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Angiopoietin-2 and Vascular Endothelial Growth Factor promote migration and invasion in hepatocellular carcinoma- and intrahepatic cholangiocarcinoma-derived spheroids

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ABSTRACT

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) represent the most common primary liver cancers. Although they arise from different backgrounds, they seem to share some similarities when the comparison is made between HCC and CCA subgroups. We recently identified a neoangiogenic transcriptomic signature that was specifically expressed in aggressive HCC. In this signature, angiopoietin-2 (ANG-2) (a protein related to angiogenesis, proliferation, and epithelial-mesenchymal transition [EMT]) is the most upregulated gene. HCCs carrying this signature share 95% of upregulated genes with intrahepatic CCA (iCCA) patients of the proliferative subgroup. Transcriptomic signature-positive HCCs and iCCAs have a significantly worse prognosis than patients without it.

The working hypothesis of this research was that these molecular and clinical similarities may reflect possible common patterns of response to proangiogenic stimulation. Thus, given the known role of ANG-2 and its main regulator, vascular endothelial growth factor (VEGF), in synergistically promoting cancer progression, we analyzed the pro-invasive effect of these pro-angiogenic factors on HCC and CCA spheroids derived from four cell lines: Hep3B and HepG2 (derived from "aggressive" and " bland" HCC, respectively), HuCC-T1 (intrahepatic CCA) and EGI-1 (extrahepatic CCA). Spheroids were stimulated with recombinant (rh)-ANG-2 or recombinant (rh)-VEGF or their combination. They were analyzed at baseline and after 24 and 48 hours. Our results showed that proangiogenic stimuli increase migration and invasive capacity in HCC- and iCCA-derived spheroids, albeit with different proteins, and are associated with a change in EMT phenotypic markers (decrease in E-cadherin and increase in N-cadherin and Vimentin), especially on the migration front. Furthermore, by using specific inhibitors of ANG-2 (Trebananib) and VEGF (Bevacizumab), the increased migratory capacity acquired by spheroids following ANG-2/VEGF stimulation was abrogated. Taken together, these data demonstrate the central role exerted by ANG-2 and VEGF in promoting migration and invasion in HCC- and iCCA-derived spheroids.

INTRODUCTION

1. Hepatocellular Carcinoma

1.1 Epidemiology and risk factors

Hepatocellular carcinoma (HCC) represents the most common of primary liver cancers and the third most common cause of cancer-related death worldwide (Vogel et al., 2022). The Global Cancer Observatory reported that in 2020 the incidence rate, with 905.677 new cases, was similar to mortality (830.180 deaths), highlighting the poor prognosis of this disease. By 2040, the incidence is expected to increase by 55% and deaths by 56.4% if current rates do not change (Rumgay et al., 2022). The highest incidence rate was recorded in Mongolia (age-standardized rate 85.6), and the lowest in Sri Lanka (age-standardized rate 1.2) (https://gco.iarc.fr/today, accessed [15 Dec 2023]). Both incidence and mortality rates have decreased in some high-incidence Eastern Asian countries (Japan, China, and the Republic of Korea), while they have increased in some low-incidence countries such as the United States, Australia, and some European countries (Arnold et al., 2020). More than 90% of HCCs result from chronic liver disease, in a setting of liver cirrhosis triggered by viral infection with the hepatitis B virus (HBV) or the hepatitis C virus (HCV), alcohol abuse, or alcoholic steatohepatitis (ASH), and non-alcoholic fatty liver disease (NAFLD) or non-alcoholic steatohepatitis (NASH) (Vogel et al., 2022). HBV and HCV remain the major risk factors for HCC development, although the HBV vaccination for newborns and the most effective anti-viral treatments have contributed to a decline in viral-related HCC over the 2000s (McGlynn et al., 2021). On the contrary, the prevalence of NAFLD/NASH-related HCC is increasing, due to the rise in obesity and metabolic-related conditions (Younossi et al., 2018). Overall, the incidence varies among countries and ethnicities depending on the distribution of risk factors and the time of exposure to them. Chronic HBV infection and aflatoxin exposure remain the major factors for HCC development in China, as well as HCV infection in Japan. All metabolic-related morbidities, such as obesity, diabetes, and alcohol are leading causes in Western countries (Valery et al., 2018). At the latest EASL Congress held in Vienna in June 2023, the leading hepatological societies introduced a new nomenclature that better describes all the steatotic diseases with different aetiologies. Thus, NAFLD was modified in “metabolic dysfunction-associated fatty liver disease” (MAFLD), as well as “metabolic dysfunction-associated steatohepatitis” (MASH) replaced NASH. Moreover, among MAFLD patients, those who consume greater amounts of alcohol (140g/week for females and 210g/week for

males) were categorized as MetALD (Rinella et al., 2023). The diagnosis of HCC occurs mainly in people aged between 60 and 70 and predominately affects men, 2- to 4-fold more than women (Petrick et al., 2020). The diagnosis of HCC is usually based on noninvasive criteria, although molecular characterization of the tumor by tissue biopsies is increasingly necessary in clinical practice (Galle et al., 2018; Marrero et al., 2018).

2.1 Pathophysiology and classification

The pathophysiology of HCC is a complex multistep process in which various factors (genetic predisposition, viral and non-viral inflammation risk factors, an altered microenvironment, and the severity of chronic liver disease) predispose for the formation of dysplastic nodules, i.e., preneoplastic lesions. Subsequent molecular alterations give the altered cells a proliferative and survival advantage that leads to complete transformation into HCC (Torrecilla et al., 2017). The cellular origin of HCC is still under debate. Some experimental evidence sustains that HCC may originate from hepatic progenitor cells, also resulting in mixed tumor HCC and cholangiocarcinoma that have progenitor cell-like features (Sia et al., 2017). However, it has also been proposed that mature hepatocytes can transform directly in HCC after a series of genetic alternations, or de-differentiate into precursor cells which then become HCC, or trans-differentiate into biliary-like cells which give rise to cholangiocarcinoma (Sia et al., 2017).

Aberrant telomerase reverse transcriptase (*TERT*) activation, by viral genome insertion, promoter mutation or gene amplification, accounts for around 70 % of cases of genetic alteration observed in HCC, representing the most frequent in this cancer (Schulze et al., 2016). Other common mutations affect Wnt-β catenin pathway, in particular, *CTNNB1* (coding for β catenin) is the most frequently affected (around 30% of cases) followed by *AXIN1*, *AXIN2* and *APC* (inhibitors of Wnt pathway). In addition to *CTNNB1*, another most frequent somatic mutation hit the protein TP53 (around 30% of cases) (Schulze et al., 2016). The low prevalence high-level DNA amplifications involve *FGF19/CCND1*, which lead to the activation of the PI3K/Akt/mTOR and RAS/RAF/mitogen-activated protein kinase pathways, and vascular endothelial growth factor A (VEGF-A) loci respectively (Zucman-Rossi et al., 2015). Additionally, other various alterations in genes implicated in epigenetic regulation and oxidative stress pathways are identified.

Molecular classification of HCC patients became necessary not only to better understand the biological pathogenesis of this tumor but also to provide potential therapeutic targets.

HCCs are commonly classified into two molecular classes: “proliferative class”, generally characterized by an activation of signals involved in cellular proliferation and cell cycle progression, and “non-proliferative class”, with molecular features common to normal hepatocytes (Rebouissou & Nault, 2020; Zucman-Rossi et al., 2015). The former type, which accounts for around 50% of patients, is more common in patients with HBV infection. It shows activation of oncogenic pathways such as Akt/mTOR, RAS/mitogen-activated protein kinase, and MET (a hepatocyte growth factor receptor) among others, along with mutation in *TP53*, amplification in *FGF19/CCND1* and a global hypomethylation profile (Rebouissou & Nault, 2020; Zucman-Rossi et al., 2015). Patients belonging to the proliferation class present aggressive clinical behavior, with high serum levels of alfa-fetoprotein (AFP), poor cell differentiation on histology, and frequent vascular invasion (Rebouissou & Nault, 2020; Calderaro et al., 2019; Zucman-Rossi et al., 2015). On the contrary, the “non-proliferative class”, which includes patients with HCV- and alcohol-related HCC, is characterized by transcriptomic features resembling normal hepatic physiology and normal Wnt pathway activation. From a clinical point of view, these HCCs show better histological differentiation and less aggressive phenotype (J. H. Choi & Thung, 2023; Rebouissou & Nault, 2020; Zucman-Rossi et al., 2015).

Further classifications of HCC, including the immune cell status evaluation, have been proposed by different groups. Sia and colleagues (Sia et al., 2017) identified an “immune class” showing the expression of programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PDL-1), cytolytic T cell activity and the presence of immune cell infiltration. Within the “immune class”, they identified two distinct tumor microenvironment (TME)-based subclasses with either active or exhausted traits. The "active immune" subclass was enriched in effectors of T-cell response (IFNγ and granzyme B signatures), while the "exhausted immune" subclass is characterized by signatures of T-cell depletion, immunosuppressive macrophages, and tumor growth factor β (TGFβ) signaling (Sia et al., 2017). The immunological environment of HCC and its association with molecular classification were further analyzed by Kurebayashi et al. (Kurebayashi et al., 2018). They classified HCC into three immune subtypes based on the number of infiltrating immune cells: "immune-high," "immune-medium," and "immune-low." In agreement with Sia et al (Sia et al., 2017), the "immune-high" subtype, enriched in T cells and B cells/plasma cells, was associated with a good prognosis (Kurebayashi et al., 2018). An improvement of these classifications was made by Zhang et al (Q. Zhang et al., 2019) which identified three different HCC subtypes with immunocompetent, immunodeficient and immunosuppressive

features, expressing different levels of cytokines/chemokines and metabolic characteristics. The former had normal T-cell infiltration, whereas the immunosuppressive subtype was enriched by high expression of T and B regulatory cells, along with immunomodulatory molecules, such as PD-1, PDL-1, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4). Finally, the immunodeficient type showed reduced lymphocyte infiltration (Q. Zhang et al., 2019).

Regarding the molecular-based prognosis prediction, more than 40 prognostic gene signatures have been described (Hoshida et al., 2012). However, despite their potential utility, none has entered routine clinical practice (European Association for the Study of the Liver & European Organisation for Research and Treatment of Cancer, 2012). One possible reason is the origin of the samples studied. Most studies have used specimens from surgical resections. As not more than 10% of HCC patients are liable to resection, resection-based signatures have limited general applicability (R. M. Critelli et al., 2015; Zucman-Rossi et al., 2015). Most prognostic signatures were derived from transcriptomic data, such as G3 (Villanueva et al., 2011; Boyault et al., 2007), 5-gene (Nault et al., 2013), iCluster grouping (Abeshouse et al., 2017), survival signature (Hoshida et al., 2009), or cluster A (J.-S. Lee et al., 2004).

Recently, our group identified a five-gene transcriptomic hepatic signature including angiopoietin-2 (*ANGPT2*), delta-like ligand 4 (*DLL4*), neuropilin (NRP)/tolloid (TLL)-like 2 (*NETO2*), endothelial cell-specific molecule 1 (*ESM1*) and nuclear receptor subfamily 4, group A, member 1 (*NR4A1*), which identifies, with high specificity and sensitivity, rapidly growing HCCs (Villa et al., 2016). All these genes are involved in processes related to neoangiogenesis, i.e. endothelial cell migration and vascular remodeling. Together with the upregulated genes involved in proliferation and cycle control, these five genes signature is an independent predictor for survival in patients with HCC (Villa et al., 2016). The prognostic information provided by this signature together with the clinical and radiological parameters already in use, would have significant implications for the therapeutic management of HCC patients.

The aggressive and "fast-growing" HCCs mentioned above, expressing the five-gene proangiogenic signature (Villa et al., 2016), were further analyzed regarding TME to better understand the biological and immune reasons for aggressiveness and subsequent clinical outcomes. They exhibited immunosuppressive features, i.e., PD-1/PDL-1 expression, enrichment of FoxP3-positive lymphocytes, as well as loss of E-cadherin expression, transition to epithelial-mesenchymal (EMT) phenotype, and poor differentiation on histological analysis. In addition, significant TGFβ1 activation signals a permanent

inflammatory state (R. Critelli et al., 2017). Therefore, a thorough knowledge of inter- and intra-tumor differences, especially considering the immune microenvironment, may be crucial for the right therapeutic choices.

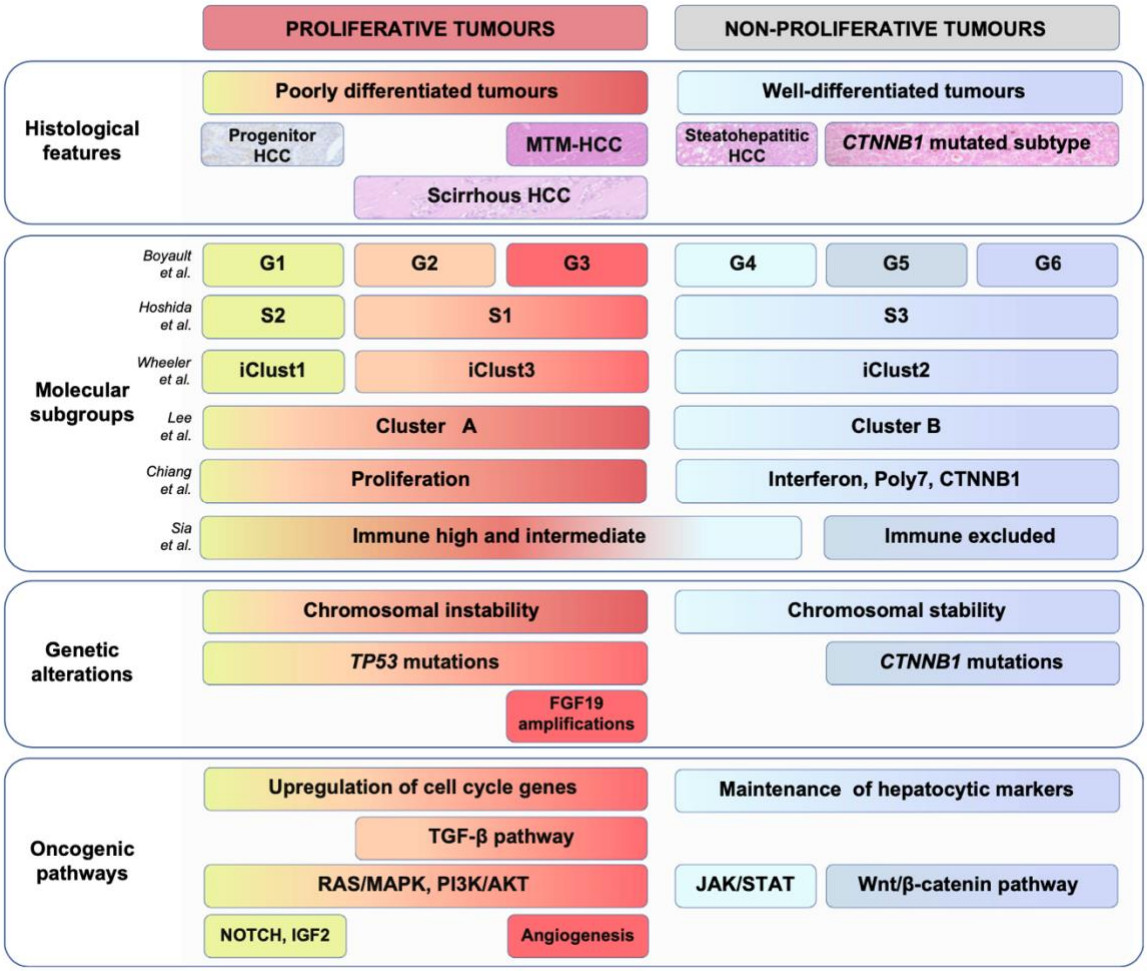

Figure 1 - Principal genetic alteration, molecular classification, and histological features of HCC. Here are summarized the main mutations along with histologic and genomic features of the two major molecular subclasses of HCC. Adapted from (Calderaro et al., 2019).

3.1 Diagnosis and treatment

Considering that most cases of HCC arise on a cirrhosis background due to HBV or HCV infections, many patients are diagnosed through surveillance. Cancer surveillance aims to identify cancer lesions at early stages to increase treatment possibilities and, consequently, to improve survival (Prasad et al., 2016). A recent meta-analysis pointed out the strong clinical benefits of surveillance in HCC patients, with two-thirds of cases detected at an early stage

(Singal et al., 2022). Abdominal ultrasonography (US) every 6 months with or without the serum measurements of AFP levels represent the recommended strategies for HCC surveillance in cirrhotic patients (Galle et al., 2018; Heimbach et al., 2018). The addition of AFP improves the sensitivity by 6-8% compared to the US alone, but its measurement is not considered cost-effective in a surveillance setting by EASL (Galle et al., 2018; Tzartzeva et al., 2018). Alternative imaging strategies, such as computed tomography (CT) and magnetic resonance imaging (MRI), are not cost-effective for surveillance. The need for contrast agents to achieve adequate sensitivity and the high rate of false positives preclude their widespread use (Pocha et al., 2013). Other biomarkers or biomarkers panels tested in case-control studies (e.g., GALAD score) showed promising results, but still need validation in large cohort studies (Marrero et al., 2009; Pepe et al., 2001). GALAD was tested in our cohort of patients with cirrhosis surveilled for HCC development. It was shown to be reliable and accurate in detecting HCC risk, better than aMAP and ALBI (Villa et al., 2023). None of the markers tested are recommended for surveillance in patients at risk of developing *de novo* HCC (Galle et al., 2018). However, the benefits of HCC surveillance need to be reevaluated considering changes in the epidemiology of this disease. The increasing number of patients with NASH, in whom the United States is more prone to fail, indicates the need for aetiology-driven protocols (El-Serag et al., 2020).

If a liver nodule is detected during screening by the US or if the patient has elevated AFP levels (>20 ng/mL), a diagnostic evaluation is required. Notably, AFP levels alone are not accurate enough to make a diagnosis (Singal et al., 2023). If the nodule is <1 cm in diameter, a repeated US examination is required after 3-4 months, whereas if the lesions are ≥ 1 cm in diameter, either a contrast-enhanced CT or MRI should be performed (Marrero et al., 2018). The HCC lesions appear brighter than the surrounding parenchyma in the arterial phase (wash-in) and less dense in the delayed or venous phase (wash-out) (Ayuso et al., 2018). This pattern, known as “radiologic hallmarks of HCC”, has a sensitivity between 66% and 82% and a specificity higher than 90% in identifying HCC lesions in cirrhotic patients (Roberts et al., 2018). In non-cirrhotic patients or in patients with inconclusive imaging features, a biopsy is necessary. The sensitivity of a biopsy is ~70% and is even lower in tumors < 2 cm because of the difficulty in distinguishing well-differentiated HCC from dysplastic nodules. The International Consensus Group for Hepatocellular Neoplasia has proposed the main histological features of hepatocellular carcinoma, which include stromal invasion, increased

cell density, intratumoral portal tracts, and diffuse fatty changes (International Consensus Group for Hepatocellular Neoplasia, 2009).

The management and treatment of HCC patients are evaluated and assigned according to the tumor staging established by the Barcelona Clinic Liver Cancer (BCLC) staging system (Galle et al., 2018; J. Llovet et al., 1999). To provide the best curative therapy, the BCLC staging system considers the liver function, the disease extension (considering not only the tumor size and number but also the presence of vascular invasion or extrahepatic spread), and the general health status of the patient (Díaz-González et al., 2016). In the very early and early stages (BCLC 0 and A), for patients with up to three nodules and preserved liver function, treatment options include ablation, surgical treatment, or liver transplantation (Galle et al., 2018; Heimbach et al., 2018). Ablation is employed to directly target the tumor and is performed by thermal, chemical, or electrical methods. Radiofrequency ablation (RFA), together with microwave ablation (MWA), is the established thermal technology, while other ablative techniques, such as cryoablation (CRA) and laser interstitial thermotherapy, are less widely used (J. M. Llovet et al., 2021). RFA determines tumor necrosis through the generation of a high level of heat (50-100°) that should be maintained over the entire tumor volume for 4-6 min (Lencioni & Crocetti, 2007). In some patients with solitary tumors < 2 cm in diameter and favorably located within the liver parenchyma, RFA competes with resection as a recommended option for first-line treatment (Cucchetti et al., 2013). The ideal candidates for surgical resection are HCC patients with single nodules, maintained liver function, and no clinically significant portal hypertension (Roayaie et al., 2015). For these patients, the survival rate is above 70% at 5 years (Pinna et al., 2018). However, for patients satisfying the Milan criteria (i.e., a single lesion less than 5 cm in size or 2-3 lesions no larger than 3 cm in size and without macrovascular invasion) (Mazzaferro et al., 1996), liver transplantation represents a better choice than RFA and hepatic resection, since they show a high risk of recurrence (Pompili et al., 2013; Shiina et al., 2012; N'Kontchou et al., 2009). Liver transplantation achieves a 5-year survival rate of 75-80%, with a risk of recurrence in 8-20% of cases (Straś et al., 2022; Vogel et al., 2022). It still represents the best treatment option since it may cure the tumor and the underlying liver disease (Straś et al., 2022; Vogel et al., 2022). However, the scarce availability of organs and the long waiting list represent the major limitations of liver transplantation. When the waiting time is longer than 6 months, neoadjuvant therapies (ablation or transarterial therapies) are administered, which is a cost-effective strategy to prevent patients from leaving the Milan criteria because of tumor progression (J. M. Llovet, 2002).

