The analysis of the tumor microenvironment and laboratory parameters as potential prognostic factors in patients with hepatocellular carcinoma.

Summary

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers and is the fourth leading cause of cancer-related deaths (Globoscan 2020). The incidence of HCC varies significantly geographically, with the highest rates in Asia and Africa, which is associated with infections by hepatotropic viruses HCV and HBV (Ferlay et al. 2015). Currently, it is predicted that the incidence in Western European countries will increase. Despite overall progress in medicine and oncology care, the prognosis for HCC patients remains poor. HCC therapy can vary depending on the stage of the disease. The European Society for Medical Oncology (ESMO) recommendations have been updated several times in recent years due to the emergence of new therapeutic options.

Treatment Recommendations for HCC Based on Disease Stage

Current recommendations emphasize the division of the disease based on its stage according to the Barcelona Clinic Liver Cancer (BCLC) scale. Very early disease (stage 0) is characterized by the presence of a single tumor smaller than 2 cm, preserved liver function (Child-Pugh A), and good clinical condition of the patient (performance status, PS 0). The early stage (BCLC A) is defined as the presence of a single tumor or up to 3 lesions up to 3 cm, partially impaired liver function (Child-Pugh A-B), and good overall condition. Intermediate disease (BCLC B) is diagnosed with multiple liver lesions and still preserved partial liver function and good patient condition. Advanced disease (BCLC C) is characterized by metastatic changes outside the liver and portal vein invasion; the patient's condition should still be relatively good (PS 1-2). Severe liver function impairment (Child-Pugh C) and poor overall condition (PS 3-4) are indicators of the terminal stage of the disease (BCLC D) (Pons et al. 2005).

Depending on the stage of the disease, patients should be qualified for different therapeutic methods. It should be noted that in 2025, new ESMO guidelines for the treatment of HCC were published. According to these guidelines, patients in stages BCLC 0–B can be qualified for resection - recommended for patients with a single tumor >2 cm, without portal hypertension, and possible for selected patients with Child-Pugh B; ablation (radiofrequency ablation, RFA/microwave ablation, MWA), which can be an alternative to resection in BCLC

0–A, for tumors 3–5 cm or as a bridge to transplantation. Liver transplantation (LT) can be considered if the Milan or University of California, San Francisco (UCSF) criteria are met. It is worth noting that bridging therapies (transarterial chemoembolization, TACE; transarterial radioembolization, TARE; stereotactic body radiotherapy, SBRT) can be considered for long waiting times. Importantly, tyrosine kinase inhibitors (TKI) or immunotherapy are not recommended after resection/ablation or LT. Significant changes in the new recommendations concern systemic treatment in advanced disease stage - BCLC C. First-line treatment regimens include atezolizumab (anti-PD-L1 immunotherapy) combined with bevacizumab (anti-VEGF) or durvalumab with tremelimumab. Alternatively, camrelizumab with rivoceranib (not approved by EMA/FDA), dual immunotherapy, nivolumab with ipilimumab (anti-CTLA-4, approved by the European Medicines Agency, EMA); durvalumab or tislelizumab monotherapy, and lenvatinib or sorafenib in case of contraindications to immunotherapy. In second-line treatment after sorafenib, regorafenib, cabozantinib, or ramucirumab (with alpha-fetoprotein level, AFP ≥400 ng/ml) can be used, while after immunotherapy or lenvatinib, sorafenib (off-label) can be considered (Vogel et al. 2025).

Although liver transplantation or resection are considered radical treatment options, some patients experience disease recurrence. Therefore, there is a strong need to identify prognostic factors that allow for the adjustment of treatment and oncological surveillance to the needs of patients and tumor characteristics. Moreover, in the likely upcoming era of adjuvant treatment and immunotherapy, where response is observed only in some patients, identifying predictive factors is crucial.

