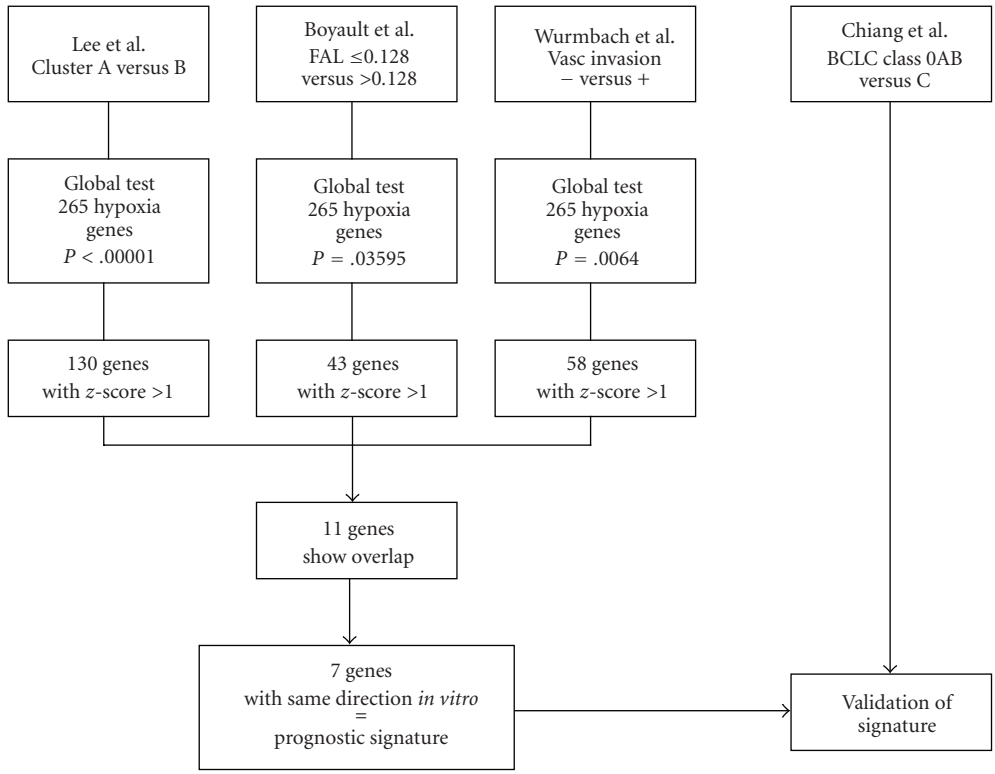

Figure 3: Selection procedure of 7-gene prognostic hypoxia gene set. We started with the 265 genes that were identified from the microarray experiments with HepG2 cells. After several selection steps, we could identify a 7-gene set (Table 1) with prognostic value from the studies by Wurmbach et al. [75], Lee et al. [50, 51], and Boyault et al. [48]. The prognostic value was subsequently confirmed when we tested this set in the study of Chiang et al. [49].

survival ($P = .02$) and early recurrence ($P = .008$) (for full discussion see van Malenstein et al. [79]).

Recently, there was a study by Lui et al. that investigated the activation of b-catenin by chronic hypoxia in HCC [80]. The Wnt/β-catenin pathway is identified as an important signaling pathway in cancer and used for the molecular classification of HCC [81]. In around 30% of HCCs, there is an aberrant expression of β-catenin [47, 81] and β-catenin has a prominent place in the transcriptome classification by Zucman-Rossi and Boyault [48]. Lui et al. found that exposure for 72 hrs to 1% O_2 induced the upregulation of Hif1α and β-catenin protein in PLC/PRF/5 and HepG2 cells but not in other HCC cell lines Hep3B or MHCC97. Under these conditions hypoxia also activated the PI3K/Akt pathway. Both *in vitro* and *in vivo* studies using HCC xenograft model showed that β-catenin depletion through shRNA suppresses the hypoxia-induced increased metastatic potential. This further showed that β-catenin is critically involved in hypoxia-induced epithelial-mesenchymal transition. There was a shift in expression of epithelial markers (E-cadherin and plakoglobin) to mesenchymal markers (vimentin and N-cadherin).

Using immunohistological staining for β-catenin and Hif1α, they further examined the correlation with prognosis in HCC patients. Hif1α was found positive in 63% of patients and was associated with a high incidence of intrahepatic metastasis ($P = .035$). β-catenin was positive in 43.5% and this correlated with microvascular invasion ($P = .001$), poor tumor differentiation ($P = .041$), and high tumor-node metastasis stage ($P = .018$). Both Hif1α and β-catenin alone are associated with reduced overall survival and with time to recurrence. Coexpression of Hif1α and β-catenin resulted in a higher incidence of metastasis and was associated with a more reduced overall survival and time to recurrence than if only one marker was positive.

Also, Dai et al. has found in a set of 110 HCC patients by analyzing mRNA levels that Hif1α plays an important role in predicting patient's outcome [82]. In the study by Simon et al. [54], the surrounding tissue was investigated and they observed a link between deregulation of Hif1α and recurrence after curative resection.