Multinodular liver disease without extrahepatic invasion, but with preserved liver function, characterizes BCLC stage B (intermediate stage) patients. These patients are preferred candidates for transarterial chemoembolization (TACE) (Galle et al., 2018; Heimbach et al., 2018). TACE requires the intra-arterial infusion of a cytotoxic agent, followed by embolization of the vessels feeding the tumor in order to achieve a strong cytotoxic and ischemic effect. The surrounding liver parenchyma is protected because its blood supply comes from the portal vein (Villanueva, 2019; Galle et al., 2018). According to patient selection, the median survival rate with TACE ranges from 26 to 40 months (Kudo et al., 2014; Burrel et al., 2012).

In the advanced stage (BCLB C), in which patients present with portal vein invasion, extrahepatic disease, or tumor-related symptoms, but preserved liver function, systemic therapy is the recommended therapy (Galle et al., 2018; Heimbach et al., 2018). Sorafenib is the first oral tyrosine kinase inhibitor (TKI) to demonstrate survival benefits in patients with advanced-stage HCC (J. M. Llovet et al., 2008). Following these results, numerous other TKI molecules were investigated. Lenvatinib, a TKI with stronger activity on the vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR) family, demonstrated non-inferiority against sorafenib in a global open-labeled phase-III trial (REFLECT) (Kudo et al., 2018). These promising results led to the approval of this drug in the first line setting after more than 10 years after sorafenib (Kudo et al., 2018). The combination of atezolizumab (anti-PDL-1 antibody) and bevacizumab (anti-VEGF antibody) has demonstrated for the first time a better overall survival (OS) in a first-line regimen compared with sorafenib in the IMbrave 150 open-labeled randomized trial (Finn et al., 2021, 2020). In patients with documented progression and who tolerate sorafenib treatment, regorafenib (a multi-kinase inhibitor targeting VEGFR 1-3 and other kinases) is the first drug to be approved in the second-line treatment (Bruix et al., 2017). Currently, other two therapies have been approved in the second-line regimen: cabozatinib and ramucirumab. Cabozatinib is a MET-, VEGFR2- and RET-inhibitor that demonstrated efficacy in advanced-stage HCC patients in second and third-line treatment (Kelley et al., 2017). Moreover, the CELESTIAL trial demonstrated an improved median OS of cabozantinib compared with placebo (Abou-Alfa et al., 2018). Ramucirumab, a monoclonal antibody targeting VEGFR2, demonstrated an improvement in OS in patients with AFP level ≥ 400ng/dl in the REACH-2 trial (A. X. Zhu et al., 2019).

Finally, patients with severe liver function impairment or severe cancer-related symptoms are grouped into the end stage (BCLB D). For these patients, the best supportive care is suggested (Galle et al., 2018; Heimbach et al., 2018).

2. Cholangiocarcinoma

2.1 Epidemiology and risk factors

Cholangiocarcinoma (CCA) is the second most common primary liver cancer which accounts for approximately 15% of all primary liver malignancy and 3% of gastrointestinal cancer (Banales et al., 2020; DeOliveira et al., 2007; Nakeeb et al., 1996). CCA represents a group of malignancies originating in the biliary tree which can be subdivided into three subtypes depending on the anatomical sites of origin: intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA) CCA (Banales et al., 2020; Rizvi et al., 2018).

iCCA can arise at the periphery of the second-grade bile ducts of the intrahepatic biliary tree; the pCCA can originate in the right and/or left hepatic duct and/or their junction or in the insertion between the cystic duct and common bile duct within the hepatic parenchyma, whereas dCCA affects the common bile duct (Kendall et al., 2019; Razumilava & Gores, 2014). A previous classification identified pCCA and dCCA as extrahepatic CCA (eCCA) because of their location outside the liver parenchyma (Marin et al., 2020). pCCA accounts for the majority of CCA cases (60%) followed by dCCA (20-30%) and iCCA (< 10%) (Banales et al., 2020; DeOliveira et al., 2007; Nakeeb et al., 1996). The incidence is highest among those aged 60-70 years, with a slight predominance in the male sex for iCCA (Qurashi et al., 2023; Sarcognato et al., 2021). The highest global incidence of CCA is in the Asian continent (Khan et al., 2019). The age-standardized incidence rate varies geographically. In Asia, the highest rates are observed in North East Thailand (85 cases per 100,000), followed by North and Central Thailand (14.5 cases per 100,000), and Gwangju, South Korea (8.8 cases per 100,000). Comparatively, in the Western regions, the incidence rates are notably lower than those in Asia, ranging between 0.5 to 3.4 cases per 100,000. Italy reports the highest incidence rate in the Western region at 3.4 cases per 100,000 (Qurashi et al., 2023; Banales et al., 2016). In general, there has been a gradual increase in the incidence of iCCA worldwide until the end of the last century, reaching a plateau in the last decade. In contrast, the incidences of pCCA and dCCA appear to be decreasing (Banales et al., 2016; Fitzmaurice et al., 2015).

As for incidence, the mortality rate is the highest in Asian countries, with South Korea, China, Taiwan, and Thailand with >4 deaths per 100,000 people (Banales et al., 2020; Bertuccio et al., 2019). Western European nations such as Ireland, the United Kingdom, Portugal, and Spain demonstrate the highest rates of mortality attributed to iCCA, surpassing 2 per 100,000 person-years among males. Moreover, outside of Europe, rising mortality rates associated with iCCA are noted in regions like the United States, Canada, and Oceania. In contrast, mortality rates for eCCA tend to be lower compared to iCCA. Nevertheless, similar to iCCA, Asian countries display the highest mortality rates for eCCA (Qurashi et al., 2023; Vithayathil & Khan, 2022).

The iCCA and eCCA shared some risk factors, while others are specific to one of the subtypes or related to specific geographical regions. Liver cirrhosis is recognized as a risk factor also for iCCA. Indeed, viral hepatitis, alcohol consumption, and NAFLD are all associated with iCCA in Western countries (El-Serag et al., 2009). On the contrary, biliary diseases (i.e. choledochal cystitis, Caroli disease, primary sclerosing cholangitis-PSC) are more closely related to eCCA (Petrick et al., 2017; Schottenfeld & Beebe-Dimmer, 2006). Choledochal cysts are a set of congenital biliary conditions that lead to the cystic dilation of bile ducts. Individuals with these cysts face a 6-30% likelihood of developing CCA in their lifetime (Petrick et al., 2017). Conversely, Caroli disease is a rare genetic disorder that affects the biliary system, resulting in biliary ectasia and bile stasis. It has been reported as one of the strongest risk factors for CCA development, conferring a higher risk for eCCA than iCCA (Petrick et al., 2017). PSC is an autoimmune disease that afflicts the bile ducts, leading to inflammation and successive obstruction of the intra- and extrahepatic bile ducts. Up to 50% of PSC patients develop iCCA within the first year of diagnosis (Alvaro et al., 2023). Infections with parasitic liver flukes, especially in South East Asia where they are endemic, represent a significant risk factor for CCA development, as they are responsible for the majority of iCCA cases (Prueksapanich et al., 2018; Shin et al., 2010).

However, in many areas, most cases of CCA remain sporadic, with no identifiable risk factors (Banales et al., 2020).

2.2 Pathophysiology and classification

Based on its growth pattern, iCCA can be subclassified as mass-forming (MF) (~ 65% of iCCA), a nodular mass in the liver parenchyma; periductal-infiltrating (PI) (~ 6% of iCCA), an iCCA that develops along the wall of the large bile ducts resulting in their thinning; or

intraductal-growing (IG) (~ 4% of iCCA), a polyploid or papillary tumor that grows toward the ductal lumen (Alvaro et al., 2023; Rodrigues et al., 2021). There are mixed growth patterns, such as MF+PI (~ 25% of iCCA), i.e., an iCCA that develops in the duct with concomitant invasion of the liver parenchyma (Alvaro et al., 2023). pCCA and dCCA can grow similarly to PI-iCCA or IG-iCCA; however, the majority of pCCA (> 80%) grow as nodular tumors with frequent periductal infiltration (Banales et al., 2016).

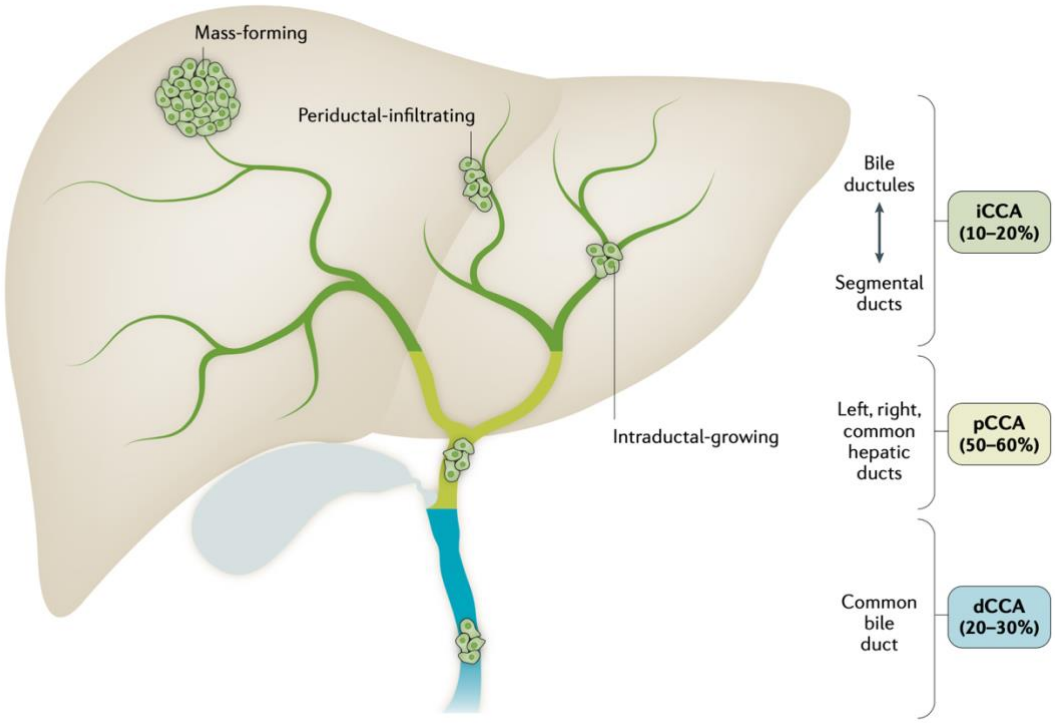

Figure 2 - Anatomical classification of CCA. Based on the anatomical site of origin, cholangiocarcinoma (CCA) can be divided into intrahepatic (iCCA) arising at the periphery of the second-order bile ducts, perihilar (pCCA) developing in the right/left hepatic duct or their junction, and distal (dCCA) originating in the common bile duct. Depending on the growth pattern, CCAs show three different models: mass-forming, a nodular mass within the liver parenchyma; peri-ductal infiltrating, which originates in the duct wall and develops along the wall; and intraductal growing, which grows toward the duct lumen. Adapted from (Banales et al., 2020).

Histologically, pCCAs and dCCAs are mainly mucinous adenocarcinomas or papillary tumors (Krasinskas, 2018; Nakanuma & Kakuda, 2015). In contrast, iCCAs are well- to moderately differentiated adenocarcinomas composed of columnar to cuboidal epithelial cells that resemble biliary epithelial cells (Kendall et al., 2019; Krasinskas, 2018; Nakanuma & Kakuda, 2015). Conventionally, iCCA are more heterogeneous in the aspect of tubular structures, acini formation, and micropapillary architecture (Kendall et al., 2019; Krasinskas,

2018; Nakanuma & Kakuda, 2015). According to the level and size of the affected bile ducts, iCCA can be further classified into two main histological types: small and large bile ducts. The former type can originate from various sources such as intrahepatic small bile ducts, progenitor cells, and mature hepatocytes. It typically presents as a small tubular or acinar adenocarcinoma with nodular growth, invading the liver parenchyma. Moreover, small bile duct iCCA often exhibits minimal or no mucin production, follows an MF growth pattern, and is predominantly localized in the peripheral regions of the liver (Hayashi et al., 2016; Nakanuma & Kakuda, 2015; Liau et al., 2014). On the contrary, large bile duct iCCA originates from larger intrahepatic bile ducts or may stem from associated peribiliary glands (PBGs) (Akita et al., 2017; Cardinale et al., 2012; Komuta et al., 2012). This particular type of CCA is characterized by mucin-producing columnar cancer cells organized in either a large duct or papillary structure. Typically, it exhibits a PI growth pattern, or less commonly, an IG pattern. Additionally, it tends to be more centrally located within the liver (Kendall et al., 2019; Akita et al., 2017; Komuta et al., 2012). In general, the histological characteristics of the large bile duct type closely resemble those of p/dCCA (Nakanuma & Kakuda, 2015).

CCA is also highly heterogeneous at the genomic level. Large duct iCCA shows a high mutation frequency of *KRAS* (15%-30%) and *TP53* (10%-40%) (Nakamura et al., 2015; Liau et al., 2014), whereas small duct type shows *IDH1/2* mutations (10%-30%) and *FGFR2*-fusions (10%-25%) (Arai et al., 2014; Borger et al., 2012; Kipp et al., 2012). Concerning eCCA, it mostly shares the mutational molecular pattern with the large duct iCCA, although with some significant variation (e.g., the frequency of *KRAS* mutation seems to be higher in pCCA and dCCA than in iCCA) (Liau et al., 2014; P. Wang et al., 2013; Borger et al., 2012; Kipp et al., 2012). In addition, *IDH1/2* and *BAP1* mutation together with *FGFR2* fusions are absent in eCCA (Nakamura et al., 2015; P. Wang et al., 2013; Borger et al., 2012; Kipp et al., 2012). Remarkably, dCCA is also associated with mutations in *ELF3* (Yachida et al., 2016).

One of the first molecular characterizations performed by Sia and colleagues (Sia et al., 2013) identified two distinct gene signature classes of iCCA: a proliferation class and an inflammatory class. The proliferation class which accounted for 62% of the cases analyzed, showed different copy number variations in several oncogenes, particularly those involved in receptor tyrosine kinase (RTK) pathways such as *EGF*, *RAS*, *AKT*, *MET*, which promote proliferation and cell survival, as well as VEGF and platelet-derived growth factor (PDGF) related to angiogenesis (Sia et al., 2013). Of note, these gene alterations identified overlapped with those previously identified in different HCC profiles associated with poor prognosis (Hoshida et al., 2009; Boyault et al., 2007; J.-S. Lee et al., 2004). This finding implies a

possible common origin for both cancer types. The inflammatory class, instead, is associated with the activation of pathways related to inflammatory response, such as the cytokines pathway (Sia et al., 2013). In particular, the signal transducer and activator of transcription 3 (STAT3) is a key regulator for cytokine signaling, but it is also implicated in carcinogenesis mechanisms (Sansone & Bromberg, 2012).

Conversely, for eCCA, the molecular landscape profile has been less studied than for iCCA, especially in the Western population. To fill this gap, Montal and colleagues (Montal et al., 2020) performed a molecular characterization of a large eCCA cohort both at genomic and transcriptomic levels. They identified four molecular classes with specific molecular and clinical-pathological features: Metabolic, Proliferation, Mesenchymal, and Immune. The Metabolic class is characterized by a gene expression profile that results in the deregulation of bile acid, fatty acid, and xenobiotic metabolism (Montal et al., 2020). The Proliferation class is defined by cell cycle activation, with enrichment of RAS/MAPK and AkT/mTOR pathways and *ERBB2* mutations. This class contained the main proportion of dCCA compared to other subclasses (Montal et al., 2020). Of note, this is the only class that overlaps with the subclasses described by Sia et al. (Sia et al., 2013). The Mesenchymal class, the most prevalent one, showed activation of signals related to EMT, TGF-β activation, and desmoplastic features, altogether translating into poor clinical outcomes. This subclass is particularly prevalent in pCCA. The Immune class, instead, is characterized by upregulation of lymphocytic response genes and immune checkpoint expression (Montal et al., 2020).

2.3 Diagnosis and treatment

iCCA is found incidentally in 25-30% of patients (Alvaro et al., 2011). Indeed, it is frequently diagnosed at an advanced stage, typically when patients begin to manifest symptoms or signs. These may include abdominal pain or, less frequently, jaundice (Brindley et al., 2021). In contrast, pCCA and dCCA are usually detected at earlier stages because biliary obstructions caused by extrahepatic lesions result in earlier clinical presentation than iCCA (Izquierdo-Sanchez et al., 2022). The serum biomarkers carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are associated with CCAs (Macias et al., 2019). However, their variable sensitivity (Tshering et al., 2018; Sinakos et al., 2011; Qin et al., 2004) and lack of specificity (Scarà et al., 2015; Morell, 1992), make them an inconsistent tool for early diagnosis. CA19-9 levels >1,000 U/ml are still of concern for the presence of metastatic CCA (Patel et al., 2000). Imaging modalities are widely used for iCCA diagnosis

including conventional US, CT, MRI, and contrast-enhanced ultrasonography (CEUS) (Brindley et al., 2021). Distinguishing an iCCA from an HCC in the context of a cirrhotic liver can be difficult. Conducting regular surveillance for HCC in patients with cirrhosis might aid in the earlier detection of iCCA (Wildner et al., 2015). However, it is difficult to distinguish HCC and iCCA in this context. Certainly, CEUS compared with CT or MRI is more likely to erroneously diagnose iCCA as HCC (Vilana et al., 2010). Histological investigation of a biopsy remains the only way to confirm a diagnosis of iCCA (Brindley et al., 2021). As for iCCA, also for dCCA, and pCCA, CT, and MRI are fundamental for the diagnosis of disease. Because of direct compression, dCCA shows abrupt cutting of the biliary tree on CT scan, whereas pCCA may be evident only when dilated segmental bile ducts appear (Joo et al., 2018). In this case, a specific type of MRI, called magnetic resonance cholangiopancreatography (MRCP), is the imaging procedure with the highest diagnostic accuracy to localize and size the stenosis (Beyer et al., 2023; Jhaveri & Hosseini-Nik, 2015). The accuracy of MRCP in differentiating between benign and malignant causes of hilar obstruction stands at 87% for sensitivity and 85% for specificity (Saluja et al., 2007). Another primary modality, endoscopic retrograde cholangiopancreatography (ERCP), allows the detection of malignant stenosis for evaluation of the biliary tree and the acquisition of biliary brushing for cytology and biopsies with high specificity but low sensitivity (Brindley et al., 2021; M.-S. Park et al., 2004). In addition to the primary modalities, endoscopic ultrasound (EUS) can be complementary and useful for the assessment of biliary stenosis and for the evaluation of eCCA or regional lymph nodes (Coronel et al., 2022).