Tumor Microenvironment and Its Impact on Disease Progression

The tumor microenvironment (TME) plays a key role in tumor development. Continuous processes of hepatocyte damage and regeneration within the TME are associated with the development of chronic inflammation. Complex interactions between hepatocytes and immune cells largely depend on the TME. The TME includes the extracellular matrix, tumor cells, fibroblasts, and immune cells. Immune cells include tumor-associated macrophages (TAM), tumor-infiltrating lymphocytes (TIL), tumor-associated neutrophils (TAN), and bone marrow-derived suppressor cells (BMDSC). All these cell types are capable of releasing signaling molecules that exert various effects, such as inducing immunity, tumor cell tolerance, angiogenesis, increased proliferation, or preventing tumor growth and metastasis (Greten and Grivennikov 2019; Sas et al. 2022).

Within the TME, growth factors, cytokines, extracellular matrix proteins, and enzymes are present, which can contribute to or prevent carcinogenesis. TAMs are particularly interesting. TAMs are immune cells that not only regulate primary tumor growth but also participate in anti-tumor acquired immune response, tumor angiogenesis, and extracellular matrix remodeling. TAMs are characterized by the expression of CD68 molecules (cluster of differentiation, CD68). Depending on their characteristics, they are divided into two main classes: M1 and M2.

M1 TAMs are essential for pro-inflammatory and anti-tumor responses. These cells can be activated by cytokines such as interferon-gamma (IFN-gamma), tumor necrosis factor-alpha (TNF-alpha), granulocyte-macrophage colony-stimulating factor (GM-CSF), or in response to bacterial infection. The production of interleukin (IL)-12 and other pro-inflammatory cytokines leads to the initiation of a Th-1 dependent immune response. M1 macrophages are also capable of cytotoxic activity against tumor cells (Martinez and Gordon 2014).

M2 macrophages are associated with tumor progression and are more frequently observed in HCC tissue than M1 macrophages. M2 macrophage markers include CD163, CD204, CD206, and MARCO (Larionova et al. 2020). M2 TAMs can be activated by IL-4, IL-10, IL-13, or macrophage colony-stimulating factor (M-CSF) and glucocorticoid hormones. M2-like macrophages exhibit various functional phenotypes and are further divided into four other types (Zhou et al. 2014; Solinas et al. 2009; Zhang and Sioud 2023).

Several studies suggest that CD68 expression may have a negative prognostic value. On the other hand, some studies suggest it is a positive prognostic factor. A recently published meta-analysis showed that CD68 TAMs are not associated with patient prognosis (Zhang et al. 2021). However, the available data have various limitations. Most studies analyzing CD68 expression were conducted among patients after resection, and only two analyzed populations after liver transplantation, with one study suggesting no impact on prognosis (n=206) (Gao et al. 2012), while the other suggested a negative impact (n=88) (Atanasov et al. 2020).

In another published study, CD68 M1 TAMs were associated with the induction of programmed death ligand 1 (PD-L1) in HCC cells, suggesting their role in tumor development. When PD-L1 expression in HCC was evaluated together with CD68, survival analysis showed that the presence of PD-L1 on tumor cells was correlated with tumor progression, while PD-L1 expression on macrophages played a protective role in HCC patient prognosis. Moreover, in this study, CD68/PD-L1+ cells were associated with an activated

immune microenvironment with intense CD8 T cell infiltration (Zong et al. 2019; Liu et al. 2018).

Immune checkpoint molecules control the immune response. One of the most important seems to be the PD1 receptor, which is typically induced on activated T cells, NK cells, B cells, and antigen-presenting cells. Conversely, PD-L1 is present on tumor cells, hepatocytes, liver stellate cells, Kupffer cells, or liver sinusoidal cells (Wang et al. 2011). PD-L2 is another known PD-1 ligand present on dendritic cells.