Also in models of breast cancer [83, 84] and in prostate cells [85, 86], the association of chronic hypoxia with EMT transition, resistance to cell death [87], and cancer growth has been demonstrated.

Together with the reduction in blood supply, the supply of nutrients and the removal of waste products will be affected in a tumor, therefore an additional role for the metabolism is expected in cancer possibly in combination with the hypoxia response [88].

6. Conclusions and Perspectives

Current treatment options for HCC patients have benefitted from the development of a rational staging system. This has

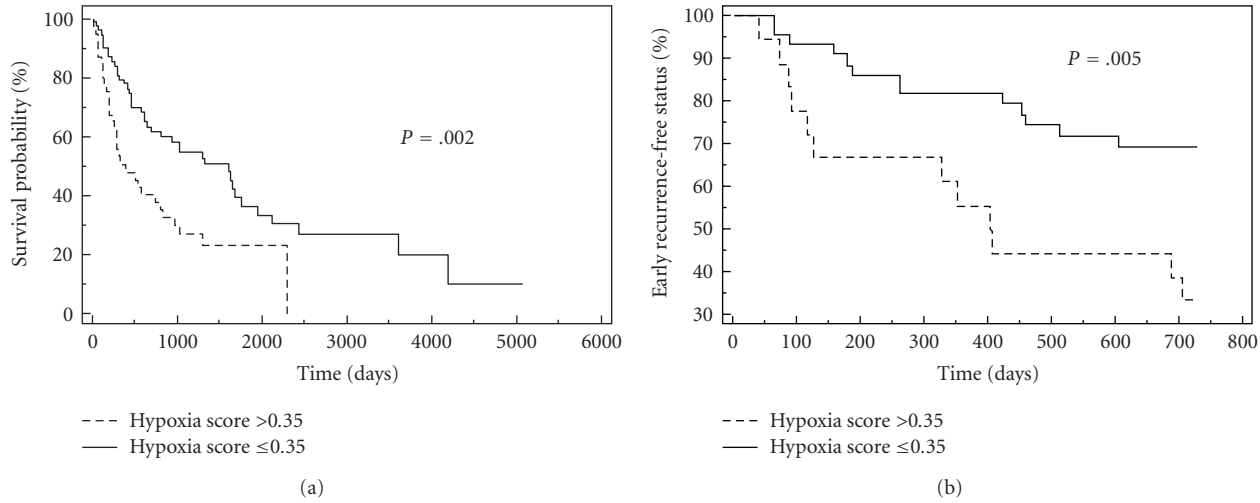

Figure 4: Survival and early recurrence. (a) Kaplan-Meier survival curve for 135 patients [50, 51]. The hypoxia score was used in a dichotomous matter (>0.35 versus ≤0.35). Patients with a score >0.35 had a significantly poorer survival. (b) Kaplan-Meier curve for early recurrence (<2 yrs) in 135 patients. Again the hypoxia score was used in a dichotomous matter (>0.35 versus ≤0.35). Patients with a score >0.35 had a significantly increased risk of early recurrence.

also led the way to an integrated molecular classification based on gene expression and mutational analysis. Due to the nature of this disease, with HCC developing in around 80% of cases in a cirrhotic lever, systemic therapy in more advanced stages of the disease has had limited success until now.

Gene analysis has already revealed some of the more important molecular pathways and that has helped to identify rational targets for therapy and initiated the development of new classes of drugs.

The relevance of chronic hypoxia has long been overlooked due to the attention for acute events. With the better understanding of neoangiogenesis and the recognition of suboptimal quality of the newly formed blood vessels, chronic hypoxia has now been recognized as a clinical entity. Here, we have three studies that show the importance of gene or protein expression in the chronic situation for patients that may have implications for the prognosis for these patients [79, 80, 82]. These results suggest that chronic hypoxia and the corresponding gene expression are important for the aggressiveness of HCC. The molecular processes need to be studied further which could help to identify on a rational basis additional drug targets. Some overlap has been found between chronic hypoxia and the β-catenin cluster in the molecular classification; it should be further investigated whether hypoxia should be incorporated in the molecular classification of HCC.

Gene expression under chronic hypoxia has also been linked to drug resistance; perhaps factors responsible for reduced efficacy of anticancer drugs (transport, metabolism) can be identified from these studies.

The integrated approach of strictly controlled cell culture experiments and clinical validation in large sets of patients shows its strength. With the development of a hypoxia gene set not restricted to a single center and that can predict prognosis for individual patients, a promising tool might be at hand. Furthermore, the demonstration of the role of chronic hypoxia in the progression of HCC suggests that it could also play a role in other types of cancer and is worthwhile to be investigated further. In addition, the study by van Malenstein et al. can also be important for other types of cancer for it presents a method to combine the information from different clinical studies in cancer, overcoming methodological differences and validation in the largest available set of clinical and microarray data.

Conflict of Interests

The authors declared that there is no conflict of interests.

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