Surgical resection remains one of the most feasible treatment options for iCCA, with disease-free survival (DFS) between 12 and 36 months reported in various patient series (S.-B. Choi et al., 2009; Endo et al., 2008). Surgical procedures are designed to achieve complete resection with clear margins both at the macroscopic and microscopic levels (R0 resection). Additionally, these procedures aim to ensure an adequate remaining portion of the liver after surgery, i.e. the liver remnant, to maintain proper liver function (Radtke & Königsrainer, 2016). Large tumor size, multifocal disease, and regional lymph node involvement are predictive of unsuccessful surgical resection and short DFS (Endo et al., 2008). Moreover, iCCA is considered *per se* a contraindication to liver transplantation because of poor survival and high recurrence rate (Rizvi et al., 2018). In patients with localized, unresectable tumors, locoregional therapy, especially TACE, is associated with a median OS of 12-15 months (Vogl et al., 2012; Kiefer et al., 2011; S.-Y. Park et al., 2011). Patients with resectable pCCA undergo pre-operative biliary drainage, preceded by imaging to determine tumor stage,

optimize liver function, and prevent post-operative liver failure (S.-B. Choi et al., 2009). In cases of dCCA, surgical resections typically involve a procedure known as pancreatoduodenectomy. This surgery involves the removal of the head of the pancreas, a portion of the duodenum, the gallbladder, and the bile duct (Rodrigues et al., 2021). The 5-year survival for both cancers following surgical resection is no more than 40 % (Dickson & Behrman, 2014; Nagino et al., 2013; DeOliveira et al., 2007). Liver transplantation becomes a viable option for patients with early-stage, unresectable pCCA or dCCA in the context of PSC. This choice arises due to PSC's association with chronic parenchymal disease, which can only be resolved through liver transplantation after neoadjuvant chemoradiation therapy (Darwish Murad et al., 2012; Rea et al., 2005; Sudan et al., 2002). For the advanced-stage CCAs that are not eligible for surgical or locoregional treatments, chemotherapy with cisplatin and gemcitabine (GEM-CIS) is the first-line treatment option (Valle et al., 2010). In patients whose disease progressed because they developed resistance to GEM-CIS, the ABC-06 study demonstrated the benefit of folinic acid, 5-fluorouracil, and oxaliplatin (FOLFOX) in second-line treatment (Lamarca et al., 2021).

Knowing the molecular landscape of CCA is useful for developing new tailored therapies, especially when conventional treatments have failed.

As aforementioned, iCCA is characterized by *IDH1/2* mutations, together with FGFR and BAP1 alterations (Nakamura et al., 2015). Numerous trials obtained promising results from the treatment with specific inhibitors of IDH and FGFR. In a multicenter, phase III, double-blind, randomized, controlled trial of 185 patients with iCCA refractory to chemotherapy and carrying *IDH1* mutations, patients who received ivosidenib, a mutant IDH1 inhibitor, showed statistically significant improvement in progression-free survival (PFS) compared with those who received placebo (Abou-Alfa, Macarulla, et al., 2020). Several FGFR inhibitors have been investigated (Abou-Alfa, Sahai, et al., 2020; Javle et al., 2018; Rizvi & Gores, 2017). Nonetheless, among these treatments, only pemigatinib obtained accelerated approval from the FDA for addressing previously treated, unresectable, locally advanced, or metastatic CCA with FGFR2 gene fusions or rearrangements. This decision followed promising results in a phase II study in which ~ 35 % of patients showed an objective response to pemigatinib (Abou-Alfa, Sahai, et al., 2020).

Alongside personalized therapy directed at molecular alterations, targeting the immune compartment, especially when it becomes an ally of tumor growth, may be another valuable therapeutic strategy. In a phase II trial, KEYNOTE-158, patients with advanced biliary tract cancer treated with pembrolizumab, a PD-1 inhibitor, the objective response rate was less

than 5.8% (Piha-Paul et al., 2020). Two subsequent studies with immune checkpoint inhibitors have shown improved efficacy. Among patients who had received prior treatment for biliary tract cancer, the utilization of the PD-1 inhibitor nivolumab resulted in an objective response rate of 22% (Kim et al., 2020), while data on the combination of nivolumab and ipilimumab, a CTLA-4 inhibitor, demonstrated an OS of 5.7 months and PFS of 2.9 months (Klein et al., 2020). The immune checkpoint pathways PD-1 and CTLA-4 negatively regulate T-cell activation, promoting the escape of tumor cells from immune control (Sas et al., 2022). Of note, the above-described responses have been observed exclusively in patients with iCCA or gallbladder CCA (Brindley et al., 2021). Currently, therapeutic approaches for eCCA are still lacking. However, a better understanding of the specific TME and pathogenesis of eCCA will possibly lead to more effective treatment options for patients in the future (Y. Yang & Zhang, 2023).

3. Tumor microenvironment

3.1 HCC microenvironment

TME significantly influences the development, advancement, and treatment response of HCC. It includes tumor cells, immune cells, non-parenchymal liver cells, cancer-associated fibroblast (CAF) along with soluble cytokines that contribute to orchestrating the crosstalk among tumor and TME components (Q. Duan et al., 2020). The complex interplay between HCC and TME may in fact contribute significantly to the acquisition of the malignant phenotype and result in increased invasion, metastasis, angiogenesis, and immune escape of tumor. Among the non-immune components, CAFs are one of the most important. They originate from hepatic stellate cells (HSCs), i.e. the major source of CAF, but also from HCC cells themselves undergoing the EMT process (J. Zhang et al., 2020), mesenchymal stem cells (MSCs) (Salah et al., 2022), or endothelial cells via endothelial to mesenchymal transition (EndMT) (H. Peng et al., 2022). They are characterized by the expression of different biomarkers, including α-smooth muscle actin (α-SMA), vimentin, fibroblast activation proteins (FAPs), fibroblast specific protein 1 (FSP-1), and PDGF receptors (PDGFRs)-α and β (H. Peng et al., 2022; Biffi & Tuveson, 2021; Sahai et al., 2020). However, the marker to uniquely distinguish CAFs is still under study (Z. Zhou et al., 2022). CAFs, besides producing extracellular matrix (ECM), that contributes to liver fibrosis, secrete different growth factors, such as epidermal growth factor (EGF), PDGF, hepatocyte growth factor (HGF) along with immunomodulatory cytokines and chemokines, and

metalloproteinase (MMP) enzymes, which together promote cancer cell proliferation, escape from the immune system and thus cancer progression (Biffi & Tuveson, 2021). Specifically, HGF, by binding its specific receptor c-Met on the tumor cell, stimulates cell cycle progression, migration, and abnormal proliferation (Jafarnejad et al., 2019). In addition, CAFs foster stem cell-like properties of HCC cells through activation of the Notch signaling pathway, secreting high amounts of IL-6 (S. Xiong et al., 2018), and tumor metastasis through upregulation of chemokine (C-C motif) ligand 2, 5, 7, 26 (CCL2, CCL5, CCL7, CCL26) and chemokine (C-X-C motif) ligand 17 (CXCL17) levels (J. Liu et al., 2016). CCL5 was found to be the most significant cytokine secreted by CAFs promoting HCC metastasis, and its serum levels were relatively high in patients who developed HCC in a setting of cirrhotic liver (Xu et al., 2022). Furthermore, Song and colleagues (Song et al., 2021) recently demonstrated that CAF-derived cardiac nutrient-like cytokine 1 (CLCF1) induced the overexpression of CXCL6 and TGFβ which promotes HCC-cell stemness and tumor-associated neutrophils (TANs) infiltration and polarization. Notably, CAF produced lower levels of TGFβ, IL-6, and granulocyte-colony-stimulating-factor (GCSF), fundamental to polarizing TANs towards pro-tumorigenic phenotype, compared to the HCC cells (Lei & Lee, 2021; Song et al., 2021). Therefore, CAFs predominantly orchestrate HCC progression via paracrine pathways (Lei & Lee, 2021; Song et al., 2021).

It has been observed that high levels of TANs infiltration within the TME were correlated with worse OS in certain solid tumors (Mao et al., 2021).

TANs can exhibit anti-tumorigenic (N1), and pro-tumorigenic (N2) phenotypes shaped by different types of TME (Arvanitakis et al., 2021). An inflammatory TME, characterized by high levels of TGFβ, granulocyte-monocyte-colony-stimulating-factor (GM-CSF), and tumor necrosis factor (TNF), triggers a shift toward an N2 phenotype (Arvanitakis et al., 2021) and, by increasing their expression of PD-L1, consequently, promotes the role of N2 in T-cell suppression in HCC (He et al., 2015). Furthermore, when triggered, TANs discharge neutrophil extracellular traps (NETs). These structures, comprised of DNA and granular proteins, aid tumor progression by secreting matrix metallopeptidase-9 (MMP-9) and cathepsin G, fostering tumor growth (Boeltz et al., 2019), as well as induction of HCC cell metastasis by sequestering circulating tumor cells (Cools-Lartigue et al., 2013). Numerous studies have shown that TANs invasion and their biomarkers are involved in disease prognosis. For instance, according to Zhou et al. (S.-L. Zhou et al., 2016), CXCL5 was suggested to facilitate neutrophil infiltration, followed by the differentiation and invasion of

HCC cells. The study proposed that the overexpression of CXCL5, either alone or in conjunction with the presence of intratumoral neutrophils, could potentially serve as a new prognostic indicator for OS.

Another important key component of TME is tumor-associated macrophages (TAMs) which are known to promote malignancy and progression in several tumors (Cassetta & Pollard, 2020). In an inflammatory context, liver macrophages can be derived from circulating monocytes, recruited through chemokine signals, or from embryo-derived macrophages residing in tissues, called Kupffer cells (KC) (Tacke & Zimmermann, 2014). TAMs are classified as "pro-inflammatory" (M1) and "immunomodulatory" (M2) (Murray & Wynn, 2011), but they may show different or mixed phenotypes depending on different tumor types and differences within tumors (Noy & Pollard, 2014). M1-type macrophages typically react to acute injuries by generating lipopolysaccharide (LPS) and IFN-γ. This response prompts the release of inflammatory factors like TNF-α, IL-6, IL-12, and IL-23. Subsequently, these factors stimulate the proliferation and function of Th1 cells (Nielsen & Schmid, 2017). On the contrary, M2 macrophages can be activated by the Th2 cytokines IL-4, IL-10, IL-13, and glucocorticoids (Martinez et al., 2009). Hypoxic TME, resulting from tumor proliferation, can also trigger the polarization of macrophages into M2. These M2 macrophages, characterized by heightened levels of PD-L1 expression, through the production of immunomodulatory mediators such as IL-10, TGF-β, and chemokines (CCL2, CCL17, and CCL24), promote immune evasion (Bohn et al., 2018; Locati et al., 2020). In terms of function, TAMs drive tumor advancement via multiple mechanisms, fostering tumor cell multiplication, spread, angiogenesis, and evading the immune system (Q. Yang et al., 2020). For instance, TAMs have been identified to facilitate TGF-β1-induced EMT, thus sustaining cancer stem cell activity (Q.-M. Fan et al., 2014). Additionally, they promote the expansion of cancer stem cells and tumorigenesis through the IL-6-mediated STAT3 signaling pathway (Wan et al., 2014). TAMs also secrete TNF-α, which enhances EMT and the differentiation of cancer stem cells via the Wnt/β-catenin pathway (Y. Chen et al., 2019). Moreover, the TNF-α produced by TAMs can trigger the Raf-Erk pathway, leading to the upregulation of CXCR4 expression in tumor vascular epithelial cells, consequently promoting tumor angiogenesis (Meng et al., 2018). Furthermore, TAMs are known to express the molecule CD48. This molecule, upon binding to the natural killer (NK) cell surface receptor 2B4, initiates early NK cell activation, ultimately leading to the depletion or apoptosis of these cells. This mechanism orchestrated by CD48 contributes to immune evasion (Y. Wu et al., 2013). Furthermore, by producing the chemokines CCL17, CCL18, and CCL22, TAMs elicit the T-regulatory (Treg)

and T helper 2 (Th2) cell infiltration, dampening the activation of cytotoxic T lymphocytes (CTLs) (Guo et al., 2014; X. Li et al., 2014). Pro-inflammatory cytokines produced by TAMs in the peritumoral stroma of HCC, namely IL-6, IL-23, IL-1β, and TNF-α, prime the expansion of Th17 cells that overexpress PD-1, CTLA-4, and glucocorticoid-induced TNF related receptor (GITR) to exert immunosuppressive function (Kuang et al., 2010).

The non-immune constituents within the hepatic TME encompass liver sinusoidal endothelial cells (LSECs). These specialized endothelial cells, found along the hepatic sinusoids, lack a basement membrane and exhibit fenestrations (Shetty et al., 2018). Their unique structure reflects multifunctional properties, including innate and adaptative immunological functions and metabolic homeostasis maintenance. However, in pathological conditions, LSECs play a crucial role in processes leading to liver fibrosis and, subsequently, to HCC development (Shetty et al., 2018). Indeed, if under physiological conditions, fenestrated LSECs are gatekeepers in maintaining HSC quiescence, thus preventing hepatic fibrosis, on the other hand, during chronic liver injury, defenestrated LSECs promote HSC activation and fibrinogenesis (M. Yang & Zhang, 2021; DeLeve, 2015).

Typically, activated HSCs commonly produce pro-angiogenic factors such as VEGFA, PDGFB, and angiopoietins (ANGs). These factors facilitate angiogenesis by binding to their respective receptors on the surface of endothelial cells (ECs) (J.-Z. Lin et al., 2016; Q. Ruan et al., 2020).

LSECs possess antigen-presenting properties; however, in contrast to conventional antigen-presenting cells (APCs), they tend to induce tolerance rather than activation of CD8+ T cells (Limmer et al., 2000). Additionally, in cases of HCC, a notable observation is the upregulation of PD-L1 expression in LSECs. This upregulation aims to hinder T-cell activity, thereby assisting in the evasion of the immune system by the tumor (Gracia-Sancho et al., 2021). Thus, taken together, these events contribute to tumor immune evasion.

The HCC TME comprises crucial immune components, notably T lymphocytes. Diverse T cell subtypes, such as CD8+ CTLs and CD4+CD25+FoxP3+ T reg lymphocytes, have been identified within HCC tumors (X. Zheng et al., 2021; W. Yao et al., 2017). CTLs exhibit anti-tumor properties by releasing cytotoxic enzymes and cytokines, including perforins, granzyme B, and INF-γ, prompting apoptosis. Conversely, T regs impede the anti-tumor response by suppressing CTLs and fostering immune tolerance against cancerous cells (Bian et al., 2020; W. Yao et al., 2017; Motz & Coukos, 2013). A higher count of CTLs along with a lower count of T regs is linked to improved OS and DFS (Q. Gao et al., 2007). On the contrary, disease progression shows a positive correlation with increased T reg infiltration

due to their inhibition of CTLs' cytotoxic function and proliferation, thus hindering tumor control (Fu et al., 2007). A subset of innate T-like cells with cytotoxic function, called mucosa-associated invariant T cells (MAIT), enriched in the hepatic microenvironment, has recently been shown to exhibit impaired function in the context of HCC. These cells are reprogrammed towards HCC progression as they produce significantly lower amounts of IFN-γ and IL17, upregulate inhibitory molecules such as PD-1 and CTLA-4, and secrete protumoral cytokines (M. Duan et al., 2019). Therefore, a high density of tumor-infiltrating MAIT cells is significantly and independently correlated with adverse clinical outcomes in patients with HCC (M. Duan et al., 2019).

In HCC, TGF-β signaling is involved in almost all stages of tumor formation (Katz et al., 2016). High levels of TGF-β increase the expression of inhibitory receptors including PD-1 and CTLA-4 on T lymphocytes in HCC (Bao et al., 2021). The PD-1 and CTLA-4 pathways are immune checkpoint mechanisms responsible for suppressing T-cell activation, thus maintaining immune tolerance. These pathways facilitate tumors in evading immune cell recognition and response (Fife & Bluestone, 2008). PD-L1 is expressed on various TME cells, including tumor cells, T regs, CAFs, TAMs, and dendritic cells. The interaction between PD-1 and PD-L1 hampers T cell proliferation, differentiation, and activation by inhibiting the MAPK/ERK and PI3K/AKT pathways. These pathways play critical roles in maintaining the cell cycle (H. Zhang et al., 2021). CTLA-4 interrupts stimulatory signaling for T cell proliferation, by competing with CD28 for binding to CD80/CD86 (Buchbinder & Desai, 2016). In recent years, immune checkpoint inhibitors (anti-PD1, anti-PD-L1, and anti-CTLA-4 antibodies) have shown promising results in the treatment of advanced HCC (Zongyi & Xiaowu, 2020).

3.1.1 Tumor-associated angiogenesis in HCC

Angiogenesis, i.e., the formation of new blood vessels from pre-existing ones, is necessary for tumors to obtain nutrients and oxygen and to eliminate waste (Hanahan & Weinberg, 2011). The relationship between cancer development and its maintenance through blood supply was first demonstrated more than 10 years ago (Ferrara, 2002). However, in the presence of rapid tumor growth, hypoxia develops within solid tumors because of the high interstitial pressure and the distance between tumor cells and the nearby vasculature (Feng et al., 2020). Hypoxia is known to be closely related to tumor progression and metastasis (Muz et al., 2015). The hypoxia-inducible factor-1α (HIF-1α), the major hypoxia-induced

transcription factor, activates a sequence of target genes, including VEGFs, PDGFs, fibroblast growth factors (FGFs), and ANGs, involved in cell survival and angiogenesis (Muppala, 2021; Shah et al., 2021). Angiogenesis is also strongly promoted by hypoxia in HCC (W. Cheng et al., 2021; X. X. Xiong et al., 2017). Typically, HCC develops from small, well-differentiated tumors lacking developed vessels to progress to a broadly poorly differentiated tumor with characteristic hypervascularization (Sugimachi et al., 2002). This transition from "dormant" avascular hyperplasia to a vascularized growing tumor and finally to malignant tumor progression is called "angiogenic switch" and refers to a time-limited event during tumor progression in which the balance between multiple pro- and anti-angiogenic factors shifts toward a pro-angiogenic outcome (Baeriswyl & Christofori, 2009).

PDGFs are a family of secreted growth factors that are closely related to VEGF and crucial in the induction of angiogenesis in HCC (Tsioumpekou et al., 2020; Heldin et al., 2002). They, through binding to tyrosine kinase receptors (PDGFR α and β), promote increased VEGF levels and perivascular cell recruitment (Magnusson et al., 2007; Laschke et al., 2006). Since the PDGF/PDGFR complex is expressed in many cancers, including HCC (B. Chen et al., 2018; Papadopoulos & Lennartsson, 2018), the activation of the PDGF/PDGFR signaling pathway is also related to cancer cell proliferation and metastasis by modulating several downstream pathways. These pathways include the PI3K/PKB and MAPK/ERK pathways (X. Zou et al., 2022). In HCC, high levels of PDGFR-α are correlated with microvascular density (MVD) and, therefore, with a worse prognosis (Shah et al., 2021). In addition, some studies indicate that increased expression of PDGF-C and B subtypes correlates with liver fibrosis and progression from dysplastic nodules to HCC in mouse models (Campbell et al., 2007; Maass et al., 2011). However, although sorafenib and other TKIs target PDGFR, among others, the clinical importance of the PDGF pathway as a potential therapeutic target and its clinical benefits remain unknown (Morse et al., 2019).

FGFs are a heparin-binding growth factors family, comprising 18 ligands and 4 homologous factors (Presta et al., 2017). Among these factors, FGF-2, interacting mainly with its receptor FGFR1, mediates angiogenesis through the RAF/MAPK pathway (Y. Wang et al., 2021). It is expressed in HCC cells but is poorly detectable in nonparenchymal cells or noncancerous liver tissue (C. Yao et al., 2023). FGF-2 cooperates with VEGF-A during the early stages of tumor growth, inducing neovascularization and stimulating tumor growth, respectively (Tsunoda et al., 2007). Indeed, FGF-2 and VEGF-A are associated with increased capillarization of sinusoids in HCC tumor angiogenesis (Morse et al., 2019). However, the interplay between FGF and VEGF pathways may potentially further contribute to the

acquisition of resistance of advanced HCC tumors to sorafenib (L. Gao et al., 2017; Lieu et al., 2011). Recently, a novel monoclonal antibody targeting FGF-2 was identified that inhibits both cell proliferation and migration and angiogenesis, thus confirming the role of FGF-2 in tumor growth and angiogenesis (L. Wang et al., 2012).