The PD-L1/PD-1 axis plays an important role in the anti-tumor response. PD-1/PD-L1 binding leads to the suppression of immune cell activity against the tumor and the development of host tolerance. PD-L1 overexpression may be one of the mechanisms used by tumors to "escape" the host immune system (Keir et al. 2008; Kudo et al. 2017). Evidence from several studies indicates that patients with PD-L1 expression on tumor cells or immune cells are more likely to benefit from systemic immunotherapy (Garon et al. 2015 1; Lin et al. 2018).

The impact of the PD1/PDL1 axis on prognosis and treatment outcomes in HCC has been evaluated in several studies. However, the results have been conflicting, and the underlying mechanism is not fully understood. It has been shown that patients with strong PD-L1 expression and the presence of TILs generally have better prognoses, although if there is lower PD-L1 expression and galectin-9 and low CD8+ TIL levels, the prognosis may be worse (Sideras et al. 2017). On the other hand, the results of another study suggest that PD-L1 expression by tumor cells or immune cells present in the tumor environment may be associated with tumor aggressiveness (Calderaro et al. 2016). These conflicting results clearly indicate the need for further research on the immune environment.

Since anti-PD-1/PD-L1 immunotherapy is currently being investigated in various clinical settings in HCC patients, and data on the impact of PD-1/PD-L1 expression on patient prognosis are inconsistent, it is essential to assess the frequency of PD-L1 expression and immune cell infiltrates in HCC and evaluate their prognostic or predictive value (Kan and Dong 2015; Sideras et al. 2017).

Retrospective Analysis of Prognostic Factors in HCC

Based on the above data, a study was designed to evaluate molecular and laboratory factors associated with the prognosis of HCC patients. The study assessed the following parameters

in correlation with overall survival and recurrence-free survival: tumor size and number, treatment method (resection/transplantation/systemic treatment), HBV, HCV infection status; baseline laboratory parameters: AFP, WBC, NLR, PLR, ALBI, PALBI, bilirubin; PD-L1, CD68, TILs expression.

The study analyzed data from over 227 consecutive HCC patients treated at an academic center. Statistical analysis was conducted for the general population and two subgroups - post-liver transplantation and post-resection. A third analysis in the group of patients eligible for systemic treatment was not conducted (these patients were also excluded from the general population analysis due to different clinical characteristics). This was because, despite evaluating data from over 70 patients treated systemically for HCC, histopathological material was available for only less than 20 patients, which did not allow for reliable statistical analysis. Additionally, significant heterogeneity of the group in terms of treatment was noted: 12 patients received sorafenib, 3 received cabozantinib, 1 received gemcitabine with oxaliplatin, and 1 received zoledronic acid.

The final analysis included 111 patients meeting the inclusion criteria: 52 post-liver transplantation, 59 post-resection. Eighty-two patients were men, and 29 were women; the median age was 61.7 years. Over 42% (n=48) were HCV-infected, and 24% (n=28) were HBV-infected. Most patients had relatively small tumors (up to 50 mm); in 38% of patients (n=44), lesions of approximately 50 mm or larger were detected.

The median follow-up period was 47.95 months, ranging from 0.1 months (death due to postoperative complications) to 138 months. During the follow-up, 52 cases of tumor recurrence were diagnosed (41/59 patients post-resection (69%) and 11/52 post-liver transplantation (21%)), and 45 patients died. The median recurrence-free survival (RFS) was 20.5 months, and the overall survival (OS) after primary treatment in case of disease recurrence was 30 months.

Additional analysis was conducted in two subgroups: post-liver transplantation and post-resection patients.

Univariate statistical analysis identified several prognostic factors for HCC recurrence. The most important negative prognostic factors in the general population were: tumor size, differentiation grade, presence of microvascular invasion, pre-treatment AFP level, and bilirubin level. Other factors included PALBI and PLT assessment. PD-L1 expression in

tumor tissue was associated with a borderline significant p-value of 0.053. In multivariate analysis, only microvascular invasion (present or absent), baseline AFP level, and WBC were significant negative prognostic factors in the general population.