Among the factors that orchestrate the angiogenic process, ANGs and VEGF are widely considered the most important contributors (Z.-L. Zhang, 2006; Sugimachi, 2003). The Angiopoietin system comprises two key components: Angiopoietin-1 (ANG-1) and Angiopoietin-2 (ANG-2), both playing critical roles as mediators of vascular remodeling by interacting with their cognate tyrosine kinase receptor, TIE-2. TIE-2 is expressed constitutively on stromal cells like fibroblasts, vascular supporting cells, and various non-vascular supporting cells (Cascone & Heymach, 2012). In physiological conditions, ANG-1 and ANG-2 are in balance. ANG-1 plays a role in recruiting pericytes and smooth muscle cells, thereby stabilizing vascular networks by binding to the TIE-2 receptor (Thurston & Daly, 2012; Torimura et al., 2004). ANG-2, on the other hand, is produced and stored within the Weibel–Palade storage granules of endothelial cells (Gerald et al., 2013), and its release is induced by VEGF-A and hypoxia during endothelial cells activation and angiogenesis (Augustin et al., 2009; Pichiule et al., 2004). ANG-2, indeed, competitively inhibits the action of ANG-1 on the TIE-2 receptor, disrupting the interactions among endothelial cells, perivascular support cells, and the extracellular matrix, thereby compromising vascular integrity (Thurston & Daly, 2012; Torimura et al., 2004; Maisonpierre et al., 1997). However, some studies have demonstrated that ANG-2 can act also as a TIE-2 agonist, but with a weaker activity than ANG-1 (Yuan et al., 2009; Daly et al., 2006; Teichert-Kuliszewska et al., 2001). Even under pathological conditions, such as in the progression of many vascularized tumors, ANG-1 and ANG-2 contribute to either the stabilization or destabilization of newly formed blood vessels (Tait & Jones, 2004). Particularly in HCC, regarding ANG-1, there is no unambiguous association between its overexpression and potential role in tumor progression. Several groups have reported similar expression of ANG-1 in HCC and adjacent non-malignant liver tissue (Mitsuhashi, 2003; Tanaka et al., 1999), while others have observed overexpression of ANG-1 only in human HCC samples (Torimura et al., 2004; Moon et al., 2003). However, the upregulation of ANG-1 did not relate to angiogenesis and tumor progression (Torimura et al., 2004; Moon et al., 2003). It is noteworthy that both ANG-1 and ANG-2 interact with VEGF to exert their role in the angiogenic process but in distinct ways. ANG-1 and VEGF-A work in tandem to promote angiogenesis synergistically; ANG-1, indeed, augments the initial pro-angiogenic response of

VEGF-A, enhancing its effects in astrocytoma (Zadeh et al., 2004). Conversely, the relationship between ANG-2 and VEGF-A is more intricate. ANG-2 overexpression leads to the destabilization of vascular structures, exposing endothelial cells to VEGF. VEGF, in turn, acts on these endothelial cells, facilitating further action by ANG-2 (Moon et al., 2003). In the presence of VEGF, a close association between the mRNA ratio of ANG-2 to ANG-1 and various factors related to angiogenesis, clinicopathological parameters, and poor prognosis in HCC was observed by Mitsuhashi et al. (Mitsuhashi, 2003). Therefore, the quantitative assessment of ANG-1/ANG-2 mRNA ratio expression might potentially serve as a valuable indicator to understand the influence of ANGs signaling on angiogenesis and HCC progression (Mitsuhashi, 2003).

Since ANG-2 and VEGF play a key role in this study, the subsequent paragraphs delve into their roles and clinical significance in HCC.

3.1.1.1 Angiopoietin-2 in HCC

Physiologically, the expression of ANG-2 significantly increases exclusively in areas where active vascular remodeling occurs, as in the female reproductive tract (Moon et al., 2003; Sugimachi, 2003). In contrast to this limited physiological expression, ANG-2 is overexpressed across a broad spectrum of inflammatory conditions and various cancer types, including HCC (Refolo et al., 2020; Thurston & Daly, 2012). Tanaka et al. (Tanaka et al., 1999) were pioneers in 1999, revealing for the first time the strong correlation between ANG-2 expression and increased vascularity in human HCC. Furthermore, their results showed that ANG-2 expression accelerates tumor progression and worsens prognosis in an ectopic xenograft model of human HCC (Tanaka et al., 1999). As mentioned earlier, the relationship between ANG-2 and VEGF is highly interconnected. When VEGF is present, ANG-2 promotes endothelial cell migration, proliferation, and the development of new blood vessels. Conversely, if the function of endogenous VEGF is hindered, ANG-2 triggers endothelial cell demise and vessel regression (Augustin et al., 2009; Lobov et al., 2002). The strong dependence of ANG-2 on VEGF also supports blood vessel formation in cancer, fostering tumor growth and increasing the risk of cancer metastasis (Q. Li et al., 2006; Z.-L. Zhang, 2006; Moon et al., 2003). Notably, inhibiting VEGF action exhibits the potential to mitigate these adverse effects, especially in the context of HCC. This implies that the combined influence of ANG-2 and VEGF significantly contributes to tumor expansion and the formation of new blood vessels (Yoshiji, 2005). Due to its role as a significant trigger for

angiogenic signaling in cancer and its association with increased VEGF levels in HCC, hypoxia-induced overexpression of VEGF might potentially contribute to the elevated levels of ANG-2 in HCC (Tait & Jones, 2004). Furthermore, in endothelial cells exposed to hypoxia, there is an observed increment in ANG-2 release from Weibel-Palade bodies. This phenomenon could further contribute to the overexpression of ANG-2 within the hypoxic environment of HCC tissue (F. Wang et al., 2015). Teixeira et al. (Teixeira et al., 2018) demonstrated that there was a distinct difference in the expression of ANG-2 between HCC lesions and non-neoplastic regenerative nodules. This distinction might be useful in differentiating between malignant and non-malignant hepatocellular nodules, suggesting a potential role for ANG-2 in the diagnostic process of identifying HCC. ANG-2 seems to have implications not only within HCC tissue but also in the bloodstream of HCC patients. Elevated levels of ANG-2 have been identified not only in the tissue affected by HCC but also in the blood of patients with HCC. This increase suggests the potential use of ANG-2 as a serum biomarker for HCC (Scholz et al., 2007). Other studies, such as the one conducted by Llovet et al. (J. M. Llovet et al., 2012), have found a strong correlation between high levels of ANG-2 in the plasma and poor prognosis in HCC patients. Elevated ANG-2 plasma levels were associated with factors indicating a poor outcome, such as shorter survival time and faster disease progression. It should be underlined, however, that in this study the highest ANG-2 levels were found in large size tumors, suggesting that ANG-2, in this case, was an indicator of the advanced stage. In addition, Kuboki et al. (Kuboki et al., 2008), further reinforce the prognostic significance of ANG-2 in assessing the outcomes of patients after surgical resection. Their findings, indeed, indicated that elevated levels of ANG-2 in the hepatic venous blood before surgery correlated with a shorter postoperative survival period for HCC patients. A high level of ANG-2 in the hepatic vein before surgery might indicate a more advanced stage of HCC. This suggests that in such advanced stages, surgical resection and potentially other curative treatments might not be as effective (Balogh et al., 2016; Kuboki et al., 2008; Wada et al., 2006). After surgical resection, there is a notable decrease observed in ANG-2 levels, both in the hepatic vein and the peripheral vein (Kuboki et al., 2008). These findings imply that preoperative ANG-2 levels could serve as an indicator of the disease stage and the potential effectiveness of surgical interventions or other curative treatments. In liver transplantation, expressed ANG-2 levels also have predictive value. Indeed, endothelial ANG-2 overexpression in explanted livers predicts post-transplant recurrence (Lasagni et al., 2022). On the contrary, the effect of TACE on circulating ANG-2 levels in patients with HCC is not concordant among different studies. Hsieh et al. (Hsieh et

al., 2011) observed a significant increase in ANG-2 serum levels following TACE treatment in their study. On the other hand, Diaz-Sanchez et al. (Diaz-Sanchez et al., 2013) did not find statistically different levels of circulating ANG-2 after locoregional HCC treatments, including RFA or TACE. Growing evidence has shown the possible use of ANG-2 as a biomarker of response to treatment with sorafenib. Miyahara et al. (Miyahara et al., 2011) were the first to establish a link between circulating ANG-2 levels and the response to sorafenib treatment, indicating that higher ANG-2 levels were associated with poorer response and post-treatment PFS in HCC patients treated with sorafenib. Llovet et al. (J. M. Llovet et al., 2012) conducted a biomarker study demonstrating that sorafenib treatment can arrest the disease progression-related increase in circulating ANG-2 levels in advanced HCC patients. Increases in ANG-2 during sorafenib treatment were associated with unfavorable outcomes, suggesting the potential utility of monitoring ANG-2 serum levels during treatment. Recent research identified specific single-nucleotide polymorphisms (SNPs) in the ANG-2 gene (*ANGPT2* rs55633437 SNPs) that were found to correlate with both patient survival and response to sorafenib. These *ANGPT2* polymorphisms hold promise as potential genetic biomarkers for stratifying HCC patients undergoing sorafenib treatment (Marisi et al., 2019). Combining sorafenib treatment with agents that target ANGs, and in particular ANG-2, like the peptibody trebananib, could be a strategic approach to enhance the efficacy of treatments. The results from a phase II trial comparing trebananib plus sorafenib treatment vs sorafenib alone in patients with high ANG-2 levels showed no improvement in PFS at 4 months. Noteworthy, trebananib treatment in lower baseline ANG-2 at the study entry demonstrated improved OS to 22 months, suggesting that further exploration is needed in the context of low baseline ANG-2 (Abou-Alfa et al., 2017). The studies by Kaseb et al. (Kaseb et al., 2012) and Kang et al. (Kang et al., 2015) provide further evidence supporting the role of circulating ANG-2 levels as predictive or prognostic biomarkers in advanced HCC patients undergoing treatment with TKIs or anti-angiogenic agents. Kaseb et al. (Kaseb et al., 2012) reported that elevated ANG-2 plasma levels were associated with a less favorable outcome in advanced HCC patients treated with a combination of bevacizumab and erlotinib (an oral TKI). Conversely, Kang et al. (Kang et al., 2015) findings implied that elevated ANG-2 levels at baseline might serve as an indicator of a less favorable response or prognosis in the context of the TKI axitinib treatment for advanced HCC. Overall, all these findings illustrate the complexity of ANG-2 value in the HCC landscape. While ANG-2 can be a valuable prognostic marker in HCC across different treatment approaches and stages, its utility in early

diagnosis might be limited due to its association with chronic liver disease. However, further research is needed to explore its potential as a therapeutic target.

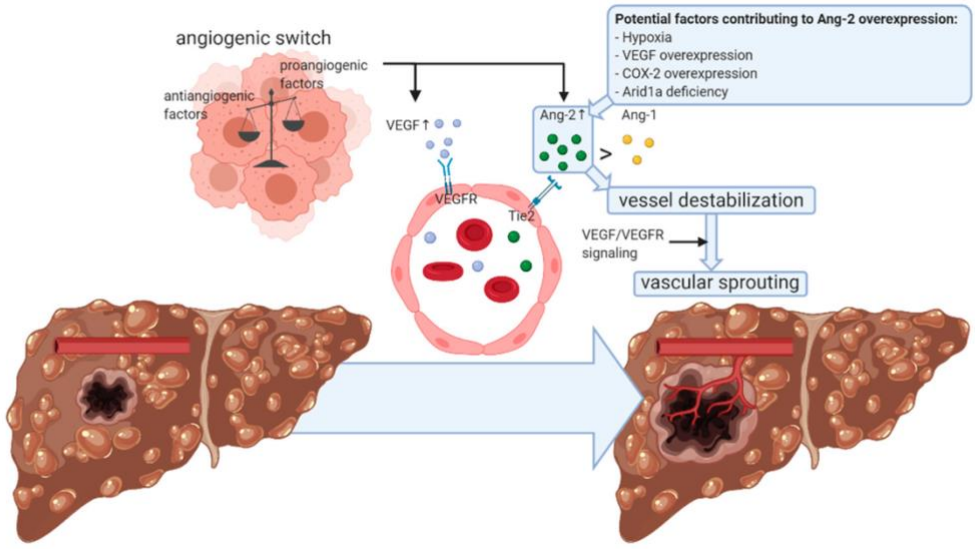

Figure 3 - The angiogenic switch in HCC growth and progression. Vascular remodeling, the process of altering blood vessel structure, is crucial for hepatocellular carcinoma (HCC) growth. The angiogenic switch signifies a shift within the tumor favoring proangiogenic factors, such as vascular endothelial growth factors (VEGFs) and angiopoietin-2 (ANG-2). Overexpression of ANG-2, triggered by factors like hypoxia and increased VEGF, alters the balance between ANG-1 and ANG-2 expression in favor of ANG-2. This imbalance can lead to vessel destabilization. ANG-2 interacts with its receptor, tyrosine kinase with Immunoglobulin (Ig) and Epidermal Growth Factor (EGF) homology domains 2, Tie-2, which is primarily expressed on endothelial cells' surfaces. This interaction destabilizes blood vessels, making them more susceptible to the actions of other proangiogenic factors, like VEGF. ANG-2's effects on HCC angiogenesis and progression are reliant on VEGF/VEGF receptor (VEGFR) signaling. The presence of ANG-2 facilitates the actions of VEGF in inducing vascular sprouting, contributing to the formation of new blood vessels within the tumor and cancer progression. Adapted from (Vanderborght et al., 2020).

3.1.1.2 VEGF in HCC

VEGF and its receptors, particularly VEGFR-2, play a crucial role in governing angiogenesis, which is the formation of new blood vessels (Arciero & Sigurdson, 2006). These growth factors, specifically VEGF-A, VEGF-C, and VEGF-D, activate VEGFR-2 upon binding (Amini et al., 2012). While VEGFR-2, along with VEGFR-1, are pivotal receptors in the angiogenesis process, VEGFR-3 is primarily confined to lymphatic endothelial cells (Ferrara et al., 2003). Among the various VEGF isoforms, VEGF-A stands out as the most critical in prompting angiogenesis and fostering vascular restructuring. Its interaction with VEGFR-2

initiates significant responses in endothelial cells, contributing significantly to new blood vessel formation (Bayona-Bafaluy et al., 2019; Abhinand et al., 2016). The growth of HCC, along with other vascularized tumors, heavily depends on the formation of new blood vessels, a process in which VEGF plays a crucial role. Increased levels of VEGF expression have been identified in cirrhotic and dysplastic liver tissues, suggesting a potential involvement of VEGF-triggered angiogenesis in hepatocarcinogenesis (El-Assal et al., 1998). Furthermore, the increased expression of VEGF is positively related to unfavorable outcomes in HCC (S. B. Choi et al., 2017; Zhan et al., 2013; Schoenleber et al., 2009). Notably, in patients with HCV, serum VEGF, and VEGF/platelet levels, separately or in combination with AFP, are reliable biomarkers for early and accurate diagnosis of HCC (Alzamzamy et al., 2021). Multiple studies have also indicated a direct non-endothelial cell effect on HCC cell proliferation and migration as a result of VEGF overexpression (S. Peng et al., 2014; L. Zhang et al., 2012). TACE causes local hypoxia in HCC, triggering a series of adaptive responses among which the main one is overexpression of HIF-1α, which, in turn, stimulates VEGF expression. Assessing serum and tissue levels of HIF-1α and VEGF after TACE could be useful in determining possible tumor recurrence and metastasis. For example, Jia et al. (Jia et al., 2011) showed elevated serum levels of HIF-1α and VEGF after TACE correlated with tumor thrombi in the portal vein and metastasis. Regarding tissue expression, several other studies confirmed that TACE stimulated tumor angiogenesis through upregulation of tissue expression of VEGF (Xiao et al., 2009; B. Wang et al., 2008). Elevated levels of circulating VEGF were also observed during DAA therapy, as indicated by Villani et al. (Villani et al., 2016), playing a crucial role in *ANGPT2* overexpression. Faillaci et al. (Faillaci et al., 2018) demonstrated that increased VEGF due to direct-acting antivirals (DAA) treatment acted as a catalyzer for HCC recurrence or onset in predisposed patients, particularly those with severe fibrosis and pre-existing abnormal activation of neo-angiogenic pathways in liver tissue. Due to its involvement in HCC development and progression, angiogenesis, specifically targeting VEGF, is a primary focus of various treatments. As mentioned earlier, sorafenib, a TKI, targets the VEGF signaling pathway among several others involved in angiogenesis. Sorafenib serves as the inaugural systemic therapy sanctioned for the treatment of advanced HCC patients (A.-L. Cheng et al., 2009; J. M. Llovet et al., 2008). During the SHARP trial, it was found that the baseline plasma concentrations of VEGF and ANG-2 independently predicted the survival of patients receiving sorafenib. However, neither of these factors could predict the response to the treatment itself (J. M. Llovet et al., 2008). Conversely, Horwitz et

al. (Horwitz et al., 2014) proposed that the amplification of VEGF-A in HCC might serve as a valuable predictor of response to sorafenib. Their observations indicated that patients exhibiting VEGF-A amplification displayed heightened tumor sensitivity to this treatment. Despite this promising finding, the connection between VEGF-A amplification and a survival advantage was not validated when sorafenib was used in an adjuvant setting (Pinyol et al., 2019). However, the survival advantages achieved with TKIs like sorafenib have been limited, although the initial revolution in systemic HCC treatment. Several other TKIs or monoclonal antibodies were tested in the first-line treatment. Bevacizumab, a monoclonal antibody directed against VEGF (Ferrara et al., 2005), functions by impeding angiogenesis and suppressing tumor growth (Finn et al., 2009). Phase II studies demonstrated response rates ranging from 13% to 14% in patients with advanced HCC (Boige et al., 2012; Finn & Zhu, 2009; Siegel et al., 2008). Circulating VEGF-A was initially suggested as a potential prognostic and predictive biomarker among patients receiving treatment with bevacizumab for various tumor types. However, subsequent findings revealed its inadequacy as a reliable predictive biomarker, making its successful implementation in clinical practice unlikely (Hegde et al., 2013). Most recently, the combination therapy involving the antiangiogenic drug targeting VEGF (bevacizumab) alongside the immune checkpoint inhibitor anti-PD-L1 (atezolizumab) demonstrated superiority over sorafenib in terms of both tumor response and providing a survival benefit (A.-L. Cheng et al., 2022; Finn et al., 2020). Another multiple TKI, lenvatinib, demonstrated non-inferiority to sorafenib in unresectable HCC patients. However, lenvatinib-treated patients experienced more adverse events than sorafenib-treated (Kudo et al., 2018). In patients with HCC progressing on sorafenib, regorafenib was the first drug to demonstrate survival benefits (Bruix et al., 2017). Recently, ramucirumab, a monoclonal antibody against VEGFR-2, showed an increase in survival only in patients with AFP level ≥ 400ng/dl intolerant to sorafenib (A. X. Zhu et al., 2019, 2015). Overall, although VEGF overexpression appears to have a predictive and prognostic role in patients with HCC, its expression is not currently validated as a biomarker of response to systemic treatment. However, nowadays, inhibition of the angiogenic pathway is still the mainstay of advanced HCC treatment.

3.2 CCA microenvironment

In CCA, as cancerous lesions develop, there is a progressive pathological transformation of the surrounding stroma. This transformation gradually becomes a crucial factor influencing

tumor growth and spread. The diverse cell types within the TME, recently termed the tumor-reactive stroma (TRS), contribute significantly to the development of an aggressive phenotype in CCA cells by secreting various soluble factors (Brivio et al., 2017). These factors, in turn, may directly stimulate the occurrence of malignant phenotypes or support the migration and abnormal activation of other stromal cells (Hui & Chen, 2015; Leyva-Illades et al., 2012).

The TRS is prevalently populated by a high number of activated CAFs which secrete a huge number of biochemical signals and ECM components, such as fibronectin and collagen type I (Brivio et al., 2017; Cadamuro, Morton, et al., 2013; Kalluri & Zeisberg, 2006). CAFs are typically recruited by CCA through the production of various mediators, including PDGF-D and TGF-β1 (Gentilini et al., 2018; Cadamuro, Nardo, et al., 2013), and they express different phenotypic markers, such as α-SMA and PDGFR-β (Montori et al., 2022; M. Zhang et al., 2020). PDGFR-β on CAFs, upon interaction with PDGF-D, triggers the release of VEGF-C and VEGF-A resulting in the expansion of the lymphatic vasculature and intravasation of tumor cells, thereby promoting metastatic spread (Cadamuro et al., 2019). It is noteworthy that the inhibition of PDGFR-β using imatinib led to a significant reduction in PDGF-D-induced fibroblast migration. Consequently, this inhibition also impacted the trans-endothelial migration of CCA cells (Cadamuro et al., 2019; Cadamuro, Nardo, et al., 2013). High α-SMA expression on CAFs induces a more pronounced proliferation effect on both CCA cells and non-tumor biliary epithelial cells compared to normal liver fibroblasts characterized by low α-SMA expression. Therefore, CAFs with high α-SMA expression were associated with larger tumor size and reduced OS (Chuaysri C. et al., 2009). Coherently with these results, both incubation of CCA cells with CAF conditioned medium and coculture of CCA cells with CAF determined increased *in vitro* proliferation and migration of tumor cells (Heits et al., 2016; Chuaysri C. et al., 2009). Additionally, the phenotype of CAFs may act as an independent prognostic factor for OS in resected iCCA (X. Zhang et al., 2017). Crosstalk between CCA and CAF cells plays a crucial role in sustaining and amplifying the malignant traits of cancer cells. This mutual reliance is supported by the production of heparin-bound (HB) EGF by CAFs. HB-EGF, upon binding to the EGF receptor (EGFR) on CCA cells, triggers the transcriptional activation of β-catenin signaling via ERK 1/2 and STAT3 pathways, subsequently promoting tumor cell motility and invasion (Clapéron et al., 2013). Upon EGFR activation, CCA cells release TGF-β1, further stimulating myofibroblast activation and HB-EGF secretion, creating a perpetuating "vicious cycle" (Clapéron et al.,

2013). Additionally, stroma cell-derived factor 1 (SDF-1) serves as another mediator released by CAFs. SDF-1 interacts with CXCR4 expressed by CCA cells, thereby stimulating tumor cell invasion through ERK1/2 and AKT signaling pathways (Gentilini et al., 2012). Molecules secreted by CAFs may also act as predictors of prognosis in patients with CCA. Indeed, a high IL-33 content in CAFs was associated with a significantly better prognosis than that of patients with a low IL-33 content, suggesting that IL-33 is a marker of good prognosis in CCA and that enhancing IL-33 in CAFs is a promising therapeutic direction (Yangngam et al., 2020). On the contrary, high SDF-1 predicted poor prognosis in CCA patients (Okamoto et al., 2012). CAFs also exert immunomodulatory functions. A subset of CAFs that overexpress FAP, through the release of CCL2 in a STAT3-dependent manner, are responsible for the inhibition of T-cell proliferation and infiltration of myeloid-derived suppressor cells (MDSCs) in mouse models of CCA (X. Yang et al., 2016). In addition, CAFs promote immunosuppression by attracting dendritic cells (DCs) and attenuating the expression of antigen-presenting HLA molecules, thus impairing the ability to activate tumor-infiltrating lymphocytes (TILs) (J. Cheng et al., 2016).

Macrophages in the CCA microenvironment often present an M2 phenotype (Y. Zhang et al., 2021), and high TAMs infiltration has been associated with unfavorable outcomes (Subimerb et al., 2010), and the development of extrahepatic metastasis (Thanee et al., 2015). TAMs play a pivotal role in promoting angiogenesis by releasing pro-angiogenic factors like ANGs and VEGF (Roy et al., 2019). Additionally, they contribute to the proliferation of CCA cells by activating the Wnt/β-catenin pathway. This activation occurs subsequent to the release of Wnt3a and Wnt7b by TAMs (Boulter et al., 2015; Loilome et al., 2014). Indeed, depletion or inhibition of macrophages determines a significant decrease of Wnt7b in animal models of CCA, thereby reducing tumor burden and proliferation (M. Zhou et al., 2021). TAMs are likely involved in facilitating tumor invasion, either through direct impact on tumor cell actions or by shaping the microenvironment surrounding the tumor. TAMs play a key role in the generation of TNF-α (Ohira et al., 2006), which is known to enhance the migratory abilities of CCA cells by triggering the activation of transcription factors such as Snail and ZEB2 (Techasen et al., 2014, 2012). In contrast, in several epithelial tumors, including CCA, TAMs produce several MMPs in abundance, particularly MMP-9 (Subimerb et al., 2010). This action helps tumor cells degrade nearby ECM, a crucial process that promotes local invasion (Brown & Murray, 2015).

Although TAMs usually show an M2 phenotype, Raggi et al. discovered a unique group of TAMs influenced directly by cancer stem cells (CSCs). These TAMs subset displays a

combination of M1 and M2 markers. Notably, TAMs linked to CSCs demonstrate strong abilities to adhere and invade tissues, alongside increased expression of genes associated with remodeling the matrix, such as ADAM17 and MMP2. This indicates a pivotal involvement in ECM reorganization (Raggi et al., 2017).

Intense ECM remodeling has been widely demonstrated to support cancer progression at multiple levels, including induction of angiogenesis, activation of stromal cells, and actively fueling the aggressiveness of cancer cells (Bonnans et al., 2014; Mueller & Fusenig, 2004). The rearrangement of ECM is a characteristic feature shared by desmoplastic tumors, including CCA. This rearrangement involves the synthesis of new components such as periostin, tenascin-C, and osteopontin, alongside the secretion of proteolytic enzymes like MMPs (Fabris et al., 2020). Periostin interaction with integrin α5 on CCA cells initiates the PI3K/AKT signaling pathway, driving both tumor cell proliferation and invasion (Utispan et al., 2012). Osteopontin contributes to the expansion and dissemination of iCCA by activating the MEK/MAPK1 and Wnt/β-catenin signaling pathways (K.-Q. Zhou et al., 2019). Nevertheless, the role of osteopontin remains controversial. Reduced osteopontin expression in iCCA tissues has been linked to lymph node metastasis and lower survival rates, whereas decreased circulating osteopontin has been associated with the presence of multiple tumors (Y. Zheng et al., 2018).

In iCCAs, T lymphocytes constitute the most predominant type of inflammatory cells. Remarkably, there's a higher proportion of CD8+ T cells compared to CD4+ T cells within these tumors (Goeppert et al., 2013). NK cells and B lymphocytes are present in limited numbers, with the latter being less represented (Goeppert et al., 2013). In general, cytotoxic T cells and NKs are reduced while T regs are increased, promoting an immunosuppressive environment (G. Zhou et al., 2019). CCA cells play a fundamental role in the production of immunomodulatory molecules, such as TGF-β and IL10 (Fabris et al., 2019). Concurrently, MDSCs and TAMs release IL10 and TGF-β, inducing the transformation of DCs into regulatory DCs. Tregs and regulatory DCs themselves sustain, in turn, the recruitment of more immunosuppressive immune cells, consequently compromising the anti-tumor defenses (Veglia & Gabrilovich, 2017; W. Zou, 2005). Moreover, CAFs, TANs, and TAMs secrete CCL2, recruiting Tregs (Fabris et al., 2019; X. Yang et al., 2016; S.-L. Zhou et al., 2014), thereby feeding this immunosuppressive condition.

Within the TIL population, CD8+, CD4+, and CD3+ T lymphocytes are predominantly localized in the peritumoral area in both iCCAs and eCCAs. Conversely, PD-1+ T cells are primarily observed within the tumor region (H. Wu et al., 2021; J.-C. Lu et al., 2019;

Goeppert et al., 2013). Globally, TILs have been linked to prognosis in both iCCAs and eCCAs (Vigano et al., 2019). A larger presence of CD4+ and CD8+ lymphocytes has been linked to improved OS and reduced occurrences of lymph node metastasis, as well as decreased rates of venous and perineural invasion, indicating a better prognosis (D. Liu et al., 2022).

A wide lymphatic vasculature associated with limited CD34+ blood vasculature is a hallmark of TME in CCA (Cadamuro et al., 2019). Tumor-associated lymphangiogenesis is influenced not only by the tumor cells but also by various elements present in the TME, including TAMs and CAFs (Cadamuro, Romanzi, et al., 2022). These components significantly contribute to the invasion of tumor cells into the lymphatic system. Within both the peritumoral and intratumoral regions, there exists a complex network of lymphatic vessels (Cadamuro, Romanzi, et al., 2022). Studies indicate that a heightened presence of peritumoral and intratumoral lymphatic vessels is associated with increased occurrences of lymph invasion within the tumor, lymph node metastasis, and the recurrence of tumors in both pCCA (Thelen et al., 2008) and iCCA (Sha et al., 2019). Another notable characteristic of TME in CCA is hypoxia, a condition that triggers the expression of HIF-1α, a pivotal cellular effector under hypoxic conditions (K. Ruan et al., 2009), whose overexpression is positively related to tumor growth and decreased DFS in iCCA (Morine et al., 2011). HIF-1α activation subsequently promotes the synthesis of VEGF and PDGF growth factor families, which collaborate in various mechanisms involved in both lymph angiogenesis and angiogenesis (Hashimoto & Shibasaki, 2015). Specifically, when CCA cells experience hypoxic conditions, they release VEGF-A and PDGFB/D, attracting CAFs that play a crucial role in assembling lymphatic endothelial cells (LECs). Additionally, CCA cells, by upregulating receptor-interacting protein kinase 1 (RIPK1), activate the JNK-p38 MAPK-AP1 pathway, leading to an increased secretion of VEGF-C and pigment epithelium-derived factor (PEDF). These heightened secretions act as direct recruiters of LECs, further amplifying the formation of the lymphatic network within the TME (Cadamuro, Romanzi, et al., 2022). The density of lymphatic vessels (LVD) and the presence of specific lymphatic markers like podoplanin and VEGFR-3, expressed by LECs, have been proposed as prognostic indicators for CCA (Sha et al., 2018; Obulkasim et al., 2017). Podoplanin, a membrane glycoprotein, is consistently found in the lymphatic vasculature and plays a critical role in the proliferation and formation of tubes by LECs (Navarro et al., 2011). Moreover, podoplanin is prominently present on the surface of CAFs and has emerged as a prognostic marker in pCCA (Obulkasim et al., 2017).

Elevated podoplanin expression on activated CAFs has also been correlated with lymph node metastasis in iCCA (Aishima et al., 2008).

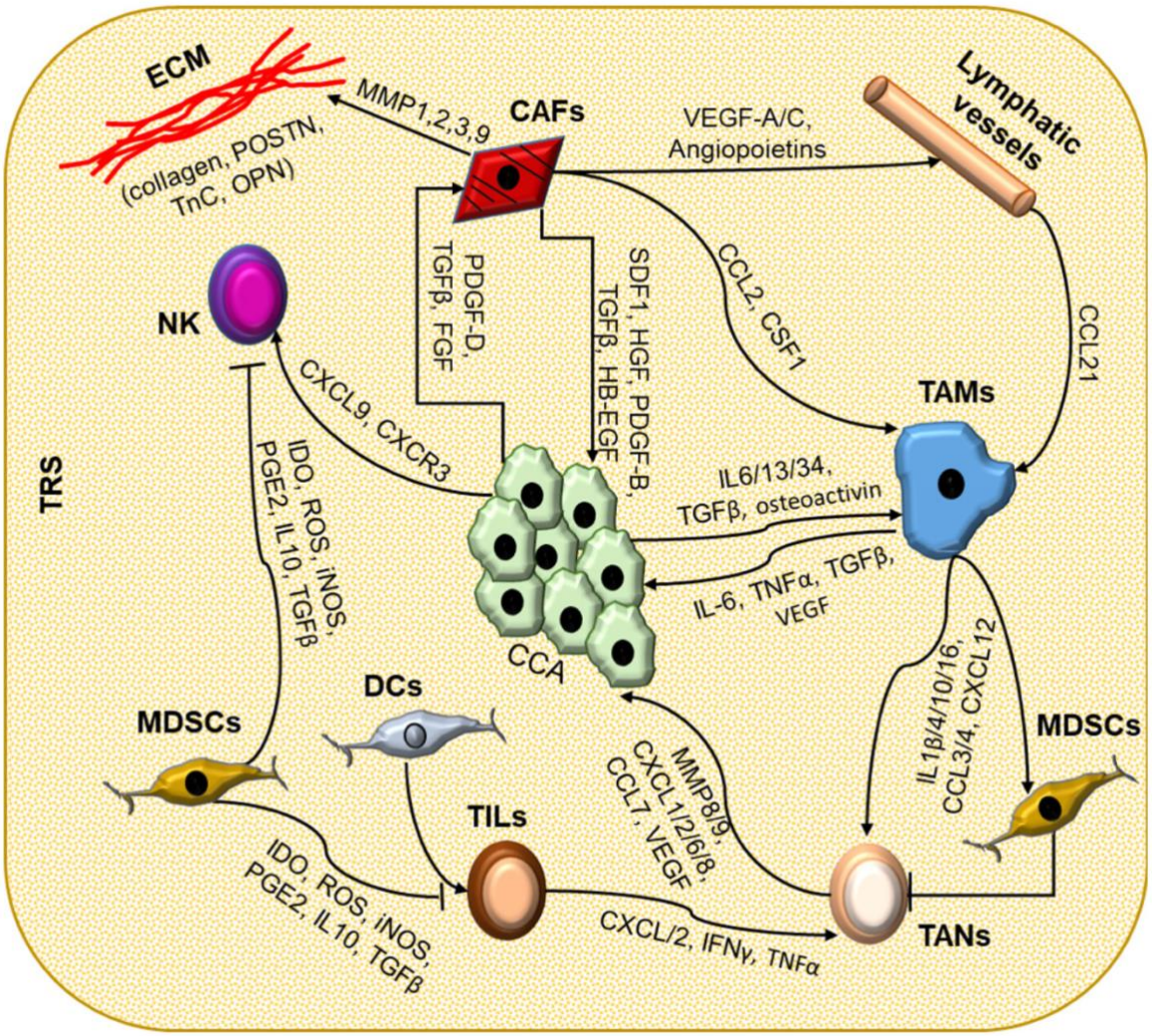

Figure 4 - The complex interplay between CCA and tumor reactive stroma cells. The tumor microenvironment (TME) undergoes significant changes influenced by cancer cells, ultimately shaping what is termed the tumor-reactive stroma (TRS). This creates a complex and dynamic milieu composed of various cell types, extracellular matrix components, and soluble factors that interact in intricate ways. CCA cells can recruit cancer-associated fibroblasts (CAFs), natural killer (NK) cells, and cancer-associated macrophages (TAMs) through the secretion of various factors such as platelet-derived growth factor (PDGF)-D, transforming growth factor (TGF) β, fibroblast growth factor (FGF), C-X-C motif ligand (CXCL) 9, IL-6, IL-13, IL- 34, and osteoactivn. These recruited cells, in turn, influence the behavior of CCA cells through the secretion of other soluble mediators, establishing a bidirectional communication network. CAFs play a role in recruiting lymphatic vessels cells and in modifying the ECM by secreting metalloproteinases (MMPs)- 1, -2, -3, and -9, collagens, and structural proteins like osteopontin (OPN), tenascin C (TnC), and periostin (POSTN). The TME

regulates immune response, with different immune cells interacting with each other. Dendritic cells (DCs) activate tumor-infiltrating lymphocytes (TILs), while TAMs recruit tumor-associated neutrophils (TANs). Myeloid-derived suppressor cells (MDSC) can inhibit the activity of various immune cells, including TILs, TANs, and NKs, contributing to immune tolerance. Adapted from (Cadamuro, Fabris, et al., 2022).

3.2.1 Tumor-associated angiogenesis in CCA

As mentioned above, a large expansion of the lymphatic vasculature is not matched by a similar increase in blood vessels (Cadamuro, Morton, et al., 2013). As known, for the sustenance of rapid growth, tumors necessitate an extensive vascular network to facilitate the arrival of a considerable amount of nutrients. Tumor-associated angiogenesis remains notably active and MVD, which serves as an indicator of the extent of tumor-driven neovascularization, has demonstrated a significant association with both survival outcomes and overall prognosis in gallbladder carcinoma, iCCA, and eCCA (Y. Chen et al., 2011; Thelen et al., 2010, 2008). However, the blood vessels within tumors exhibit structural irregularities, incomplete wall formation, and heightened permeability, resulting in local tumor hypoperfusion and subsequent hypoxia (Jain, 2005). The overexpression of HIF-1α, the key mediator associated with the effects of hypoxia, shows a positive correlation with tumor size and decreased DFS in iCCA (Morine et al., 2011). Furthermore, the upregulation of HIF-1α triggers the expression of angiogenic factors such as the VEGF family and ANG-2, which exert a pivotal role in the process of neoangiogenesis and the proliferation of cholangiocytes (Cadamuro et al., 2018; Fabris et al., 2006). The secretion of another key player involved in blood vessel formation, i.e. PDGF, was found to be positively correlated with stage, metastasis, and short survival rate among CCA patients (Boonjaraspinyo et al., 2012). However, other molecules in the TME, such as thrombospondin 1 and 2 (THBS1-2) and PEDF, have been shown to inhibit angiogenesis and promote lymph angiogenesis in iCCA (Carpino et al., 2021). Conversely, the endothelins (ETs), a class of vasoconstrictor peptides responsible for diverse mechanisms promoting cancer progression (Bagnato & Rosanò, 2008; Nelson et al., 2003), have been shown to exert an inhibitory effect on the proliferation of CCA cells. This effect is attributed to their ability to reduce the expression of VEGF proteins, thereby inhibiting potent stimulators of vascularization (Fava et al., 2009). ANG-1 also appears to have a protective effect against metastasis and tumor recurrence. Indeed, its expression appears to correlate with a lower risk of metastasis in hilar cholangiocarcinoma (Atanasov et al., 2016). Additionally, the presence of TIE2-positive

monocytes (TEMs) near the microvasculature is associated with reduced tumor recurrence (Atanasov et al., 2016). Another study by Atanasov et al. (Atanasov et al., 2018) proposed a possible influence of miR-126 in the upregulation of ANG-1. This elevation in ANG-1 levels may facilitate the recruitment of more TEMs, subsequently exerting an inhibitory influence on the progression of CCA. This implies a positive impact of ANG-1 and TEMs on CCA development. Overall, a better understanding of the "yin and yang" aspects of angiogenesis in CCA could be a good starting point for therapeutic progress (Romanzi & Villa, 2023). The following paragraphs detail the function and clinical impact of ANG-2 and VEGF in CCA, considering their relevance to the present study.

3.2.1.1 Angiopoietin-2 in CCA

The Angiopoietin system is implicated in various vascularized tumors, as in HCC, but also in CCA. However, the role of ANGs, especially ANG-2, in the progression of CCA remains a topic of ongoing debate. Indeed, unlike some other cancers that heavily rely on blood vessels for growth and metastasis, such as HCC (Z. F. Yang & Poon, 2008), CCA has shown a significant dependence on the lymphatic network for its progression (Cadamuro, Romanzi, et al., 2022; Cadamuro, Morton, et al., 2013). This reliance on lymphatic vessels rather than blood vessels for dissemination might influence the understanding of angiogenic factors in CCA. Several research studies suggest that ANG-1 and ANG-2 are essential factors in the later stages of lymphatic vessel development, contributing to the maturation, stabilization, and remodeling of the lymphatic network (Fagiani et al., 2011; Gale et al., 2002). Notably, the presence of the TIE-2 receptor on LECs justifies the significant involvement of ANGs in influencing lymphatic vessel function and development (Morisada et al., 2005). Nonetheless, only a few studies are available on the association between ANG-2 and the progression of CCA. In a study by Tang et al. (Tang et al., 2006), ANG-2 expression was observed in 57.6% of cases of CCA, with a significantly higher MVD in cases positive for VEGF and ANG-2. This finding suggests a cooperative role between VEGF and ANG-2 in promoting angiogenesis in CCA. Additionally, ANG-2 was detected in the serum and bile of CCA patients, showing a positive correlation with the disease in serum, indicating a potential utility in distinguishing CCA from PSC (Voigtländer et al., 2014). Furthermore, a study by Kimawaha et al. (Kimawaha et al., 2021) found that high levels of ANG-2 correlated with advanced cancer stages and metastasis in CCA compared to non-CCA patients. Overall, ANG-2 seems to be associated with promoting angiogenesis, advanced cancer stages, and

metastasis in CCA. Understanding the roles of ANG-2 in CCA could potentially lead to novel therapeutic approaches targeting angiogenesis and tumor progression.

3.2.1.2 VEGF in CCA

The role of VEGF and its association with clinicopathological features in the progression of CCA has received more research attention than ANGs in the same context. The VEGF/VEGFR pathway has a crucial role during bile duct maturation at the early stage, but it becomes inactive during the mature stage. Nevertheless, it can be reactivated in disease conditions (Fabris et al., 2008; Mariotti et al., 2021). Indeed, the interaction between VEGF-A and VEGF-C produced by CAFs with their cognate receptors expressed by LECs results in the expansion of lymphatic vasculature and tumor cell extravasation, thereby promoting metastatic spread (Cadamuro et al., 2019). However, there is a clear discrepancy in findings among various studies regarding the impact of VEGF expression on the prognosis of CCA patients, particularly in eCCA and iCCA. Some studies suggest that in eCCA, patients with VEGF positivity tend to have worse OS compared to those with negative VEGF expression (Hida et al., 1999). Other studies, such as Kawahara et al. (Kawahara et al., 1998) and Möbius et al. (Möbius et al., 2007), found no correlation between VEGF expression and survival in CCA patients. While the first evidenced a lower VEGF expression in CCA patients compared to control, the latter indicated a better prognosis in eCCA patients with low MVD. Conversely, Yoshikawa et al. (Yoshikawa et al., 2008) demonstrated higher levels of VEGF expression in both iCCA and eCCA compared to controls and correlated high VEGF expression with intrahepatic metastasis in iCCA. Further studies on eCCA have shown that the VEGF-A expression levels in dCCA were higher than pCCA and correlated with an increment in MVD (Guedj et al., 2009). Moreover, Cai et al. (Cai et al., 2022) linked high VEGF expression to shorter OS in iCCA patients. Overall, most studies in the literature, including Calastri et al. (Calastri et al., 2022), tend to associate higher VEGF expression with a worse prognosis in iCCA patients. Moreover, VEGF has been shown to promote cholangiocyte proliferation via an autocrine mechanism (Gaudio et al., 2006). Despite conflicting results on the prognostic significance of VEGF, the widely accepted positive association between high VEGF level and worse prognosis in CCA has suggested the use of anti-VEGF treatments alone or in combination with conventional chemotherapy to evaluate their efficacy in the management of advanced stages of this cancer. Sorafenib has shown limited benefits as a single agent in phase II studies for patients with advanced biliary tract carcinoma (El-Khoueiry et al., 2014; Bengala et al., 2010). However, combining sorafenib

with the best supportive care in a multicenter prospective study for advanced iCCA demonstrated modest PFS (3.2 months) and OS (5.7 months) (Luo et al., 2017).