Subgroup analysis showed that among post-liver transplantation patients, age, tumor size, differentiation grade, microvascular invasion, and CD68 expression were negative factors in univariate analysis. In multivariate analysis, microvascular invasion and differentiation grade were statistically significant. Similarly, in univariate overall survival analysis, AFP, tumor size, differentiation grade, microvascular invasion, PD-L1 expression, and NLR were suggested as prognostic factors, while in multivariate analysis, only microvascular invasion and NLR were statistically significant.

Among post-resection patients, univariate analysis showed that age, AFP, and WBC were prognostic factors for disease recurrence. However, multivariate analysis did not confirm statistical significance. Univariate overall survival analysis suggested that AFP, bilirubin, and microvascular invasion were prognostic factors. Multivariate analysis also confirmed this observation.

PD-L1 expression was confirmed in 4 samples in the entire population. Among post-liver transplantation patients, it was detected in 2 individuals, both of whom experienced recurrence. In one case, progression was diagnosed after 87 months, with PD-L1 expression in 10% of cells; in the other case, recurrence was diagnosed after 10.8 months, with PD-L1 expression in 100% of cells. Additionally, among post-resection patients, 2 other cases of PD-L1 expression were identified: in the first patient, PD-L1 expression was confirmed in 5% of tumor cells, and the patient died due to disease recurrence 14.75 months after surgery; in the second patient, PD-L1 expression was 10%, and recurrence was diagnosed 24 months after surgery. The patient died 3 months after recurrence diagnosis. The small number of patients with PD-L1 expression warrants caution in interpreting this observation, although statistical analysis did not show a significantly increased risk of disease recurrence ($p=0.053$) or death. Only in univariate analysis was it suggested as a negative prognostic factor in post-liver transplantation patients.

PD-L1 expression was also detected on tumor-associated macrophages (TAM) and/or tumor-infiltrating lymphocytes (TIL) in 55 patients: 17 post-liver transplantation and 31 post-resection, including 8 later treated systemically. However, this was not associated with prognosis in any patient subgroup.

CD68 staining was performed in all tissue samples, and the presence of CD68 tumor-associated macrophages was confirmed in 62 cases, ranging from 5% to 40%. Analysis showed that CD68 was not associated with the risk of HCC recurrence.

Tumor-infiltrating lymphocytes (TIL) were observed in 19 cases. The presence of TIL was not associated with prognosis in any subgroup or the general population.

According to univariate analysis results in the HCC patient population, PLT level was also a prognostic factor. Therefore, a meta-analysis and systematic review were conducted, forming the basis of the fourth publication in the series. The study aimed to investigate the impact of thrombocytopenia on the survival of patients diagnosed with HCC and assess the relationship between platelet count and clinical-pathological features of the tumor. A systematic literature review and meta-analysis were conducted according to PRISMA guidelines, searching included databases: PubMed, Web of Science, and Scopus. Based on the search, 26 studies (9403 patients) meeting the specified search criteria were identified. Hazard ratios (HR) for overall survival (OS) were analyzed depending on platelet count. The analyses indicate that thrombocytopenia ($<100 \times 10^9$/L) was associated with poorer overall survival: HR (random effects model) =1.30 (95% CI: 1.05–1.63), HR (multivariate model)= 1.47 (95% CI: 1.15–1.86). In the group treated with curative intent (e.g., resection, RFA), HR=1.62 (95% CI: 1.25–2.11), while in the palliative treatment group (e.g., TACE, systemic treatment), HR=0.81 (95% CI: 0.62–1.05) – suggesting a possible reverse trend. Additionally, low platelet count was more common in patients with smaller tumors and liver cirrhosis, while higher PLT values correlated with larger tumors and lower cirrhosis frequency. This indicates a possible role of platelets and their mediators (e.g., platelet-derived growth factor, PDGF, serotonin) in HCC progression. Platelet count may be a useful, easily accessible prognostic marker in HCC patients, particularly in curative treatment. However, further prospective studies are needed, especially in systemically treated groups, considering precise inclusion criteria.