Bevacizumab has been used in combination with standard chemotherapy (gemcitabine and oxaliplatin) and has shown a slight but significant increase in PFS in patients with metastatic carcinoma of the biliary tract (Bréchon et al., 2018). Another VEGF inhibitor, apatinib, has exhibited promising effects in blocking proliferation, migration, and angiogenesis (M.-P. Huang et al., 2021), and promoting apoptosis (H. Peng et al., 2016) in iCCA cell lines. This suggests its potential usefulness in advanced iCCA (G. Lin et al., 2020), although further clinical studies are required to establish its efficacy and safety in patients. On the other hand, a combination of pazopanib (a multikinase inhibitor of VEGFR, PDGFR, FGFR) with trametinib (a MEK inhibitor) did not yield a statistically significant enhancement in PFS for patients with refractory CCA (Shroff et al., 2017). Overall, while some trials have shown promising results in utilizing drugs targeting the angiogenic pathway involving VEGF in CCA treatment, the efficacy of these agents as single or combination therapies remains variable. These findings highlight the complexity of targeting VEGF-mediated pathways in CCA and suggest that further exploration and investigation into VEGF pathway inhibition are warranted.

4. Preclinical experimental *in vitro* models of HCC and CCA

Utilizing the appropriate research framework holds significant importance in comprehending the intricate diversity within both HCC and CCA. Traditional two-dimensional (2D) monolayers of cancer cell lines are commonly employed in cancer studies, offering valuable insights into cellular signaling pathways, metabolic processes, proliferation, and responses or resistance to therapies (Blidisel et al., 2021; Mirabelli et al., 2019; Katt et al., 2016). Moreover, their quick replication cycle and cost-effectiveness make 2D cultures convenient models for investigating tumorigenesis and ensuring high experimental consistency (Calvisi et al., 2023). However, extended serum-based culture conditions often promote the acquisition of new genetic variations and the selection of clones that do not accurately represent the genetic diversity present in the original tumor (Domcke et al., 2013; Kaur & Dufour, 2012; Chow & Rubin, 2000). Additionally, the flat 2D growth fails to replicate realistic cell-cell and cell-extracellular matrix interactions, thus lacking in mimicking tissue architecture (Saydé et al., 2021; Kaur & Dufour, 2012). Despite their limitations in replicating the complex TME, 2D cell cultures continue to represent the cornerstone of current preclinical research (Kimlin et al., 2013). Moreover, alongside immortalized 2D cell

lines, primary cultures derived from human tumor tissue present an alternative approach. While these primary cultures better retain the original traits of the tumor, they have a limitation—they reach senescence relatively quickly after derivation. This restricts their viability for long-term culture and hinders experiment reproducibility (Calvisi et al., 2023). In contrast to traditional cell cultures, three-dimensional (3D) systems offer a considerably more authentic simulation of *in vivo* conditions, particularly within the microenvironment where tumors evolve (C. Wang et al., 2014). These 3D cultures effectively represent a link between 2D cultures and animal testing, serving as a valuable tool that enhances the efficacy of anticancer drug research (Chiew et al., 2017). Indeed, these 3D systems facilitate a more comprehensive assessment of various cellular behaviors, encompassing proliferation, morphology, motility, and especially the interactions between tumoral cells or among tumoral cells and other cell types within the TME (Ravi et al., 2015).

The most common 3D models used are briefly described below.

- Spheroids, which are sphere-shaped aggregations of cells, typically originate and are sustained under low-attachment and low-serum conditions to promote their formation. They exhibit a necrotic core surrounded by a proliferative layer, effectively mirroring the principal characteristics of the tumor bulk (Massa et al., 2020; Pinto et al., 2020). Further description of spheroids will be provided in the subsequent paragraph.
- Organoids are a 3D model that faithfully maintains the characteristics of real organs, demonstrating self-organization, self-renewal, and histological features akin to their organ counterparts. They stand out as one of the more advanced systems for investigating carcinogenesis and conducting drug screenings, especially for discovering personalized treatments (Lo et al., 2020; H. Fan et al., 2019). Both HCC and CCA organoids have been derived from surgical resections obtained from patients, displaying a sustained expression of specific tumor markers and retaining the genomic traits of the original tumors (Nuciforo et al., 2018; Broutier et al., 2017). Nevertheless, organoids carry some drawbacks, such as high cost, lengthy culture periods, and demanding preparation as well as the absence of blood vessels and immune components crucial for *in vivo* conditions (Molina-Sánchez & Lujambio, 2019).
- Scaffold-based technologies are a unique 3D culture system that offers a physical framework for cells to gather, multiply, and move around (Langhans, 2018). In this setup, cells are embedded in a matrix, which can be either natural or synthetic,

designed to mimic the properties of the extracellular matrix. This scaffold can be enhanced with growth molecules to boost cell proliferation or trigger specific behaviors (Langhans, 2018). Researchers have used materials like chitosan or cellulose in scaffold-based studies involving HCC models, proving these scaffolds' effectiveness in drug screening (Ahmadian et al., 2023; Leung et al., 2010). However, the use of various material compositions, along with issues related to low reproducibility and insufficient replication of the human TME, represent significant drawbacks that need consideration (Hoarau-Véchot et al., 2018).

- 3D bioprinting is an advanced technology that involves the precise deposition of bioink, comprising living cells, ECM components, and growth factors, guided by computer-aided design (CAD) software. This process enables the creation of 3D tissue-engineered constructs that closely mimic the natural tissue architecture (Zhuang et al., 2023; Augustine et al., 2021). In both HCC and CCA, 3D bioprinting platforms exhibit elevated expression levels of tumor-related genes and demonstrate pharmacodynamics that more closely resemble *in vivo* conditions compared to 2D cultures (C. Li et al., 2022; Sun et al., 2020).
- Organ-on-chip (OOC) technology has garnered significant attention as one of the most rapidly emerging fields in recent times. It serves as a platform that integrates microfluidics, microfabrication techniques, and tissue engineering principles (Tsai et al., 2017). Essentially, an OOC represents a microfluidic device incorporating a chip designed to replicate a microenvironment similar to a real organ. It consists of living cell tissue, elements for administering stimuli or drugs (like electrical stimulation for the maturation of myocardial tissue), and sensors for detecting and gathering data (Q. Wu et al., 2020). OOCs stand out as one of the most promising tools for investigating TME and advancing drug development (Cauli et al., 2023; Deng et al., 2019). Recent studies have showcased the remarkable utility of this technology for conducting pharmacological investigations and personalized drug testing related to HCC, HCC metastasis, and CCA (Polidoro et al., 2024; Sharifi et al., 2020; S. Lu et al., 2018).

4.1 Spheroids

Spheroids have emerged as a promising model for drug discovery and the investigation of cancer initiation, invasion, and progression (Pinto et al., 2020). These structures exhibit a spherical shape featuring three distinct concentric zones housing different cell populations: an outer zone comprising highly proliferative and migrating cells, a middle zone of quiescent

cells, and an inner zone consisting of necrotic cells, as depicted in Figure 5 (Pinto et al., 2020; Hirschhaeuser et al., 2010). The well-organized arrangement within spheroids arises from gradients of nutrients, oxygen, and pH extending from the periphery to the core. The innermost region sustains significant hypoxia, triggering hypoxia-inducible factors that prompt the Warburg effect, resulting in increased lactate production and lowered pH levels. Consequently, scarce nutrient and oxygen availability, coupled with the accumulation of metabolic waste, leads to necrotic cell death within the core of the spheroid (Pinto et al., 2020). Typically, spheroids have a diameter of around 200 μm. However, those ranging from 300 to 500 μm demonstrate optimal conditions to replicate hypoxia and proliferation similar to *in vivo* environments (Pinto et al., 2020). Cancer cells possess the ability to spontaneously aggregate into spheroids, comprising either a single or multiple cell suspension (Massa et al., 2020). The size and complexity of these spheroids are influenced by the growth kinetics of individual cell types, the initial cell density during seeding, and the duration of culture. As the size and heterogeneity of spheroids can impact the efficacy of assays, it becomes crucial to produce spheroids that maintain uniform size and complexity (Nath & Devi, 2016). Commonly, 48-hour cultures yield small spheroids (about 200μm in diameter) that are uniform and homogeneous in size. In contrast, longer-term cultures (lasting more than 4 days) generate larger spheroid, exceeding 500μm in diameter. These larger spheroids exhibit heterogeneity, containing hypoxic nuclei and cells with varying rates of proliferation (Nath & Devi, 2016; Winters et al., 2006).

Numerous methods have been devised for producing spheroids, categorized into scaffold-based and scaffold-free techniques. Scaffold-based methods encompass variants such as matrix on top, matrix embedded, or matrix encapsulation, as well as approaches involving spinner flasks and micropatterned plates. On the other hand, scaffold-free methods include alternatives like ultra-low attachment plates, hanging drop techniques, magnetic levitation, or magnetic 3D printing (Pinto et al., 2020; Nath & Devi, 2016).

Among the various available methods, the extensive focus lies in elucidating the hanging drop and ultra-low attachment techniques utilized for generating spheroids in this study.

4.1.1 Hanging drop

In this technique, cells are seeded into a droplet of culture medium which is then dispensed onto the underside of a Petri lid dish and promptly flipped downward after seeding. The droplets hang suspended due to surface tension, enabling cells to naturally aggregate into

spheroids under the influence of gravity. To prevent droplet dehydration, the Petri dish contains phosphate-buffered saline (PBS) (Nath & Devi, 2016; Jørgensen et al., 2014; Achilli et al., 2012). While this method facilitates the large-scale production of spheroids, certain cell lines may not form tightly compacted spheroids using this approach (Ong et al., 2010). Furthermore, this specific culture condition does not guarantee uniform spheroid formation in terms of size and morphology, posing a risk of cell damage due to potential media evaporation (Pinto et al., 2020; Nath & Devi, 2016). Simple tasks, like changing or adding the medium or incorporating compounds, may become intricate and time-consuming within this setup (Vantangoli et al., 2015).

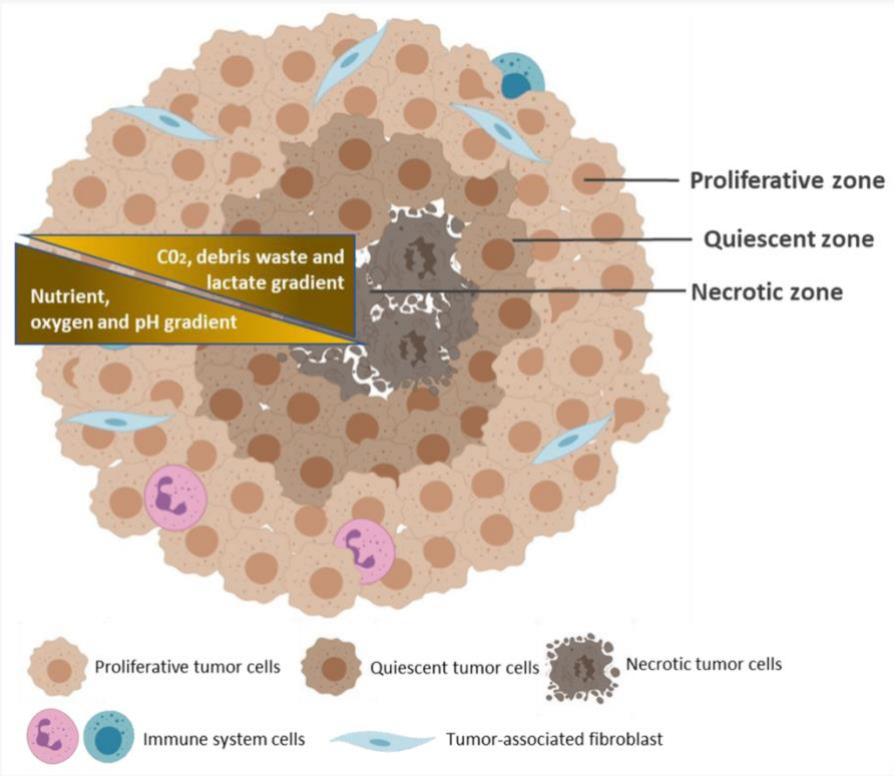

Figure 5 - Schematic representation of the spheroid structure. The varying gradients of nutrients, oxygen, and waste within spheroids result in the formation of three distinct layers composed of different cell populations: an outer layer (Proliferative zone) characterized by active proliferation, a middle layer of quiescent cells (Quiescent zone), and an innermost layer consisting of necrotic cells (Necrotic zone). Adapted from (Pinto et al., 2020).

4.2.1 Ultra-low attachment plates

In this technique, the surface of plates is treated to prevent cell adhesion, thus facilitating spheroid formation. A key advantage of this method is the ability to both generate and assay the spheroids within the same plate, thereby minimizing the risk of damage and experimental

bias (Fang & Eglen, 2017). Additionally, this approach enables the simultaneous creation of a substantial number of spheroids, enhancing experimental reproducibility (Pinto et al., 2020). Some plates feature wells with distinct shapes (such as round bottom, V-shaped, or conical), facilitating the positioning of individual spheroids (Fang & Eglen, 2017; Vinci et al., 2012). Conversely, flat-bottom plates are coated with an inert substrate, such as agarose or polyhydroxyethylmethacrylate (poly-HEMA) (Pinto et al., 2020), or are available pre-coated and ready for use. One drawback associated with ultra-low attachment plates is that certain tumor cell lines may not efficiently form tightly compacted spheroids using this method (Ekert et al., 2014).

PROJECT PURPOSE

There are substantial differences in the prognosis and development of resistance between HCC and iCCA. Typically, iCCA exhibits poorer outcomes, often with a 5-year survival rate not surpassing 50% post-liver transplantation. In contrast, current therapies for HCC yield an approximately 30% cure rate, while liver transplantation offers a 60-70% 5-year survival rate (Y. Zhou et al., 2017; J. M. Llovet, 2005). However, these discrepancies become less apparent when comparing the proliferative subtype of iCCA, as delineated by Sia et al. (Sia et al., 2013), with an aggressive subgroup of HCC identified by the previously mentioned 5-gene 'neoangiogenic' transcriptomic signature (TS), as described by Villa et al. (Villa et al., 2016). This specific subgroup shares 95% of upregulated genes and poor prognosis with the proliferative iCCA subgroup. *ANGPT2*, an upregulated gene in the neoangiogenic TS, is a crucial factor in this context. The study hypothesized that these molecular and clinical resemblances might indicate potential shared patterns of response to proangiogenic stimulation. ANG-2 and its main regulator, VEGF, were known to be key players in promoting cancer progression also in a synergistic manner (Yoshiji, 2005). Although the roles of ANG-2 and VEGF in the clinical landscape of HCC and CCA are well-documented, their direct effects, whether individually or collectively, on liver malignant cells *in vitro* have not been thoroughly elucidated. Few studies have investigated their direct impact on cancer cells. For instance, ANG-2 has demonstrated the ability to enhance the growth of tumor cells in pituitary neuroendocrine tumors (Karabid et al., 2022) and promote the invasive capabilities of glioma cells (B. Hu et al., 2003). In HCC, VEGF is crucial for cell migration and proliferation (S. Peng et al., 2014; L. Zhang et al., 2012). Additionally, recent research by Rawal et al. (Rawal et al., 2022) and numerous other studies in the field (Blidisel et al., 2021; Pinto et al., 2020), have highlighted distinct behavior of liver cancer cells when cultured in 3D models compared to traditional 2D cultures, suggesting that 3D models may better replicate the actual conditions of cancer growth and its surrounding environment. Consequently, the study aimed to analyze the pro-invasive and pro-migratory effects of these pro-angiogenic factors on HCC and CCA spheroids. Expanding on prior findings regarding the synergistic effects of ANG-2 and VEGF, the study also investigated whether combining these factors would yield a more substantial impact than their individual usage, particularly under *in vitro* conditions.

EXPERIMENTAL PROCEDURES

1. Cell lines

The experiments described in this study were conducted in two immortalized HCC-derived cell lines, HepG2 (López-Terrada et al., 2009; Aden et al., 1979) and Hep3B (Aden et al., 1979), as well as two immortalized CCA-derived cell lines: HuCC-T1 derived from iCCA (Miyagiwa et al., 1989) and EGI-1 derived from eCCA (Scherdin U GM & Klouche M., 1987). HepG2 cells were grown in Eagle's MEM (Merck Life Science S.r.l., Milan, Italy) supplemented with 10% FBS, 1% non-essential amino acids solution, 1% glutamine, and 1% Penicillin-Streptomycin. Hep3B cells were grown as HepG2 with the addition of 1% sodium pyruvate (all purchased from Gibco, Thermo Fisher Scientific, Waltham, MA, USA). HuCC-T1 cells and EGI-1 cells were grown in RPMI 1640 (Merck Life Science S.r.l.) supplemented with 10% FBS, 1% glutamine, and 1% Penicillin-Streptomycin. All cell lines were maintained at 37°C in a 5% CO_2 environment. HepG2 cells were purchased from the European Collection of Authenticated Cell Cultures, Hep3B cells from the American Type Culture Collection, HuCC-T1 cells from the Japanese Collection of Research Bioresources Cell Bank, and EGI-1 cells from the DSMZ-German Collection of Microorganisms and Cell cultures GmbH.

2. Spheroids formation

During the initial stages of our project, we experimented with various techniques to induce spheroid formation. Our first attempt involved employing the "hanging drop" method, which proved intricate as it required seeding cells in a droplet on the lid of a Petri dish, promptly inverting the dish to close it. This technique relied on gravity to prompt cell aggregation into a single spheroid (Achilli et al., 2012). However, as concerns our experience, this method presented complexities in preparation and lacked consistency in generating uniformly sized spheroids. Droplet melting posed a risk, leading to the formation of larger spheroids compared to others. Consequently, we opted for a less complicated methodology that could facilitate the simultaneous generation of numerous spheroids, ensuring greater experimental reproducibility. Among the available techniques, we decided to employ the ultra-low attachment method. This method involves treating multiwell attachment plates by coating them with an inert substrate, such as poly-HEMA. Applying this coating prevents cells from adhering to the surface of the well, thereby compelling the cells to aggregate and form spheroids (Hoarau-Véchot et al., 2018; Kelm et al., 2003). Firstly, we prepared a 120mg/mL

stock solution of poly-HEMA by dissolving poly-HEMA powder (Merk Life Science S.r.l) into 95% ethanol. Subsequently, we made the 30mg/mL working solution of poly-HEMA, which underwent filtration before use in cell culture. Typically, we prepared 6 well-coated plates a day before the seeding process. In a controlled environment, under a tissue culture hood, we applied 400µL per well of poly-HEMA working solution to each well of 6 well plates, ensuring even distribution across the entire well surface. To allow the poly-HEMA to completely dry, we left the lids fully open overnight. The subsequent day, we seeded cells at varying densities based on the specific demands of the experimental assay being conducted. After the seeding, the plates underwent an incubation period of three days under standard conditions, set at 37°C with a 5% CO2 environment. During this incubation period, the spheroids achieved the intended size and shape, ensuring their appropriate formation. To generate spheroids from HepG2 and Hep3B cells, the same growth medium used for 2D cultures was employed, with the addition of 2% FBS. Conversely, for HuCC-T1 and EGI-1 spheroids, a serum-free DMEM/F12 medium (Gibco) supplemented with 1× B27 (Gibco), 10 ng/mL EGF (Merk Life Science S.r.l), 10 ng/mL bFGF (Life Technologies, Waltham, MA, USA), and 1% Penicillin-Streptomycin was utilized. At the conclusion of the formation period, spheroids from the different cell lines exhibited varying sizes: spheres derived from Hep3B and HuCC-T1 cells were smaller compared to those from HepG2 and EGI-1, as depicted in Figure 6 (A, B, C, D).

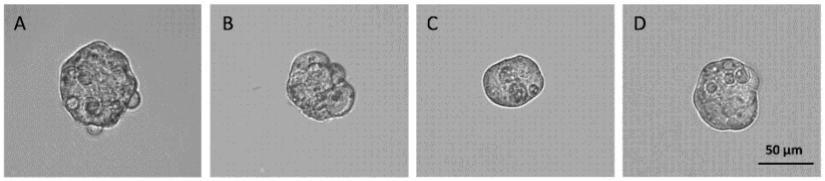

Figure 6 - Spheroids of HCC and CCA cell lines. Spheroids generated from HepG2 (A), Hep3B (B), HuCC-T1 (C), and EGI-1 (D) cell lines after formation. Images were taken at a 20× magnification simultaneously. Uniform cell numbers were seeded across all cell lines. Variations in spheroid sizes were noted, likely due to differences in cell aggregation mechanisms. HepG2 and EGI-1 spheroids appeared slightly larger, while those from Hep3B and HuCC-T1 were comparatively smaller.

3. 3D viability assay

For assessing the influence of recombinant human ANG-2 (rh-ANG-2) (623-AN, R&D Systems, Minneapolis, MN, USA) and recombinant human VEGF (rh-VEGF) (293-VE,

R&D Systems) on cell viability within 3D cultures, a 3D viability assay was conducted. Cells were seeded at a rate of two thousand HCC cells per well or one thousand five hundred CCA cells per well into polyHEMA-coated 96-well culture plates. Once spheroids had formed, they were immediately stimulated (at time 0) with increasing doses of rh-ANG-2 and/or rh-VEGF proteins (ranging from 0 to 800 ng/mL). After 48 hours from stimulation, a 3D viability assay was conducted. Spheroids were transferred to an uncoated 96-well white plate, followed by the addition of the CellTiter-Glo® 3D reagent (Promega, Milan, Italy) to elicit ATP release from the spheroids as per the manufacturer's instructions. The luminescence signal was measured after 30 minutes using the GloMax® bioluminescent reader (Promega).

4. Migration assay

To evaluate the impact of stimulation with rh-proangiogenic factors on the migration ability of our spheroids, a migration assay was conducted. The experiment involved seeding fifty-thousand cells per well into a polyHEMA-coated 6-well plate to generate spheroids. These spheroids were then transferred to an uncoated 6-well plate to allow attachment and stimulated with 200 ng/mL of rh-ANG-2 or rh-VEGF or a combination of 100 ng/mL of each protein, representing central doses within the tested viability assay range. Unstimulated spheroids were used as controls. In the migration assay, observations began 3 hours after stimulation to allow spheroid adhesion, and monitoring continued at 24 and 48 hours. The observation period ended at 48 hours, at which point all spheroids displayed a clearly visible migrated area while maintaining a reduced necrotic core. To affirm the potential involvement of proangiogenic factors in facilitating spheroid migration, inhibitors were introduced simultaneously with the stimulation of rh-ANG-2 or rh-VEGF in some experiments. Trebananib (Biogem, Ariano Irpino (AV), Italy) was utilized at concentrations of 7.5 ng/µL on HuCC-T1 spheroids and 14 ng/µL on HepG2 spheroids to inhibit the effect of rh-ANG-2, while Bevacizumab (Selleckchem, Houston, TX, USA) at 20 ng/mL was used on Hep3B spheroids to inhibit the effect of rh-VEGF. Experimental conditions were determined by calculating the IC70 for each cell line. Using a MICA microscope (Leica, Wetzlar, Germany) in widefield mode, images were captured to measure the spheroid area using the Fiji ImageJ tool, serving as an indicator of migration capability since our models did not distinctly differentiate the spheroid core from the migration area. Statistical analyses were conducted, and the measurements were represented in a bar graph.

5. Invasion assay

To investigate the potential impact of proangiogenic stimuli on invasiveness, we carried out an invasion assay. In the invasion assay, spheroids were formed following the same procedure as employed in the migration assay, utilizing the same cell count for spheroid formation. These generated spheroids were embedded in a 1:1 mixture of Matrigel 5 mg/mL (Corning, Arizona, US) and cell culture medium supplemented with rh-ANG-2 and/or rh-VEGF in similar amounts as mentioned earlier. Within each replicate, 4-5 representative spheroids were identified. Individual spheroids were tracked over time for quantitative size analysis. Images were captured immediately after stimulation (time 0, serving as the baseline), at 24 and 48 hours using a Leica DM camera. These images were processed using Fiji ImageJ, and invasiveness was quantified by measuring the area occupied by spheroids in Matrigel. Raw data were log2-transformed and presented as fold changes relative to baseline values due to our models not distinctly differentiating between the spheroid core and the invasive area.

6. Gene expression analysis

To investigate the impact of rh-ANG-2 and/or rh-VEGF stimulation on the expression of EMT markers, we performed gene expression analysis. Four hundred thousand cells/well were seeded into a 6-well plate coated with polyHEMA to obtain spheroids for gene expression analysis of E-cadherin (*CDH1*), N-cadherin (*CDH2*) and Vimentin (*VIM*) markers. Spheroids were transferred into a 6-well plate without coating and stimulated as for the migration assay. After 3 and 48 hours from stimulation (representing time points after adhesion and extensive migration of cells, respectively), total RNA was extracted from the spheroids. Following sample collection and PBS washing, RNA extraction was carried out by adding Nucleozol (Mechery-Nagel GmbH & Co. KG, Dueren, Germany). Subsequently, purification of the extracted RNA was performed using NucleoSpin columns (Mechery-Nagel GmbH & Co. KG). The purification steps were executed following the manufacturer's protocol. The quantification of RNA was performed using a Quantus Fluorometer (Promega), adhering to the manufacturer's protocol. Subsequently, cDNA synthesis was conducted utilizing the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad), which facilitated the removal of genomic DNA and the subsequent cDNA synthesis using the C-1000 Touch Thermal cycler (Bio-Rad). For gene expression analysis, digital droplet PCR (ddPCR) was employed. This multiplex PCR technique based on water-oil emulsion droplet technology segregated the samples into numerous nano-liter-sized droplets, where PCR was individually

conducted within each droplet. The ddPCR mix was prepared using ddPCR master mix (Bio-Rad, Segrate (MI), Italy), primers for each target (Thermo Fisher), and sample nucleic acid solution. The Automated Droplet Generator (Bio-Rad) facilitated the preparation of droplets, which were then transferred to 96-well PCR plates for nucleic acid amplification using the C-1000 Touch Thermal cycler (Bio-Rad). Following amplification, the droplets in each well were automatically read with the QX200 Droplet Reader (Bio-Rad). All procedures strictly followed the manufacturer's instructions. Copies/µL of each gene target were normalized against the copies of the actin gene, used as a reference. The final data were statistically analyzed and plotted on graphs to represent the findings after the completion of the analyses.

7. Western blot analysis

In addition to the gene expression analysis, the protein expression of the previously mentioned EMT markers was assessed. For this purpose, two hundred thousand cells per well were seeded into a polyHEMA-coated 6-well plate to generate spheroids, ensuring sufficient quantities of proteins upon lysis. Subsequently, these newly formed spheroids were transferred to uncoated 6-well plates and stimulated as previously described. At 3 and 48 hours post-stimulation (matching the time points used in the gene expression analysis), spheroids were harvested, lysed, and utilized for western blot analysis to detect the expression levels of E-cadherin, N-cadherin, and Vimentin.

To examine the impact of Trebananib and Bevacizumab on cell inhibition, HepG2 and HuCC-T1 spheroid cultures were treated with rh-ANG-2 and Trebananib, while Hep3B spheroids were exposed to rh-VEGF and Bevacizumab. Samples were collected at 3, 24, and 48-hour intervals following stimulation, corresponding to the time points allocated for assessing cell migration. The lysates extracted from these samples were employed to assess the expression levels of ANG-2 in HepG2 and HuCC-T1, and VEGFA in Hep3B.

Additionally, to investigate the activation of specific receptors by ANG-2 and VEGFA, spheroid cultures were stimulated following the aforementioned protocol. Samples were collected at 15, 30, and 60-min intervals post-stimulation. Lysates obtained from these samples were then subjected to western blot analysis. This enabled the measurement of ANG-2, TIE2, phospho-TIE2 (P-TIE2), VEGFA, VEGFR1, and phospho-VEGFR1 (P-VEGFR1) levels, facilitating the assessment of receptor activation dynamics.

The detailed protocol for the western blot analysis was as follows. Spheroids were lysed in a cold radioimmunoprecipitation assay buffer (RIPA) buffer supplemented with a cocktail of

protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany). Specifically, for the early time points where spheroids exhibited non-adherence to the plate, we followed two distinct protocols for the lysis process. The suspended portion (spheroids not attached) was collected into 1.5 mL Eppendorf tubes. Following a wash with cold PBS, we introduced the previously prepared lysis solution. These samples were then incubated on ice with periodic vortexing. Conversely, the attached spheroids underwent a different lysis procedure. After a cold PBS wash and the addition of lysis solution, these spheroids were detached from the plate using a scraper. Post-lysis, the samples were transferred to 1.5 mL Eppendorf tubes and incubated on ice for 30 minutes, undergoing frequent vortexing. Subsequently, all samples, both attached and suspended, were centrifuged for 20 minutes at 12,800 × g. They were combined into a single tube before conducting the assessment of protein concentration. Protein concentrations in the lysates were quantified using the bicinchoninic acid (BCA) method with the Quantum micro protein kit (Euroclone Spa, Milan, Italy). A standard curve for absorbance values was created using bovine serum albumin (BSA). 40 µg of protein lysates were loaded and run on 8-10% polyacrylamide gels based on the molecular weight of each specific protein. Subsequently, proteins were electrotransferred to nitrocellulose membranes (Bio-Rad). To block nonspecific binding sites, membranes were incubated with 5% BSA in Tween-20 0.05% in TBS (TBS-T) for 1 hour at room temperature (RT). The membranes were then probed with primary antibodies overnight at +4°C. The primary antibodies used included E-cadherin (BD Biosciences), N-cadherin (Thermo Fisher Scientific), Vimentin (BD Biosciences), ANG-2 (R&D Systems), VEGFA (Cell Signaling Technology), TIE2 (Santa Cruz Biotechnology), P-TIE2 (R&D Systems), VEGFR1 (Santa Cruz), P-VEGFR1 (R&D Systems), and β-actin (R&D Systems). After primary antibody incubation, membranes were exposed to specific secondary antibodies (R&D Systems) for 1 hour at RT. Protein bands were visualized using an enhanced chemiluminescent substrate (Cyanagen srl, Bologna, Italy) and the Chemidoc MP imaging system (Bio-Rad). Quantification of protein bands was performed using ImageLab software (Bio-Rad), normalized to β-actin content, and analyzed via densitometry. For the evaluation of phosphorylated proteins, membranes probed with antibodies against TIE2 or VEGFR1 were stripped and reprobed with antibodies targeting P-TIE2 or P-VEGFR1. The stripping protocol was based on the guidelines provided by BioRad on its website.

8. Immunofluorescence analysis

We conducted an immunofluorescence assay for the visualization of protein localization and signal intensities of E-cadherin and N-cadherin within spheroids following the stimulation.
Fifty thousand cells per well were seeded into a polyHEMA-coated 6-well plate to generate spheroids. The obtained spheroids were collected and transferred into 6-well plates containing coverslips at the bottom. These spheroids were then stimulated according to the previously described procedure. At 3 and 48 hours post-stimulation, the cells were fixed using 4% formaldehyde (Carlo Erba Reagents) for immunofluorescence assessment of E-cadherin and N-cadherin markers. Spheroids fixed in formaldehyde were permeabilized with 0.1% Triton X-100 (Merk Life Science S.r.l) in PBS for 5 min. Subsequently, spheroids were blocked with Image IT Fix (Invitrogen) for 30 min at RT. Following blocking, spheroids were incubated with specific primary antibodies targeting E-cadherin (BD Biosciences) and N-cadherin (Thermo Fisher Scientific). After three washes in 1% BSA in PBS, spheroids were incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 and Alexa Fluor 546, Life Technologies) for 1 hour at RT in the dark. Following incubation, spheroids underwent three washes in PBS and were stained for nuclei using DAPI (Thermo Fisher Scientific). Slides were mounted using Fluoromount-G (Invitrogen) on cover glasses. Visualization of the stained spheroids was performed using a Leica MICA microscope in confocal mode.

9. Statistical Analysis

The results of the experiments were presented as means along with the standard error of the mean (SEM). Each experiment was conducted a minimum of three times to ensure reliability. To determine the significance of differences between groups, statistical analyses were performed using either One-way or Two-way ANOVA (depending on the experimental design) followed by suitable post-hoc tests for multiple comparisons. The statistical analysis was conducted using GraphPad software, specifically Prism 9 from San Diego, CA, USA. Differences were considered statistically significant if the p value < 0.05.

RESULTS

1. Proangiogenic stimulation did not affect the viability of HCC and CCA spheroids.

The viability assay performed within a 3D culture setting aimed to evaluate the potential impact of rh-ANG-2 and rh-VEGF on cellular viability as a preliminary step before their application in cell stimulation experiments. Upon spheroids formation, they were promptly exposed to escalating doses of rh-ANG-2 and/or rh-VEGF proteins (from 0 to 800 ng/mL). After 48 hours, according to the manufacturer protocol, the spheroids were moved to an uncoated 96-well white plate. Then, the CellTiter-Glo® 3D reagent was introduced to prompt ATP release from the spheroids. Evaluation of ATP release occurred after 30 minutes by measuring the luminescent signal. The findings, as depicted in Figure 7 (A, B, C, D), revealed that despite variations in stimulation conditions, there were no significant differences observed in luminescence levels. These results strongly suggest that the presence of rh-ANG-2 and rh-VEGF did not exert a noticeable effect on cellular viability under the specific experimental conditions tested.

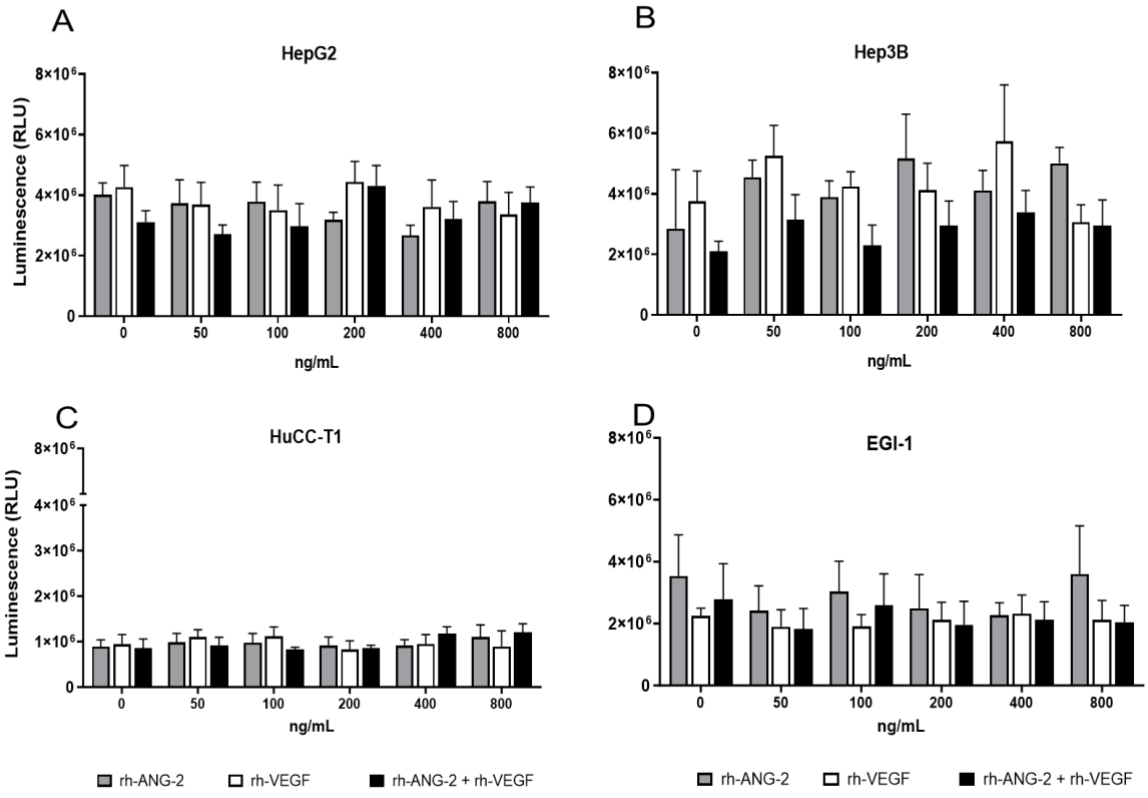

Figure 7 - Evaluation of viability in 3D culture following proangiogenic factors stimulation at increasing doses. Viability assessment was conducted on HepG2 (A), Hep3B (B), HuCC-T1 (C), and EGI-1 (D) cell lines following exposure to varying concentrations (0, 50, 100, 200, 400, and 800 ng/mL) of rh-ANG-2 and rh-VEGF, administered individually or in combination, within a 3D cell culture environment for a duration of 48 hours. Quantification of ATP levels, serving as an indicator of cellular metabolic activity, was represented as luminescence signals (RLU) in histogram plots. The luminescence levels were relatively consistent across HepG2 (3.6×10^6), Hep3B (3.8×10^6), and EGI-1 (2.4×10^6 luminescence units). Notably, HuCC-T1 exhibited comparatively lower luminescence (9.6×10^5 units). These experiments were conducted in triplicate to ensure data reproducibility. Statistical analysis using One-way ANOVA was employed to discern potential differences among groups. No statistically significant differences were observed among the various comparisons, indicating that the tested concentrations of rh-ANG-2 and rh-VEGF, whether administered alone or in combination, did not exert a significant influence on cellular viability within the confines of this specific experimental setup.

2. rh-ANG-2 and rh-VEGF stimulation increased migration capability in HepG2, Hep3B, and HuCC-T1.

After verifying the nontoxicity of stimulation, we investigated the specific effects of rh-ANG-2 and rh-VEGF on the migration patterns of HCC and CCA spheroids, leveraging the well-documented roles of these factors in promoting cellular migration (L. Zhang et al., 2012; X. S. Liu et al., 2009). In this and all subsequent experiments detailed in this study, we employed the midrange concentrations previously assessed.

After spheroids formation, they were transferred into 6 well-plates without coating and stimulated with 200 ng/mL of rh-ANG-2 or 200ng/mL of rh-VEGF or a combination of 100 ng/mL of each protein. We started observing the migration 3 hours after stimulation to let the spheroids adhesion. The observations went on until 48 hours, by which time all spheroids showed a clear migrated area and a smaller dead core. Importantly, at this point, they were still viable.

The evaluation involved comparing the migration capabilities of treated spheroids to untreated controls and examining the temporal changes in migration post-treatment.

In HepG2 spheroids, stimulation with rh-ANG-2 and rh-VEGF initiated observable bubble formation along the spheroid perimeters as early as 3 hours post-stimulation. By the 24-hour mark, all spheroids exhibited similar growth patterns. However, at 48 hours, spheroids treated with rh-ANG-2 and rh-VEGF displayed a significantly larger area occupied by migratory

cells compared to the 3-hour measurement. Notably, only rh-ANG-2 treatment led to a substantial increase in migration compared to untreated controls (Figure 8 A and B).

Hep3B spheroids demonstrated increased adherence to the plate at 3 hours post-stimulation compared to HepG2. Treated spheroids exhibited a partially visible migration zone around their perimeters, which notably enlarged at 24 and 48 hours post-stimulation, especially in groups treated with rh-VEGF or a combination of rh-ANG-2 and rh-VEGF. This increase in migration was significantly higher in these groups compared to the untreated controls (Figure 8 C, D). Control spheroids maintained their original morphology, and the migration zone remained indistinct.

In HuCC-T1-derived spheroids, a migration zone became evident at 24 hours post-stimulation, reaching complete cell migration after 48 hours. Notably, the migration zone in all spheroids expanded significantly over time under all tested conditions, except for those treated with both rh-ANG-2 and rh-VEGF. However, only the spheroids treated with rh-ANG-2 alone exhibited a significantly larger migration zone than the control after 48 hours (Figure 8 E, F).

Distinct from HuCC-T1, EGI-1 spheroids were slightly larger and better defined in shape. Initially, at 3 hours, only a few spheroids adhered, and no sprouting cells were observed under any conditions. By 24 hours, and more prominently at 48 hours, the cells dissseminated, forming a discernible migration zone. The spheroids' area increased significantly over time under all conditions (Figure 8 G, H). However, there were no noticeable differences in migration between the control and treated spheroids.

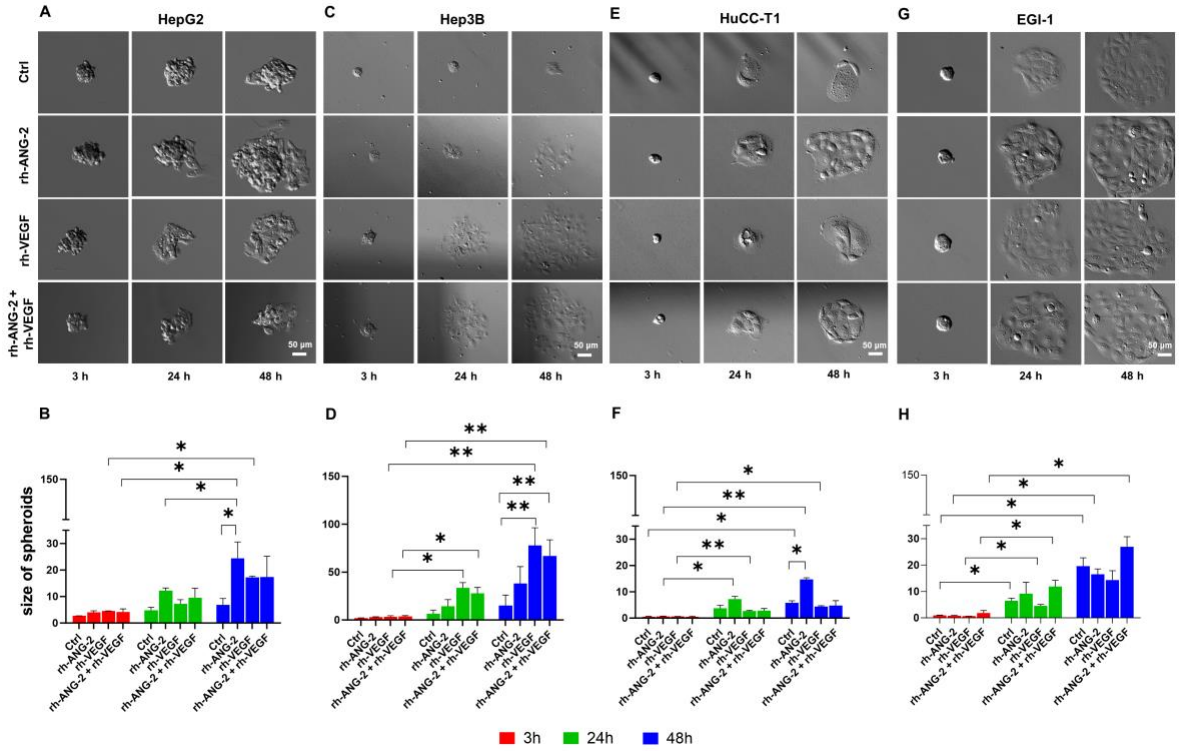

Figure 8 – Evaluation of HCC and CCA spheroids migration. Migration assays performed on spheroids derived from HepG2 (A, B), Hep3B (C, D), HuCC-T1 (E, F), and EGI-1 (G, H) cell lines subjected to various treatments: untreated (Ctrl), 200 ng/mL of rh-ANG-2, 200 ng/mL of rh-VEGF, or a combination of 100 ng/mL each of rh-ANG-2 and rh-VEGF. Images were captured at 10× magnification at 3, 24, and 48 hours post-treatment. Note that due to size reduction and cropping, images of EGI-1 spheroids may appear larger than the frame. Quantification of spheroid areas using ImageJ to assess migration capability is presented for HepG2 (B), Hep3B (D), HuCC-T1 (F), and EGI-1 (H). Comparative analyses were conducted to evaluate differences between control and treated spheroids at respective time points and among different spheroid areas at 24 and 48 hours in contrast to changes observed at 3 hours within the same treatment group. Experiments were replicated a minimum of three times for reliability. Statistical significance was determined using Two-way ANOVA for paired data followed by post-hoc tests for multiple comparisons (* $p<0.05$, ** $p<0.01$).

3. Trebananib and Bevacizumab inhibited the rh-ANG-2 and rh-VEGF-stimulated migration in HepG2, HuCC-T1, and Hep3B spheroids.

To further validate the involvement of proangiogenic factors in facilitating spheroid migration, we employed specific inhibitors, Trebananib and Bevacizumab, to block the effects of rh-ANG-2 and rh-VEGF stimuli, respectively. Migration assays, following the previously described protocol, were conducted on HepG2 and HuCC-T1 spheroids stimulated with rh-ANG-2, and Hep3B spheroids stimulated with rh-VEGF, in the presence or absence

of these inhibitors. Observations were made at 3, 24, and 48 hours post-stimulation (Figure 9 A-F).

Concurrently, we quantified the levels of ANG-2 and VEGFA proteins in cell lysates to validate the inhibition within the cells (Figure 9 G-I). Notably, at 24 hours for HuCC-T1 (Figure 9 C and D) and at 48 hours for both HepG2 (Figure 9 A and B) and HuCC-T1 (Figure 9 C and D), Trebananib significantly diminished the migration enhancement induced by rh-ANG-2. This inhibition resulted in migration areas notably smaller than those stimulated solely with rh-ANG-2. Western blot analysis indicated a substantial reduction in ANG-2 protein levels in spheroids treated with both rh-ANG-2 and Trebananib, observed as early as 3 hours in HepG2 and HuCC-T1, and additionally at 24 and 48 hours specifically in HepG2.

In Hep3B spheroids, Bevacizumab effectively attenuated rh-VEGF-dependent migration as early as 24 hours post-stimulation, with a further decrease observed at 48 hours (Figure 9 E, F). Additionally, VEGFA protein levels in Hep3B spheroids treated with a combination of rh-VEGF and Bevacizumab showed a significant reduction at 3 hours and sustained lower levels at 24 hours post-treatment (Figure 9 I).

4. rh-ANG-2 and rh-VEGF enhanced the invasion capabilities of HuCC-T1 and Hep3B spheroids.

Following the migration assay results, an invasion assay in Matrigel was conducted to assess the impact of proangiogenic stimuli on invasiveness. According to the protocol, once the spheroids were formed, they were embedded into a blend of Matrigel and culture medium containing proangiogenic factors. We tracked and snapped pictures of each spheroid at different time points: right after stimulation (time 0 that represented the baseline), then at 24 and 48 hours later.

In HepG2 spheroids, a notable increase in invasive area was observed at 24 hours post-stimulation with either rh-ANG-2 or rh-VEGF compared to the baseline. However, after 48 hours, only spheroids treated with rh-ANG-2 displayed a continued significant expansion (Figure 10 A, B).

Similarly, Hep3B spheroids displayed an increased invasive area at 24 hours post-stimulation with rh-VEGF alone or in combination with rh-ANG-2. Nevertheless, at 48 hours, significant enlargement was evident only in spheroids subjected to the individual treatments of rh-ANG-2 or rh-VEGF (Figure 10 C, D).

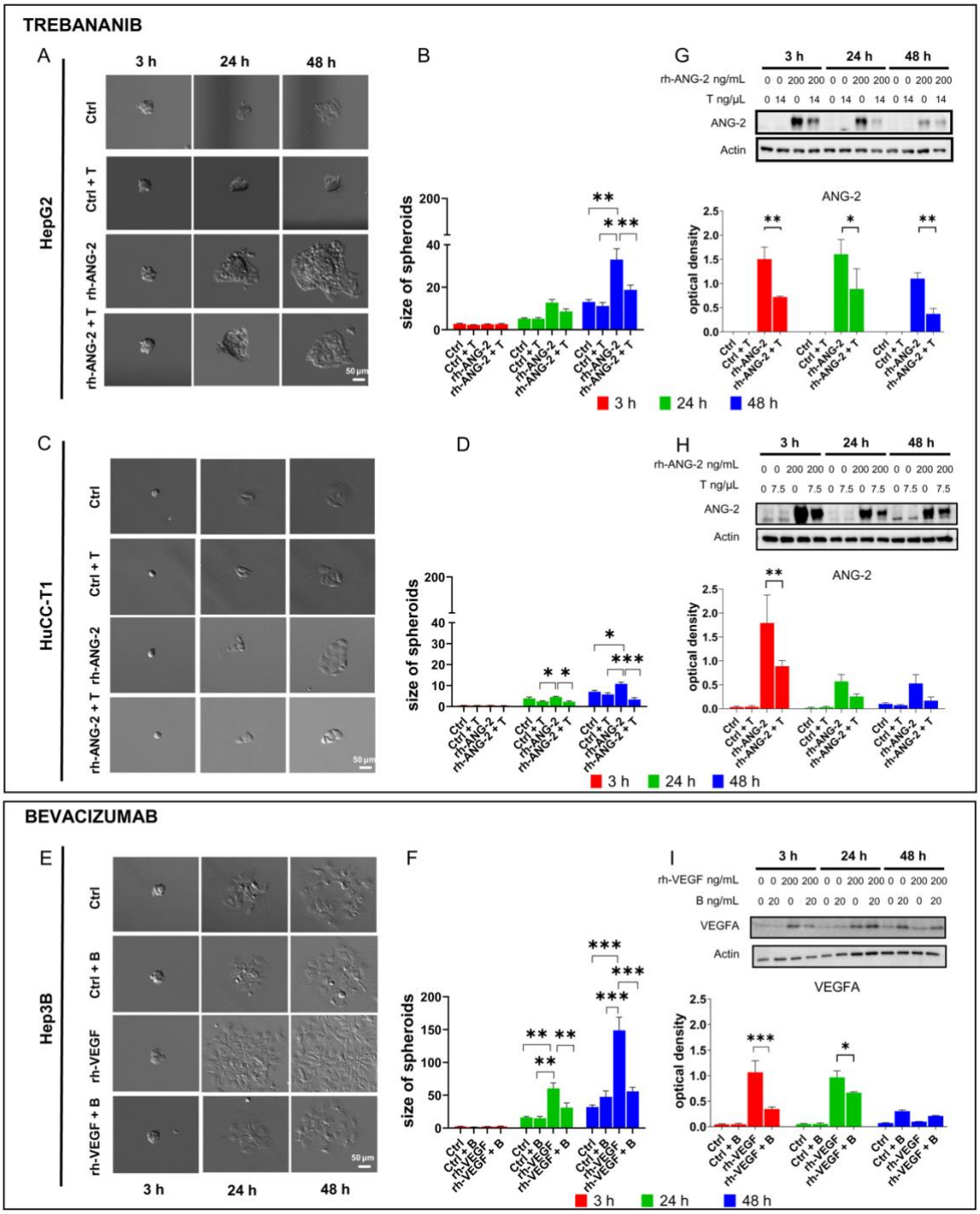

Figure 9 - Evaluation of HCC and CCA spheroids migration and ANG-2/VEGF expression with Trebananib/Bevacizumab inhibition. The effects of proangiogenic factors and their inhibition on HepG2 (A, B, G), HuCC-T1 (C, D, H), and Hep3B (E, F, I) spheroids. For HepG2 (A) and HuCC-T1 (C) spheroids, treatments included no stimulation with proangiogenic factors (Ctrl), Ctrl with Trebananib (T), or stimulation with 200 ng/mL of rh-ANG-2, alone or in combination with Trebananib. Similarly, Hep3B spheroids (E) were subjected to no stimulation with proangiogenic factors (Ctrl), Ctrl with Bevacizumab (B), or stimulation with 200 ng/mL of rh-VEGF, alone or combined with Bevacizumab. Quantification of the spheroid area using ImageJ served as an indicator

of migration capability (HepG2 [B], HuCC-T1 [D], Hep3B [F]). Observations were made at 3, 24, and 48 hours post-treatment, and images were captured at 10× magnification. Due to image reduction, Hep3B spheroids treated solely with rh-VEGF at 48 hours may exceed image boundaries. Protein expression levels in treated spheroids were assessed for rh-ANG-2 alone or with Trebananib in (G) HepG2 and (H) HuCC-T1, and for rh-VEGF alone or with Bevacizumab in (I) Hep3B, at 3, 24, and 48 hours post-stimulation. Protein bands quantified using ImageLab software, normalized to β-actin, facilitated comparative analyses. Statistical analyses (Two-way ANOVA with post-hoc tests) highlighted significance levels (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). Experiments were replicated at least three times for reliability and consistency.

In HuCC-T1 spheroids, a significant increase in area was observed 24 hours after stimulation with rh-ANG-2. At 48 hours, spheroids exposed to rh-ANG-2, either alone or combined with rh-VEGF, exhibited significantly larger areas compared to the baseline (Figure 10 E, F).
Contrarily, in EGI-1, both control and stimulated spheroids displayed a significant increase in area at 24 hours, persisting at 48 hours, regardless of the stimulation condition (Figure 10 G, H).
Notably, among the examined cell lines, only Hep3B and HuCC-T1 demonstrated notable effects on invasive capability when comparing treated spheroids with controls, each responding to different proteins. Specifically, rh-VEGF enhanced invasiveness in Hep3B spheroids at 48 hours post-stimulation (Figure 10 C, D), while in HuCC-T1, this effect was attributed to rh-ANG-2 (Figure 10 E, F). These findings align with the migration assay results, corroborating the role of rh-ANG-2 in HuCC-T1 and rh-VEGF in Hep3B as facilitators of cellular migration and invasion.

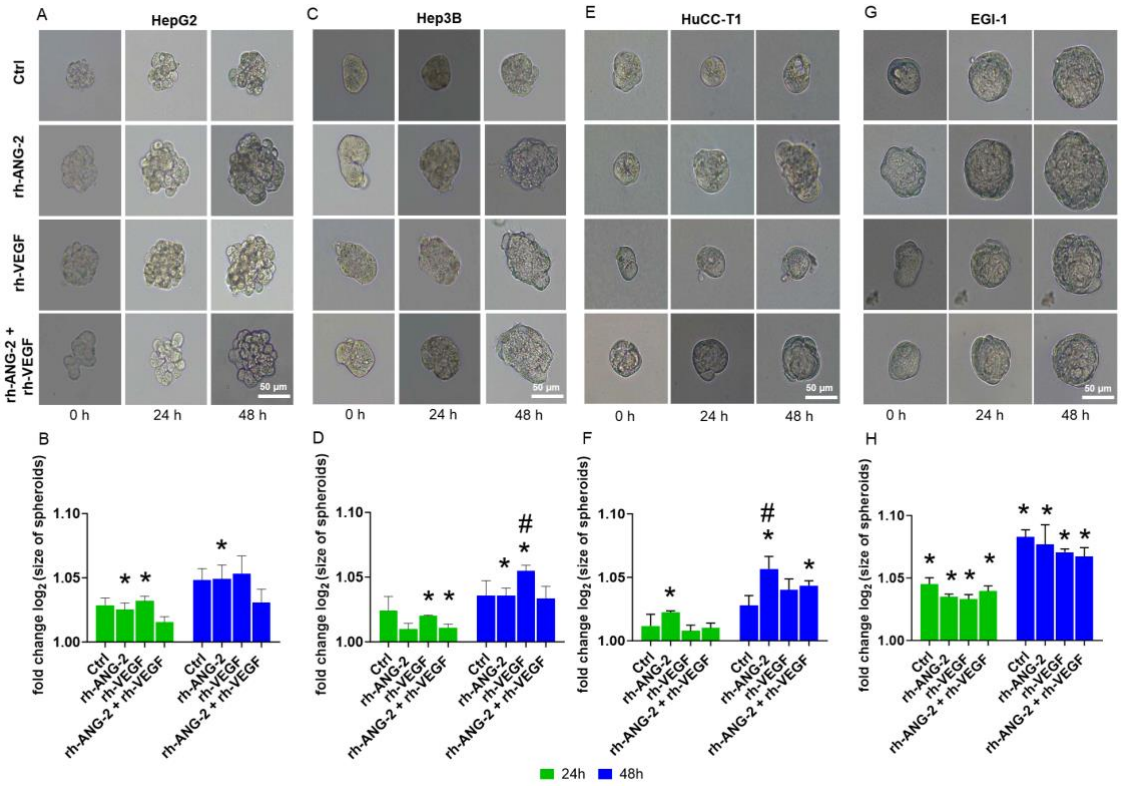

Figure 10 - Assessment of HCC and CCA spheroids invasiveness in Matrigel in response to proangiogenic stimulation. Spheroids derived from HepG2 (A, B), Hep3B (C, D), HuCC-T1 (E, F), and EGI-1 (G, H) were subjected to treatments with 200 ng/mL of rh-ANG-2 or rh-VEGF independently, and a combination of 100 ng/mL each of rh-ANG-2 and rh-VEGF. Imaging was performed at 0, 24, and 48 hours post-stimulation to monitor the invasion process. The extent of invasiveness was quantified using ImageJ software by measuring the area covered by the spheroids after embedding in Matrigel. Comparative analyses were conducted between control (Ctrl) spheroids and treated groups at corresponding time points. Additionally, within each treatment group, comparisons were made between the spheroids' areas at 24 and 48 hours and their initial size at time 0. To ensure data robustness, experiments were executed in triplicate. Results are presented as the ratio of treated spheroids' area to the baseline, with log2 transformation applied to raw data for normalization. The baseline value was standardized at Y=1.0. Statistical assessments involved Two-way ANOVA for paired data, accompanied by post-hoc tests for multiple comparisons. Significance is indicated by asterisks (*) for $p<0.05$ compared to the baseline within each treatment group and by hash (#) for $p<0.05$ compared to the control at the corresponding time points.

5. Proangiogenic factors activated their respective receptors solely within the HCC models.

In our migration assays, with and without inhibitors, the pivotal role of rh-ANG-2 and rh-VEGF in augmenting the migratory potential of HepG2 and HuCC-T1 spheroids, and Hep3B spheroids, respectively, was evident. In addition, the involvement of rh-ANG-2 and rh-VEGF as enhancers of invasive capabilities in HUCC-T1 and Hep3B, respectively, has also been demonstrated. Consequently, we proceeded to investigate the expression and activation of their respective receptors, TIE2 and VEGFR1, along with their phosphorylated forms (P-TIE2 and P-VEGFR1). We extracted protein samples from HepG2, HuCC-T1, and Hep3B spheroids that were placed in uncoated 6-well plates. These samples were collected at 15, 30, and 60-minute intervals of stimulation, as illustrated in Figure 11. We selected these particular time points because they have the potential to capture and reveal any immediate activation of the receptors.

In HepG2 spheroids, TIE2 expression remained relatively stable across treatments at 15 minutes, with a slight increase noted at 30 and 60 minutes, particularly noticeable in spheroids treated with rh-ANG-2 compared to the control at the 30-minute mark. Notably, the activation of TIE2, indicated by its phosphorylation, increased in rh-ANG-2-treated spheroids from 15 minutes onwards. This heightened activation was confirmed by evaluating the ratio of phosphorylated TIE2 to total TIE2 (Figure 11 A and B).

In HuCC-T1 spheroids, TIE2 expression showed no significant change at 15 and 30 minutes but demonstrated a substantial increase at 60 minutes upon stimulation with rh-ANG-2. Despite steady TIE2 levels, phosphorylation levels (indicating receptor activation) showed no marked alterations at 15 and 30 minutes. However, they significantly decreased at 60 minutes post-stimulation with rh-VEGF, both independently and combined with rh-ANG-2, compared to controls. This reduction was even more pronounced when both pro-angiogenic factors were utilized simultaneously, as evident in the P-TIE2 to total TIE2 ratio. Interestingly, a significant decrease in P-TIE2 to total TIE2 ratio at 60 minutes was observed with rh-ANG2 stimulation, attributed to increased receptor expression without a corresponding rise in phosphorylation status (Figure 11 C and D).

In Hep3B spheroids, VEGFR1 expression remained consistently unchanged across various conditions and time points. VEGFR1 activation, indicated by phosphorylation, was evident only at 60 minutes in spheroids treated with rh-VEGF, compared to untreated controls. This observation was further supported by the significant ratio of P-VEGFR1 to total VEGFR1 (Figure 11 E and F). It's important to note that we also performed Western blot analysis of

VEGFR2 expression in Hep3B spheroids. However, it produced inconclusive outcomes, marked by non-specific signals that were unquantifiable. Therefore, this data has not been presented.

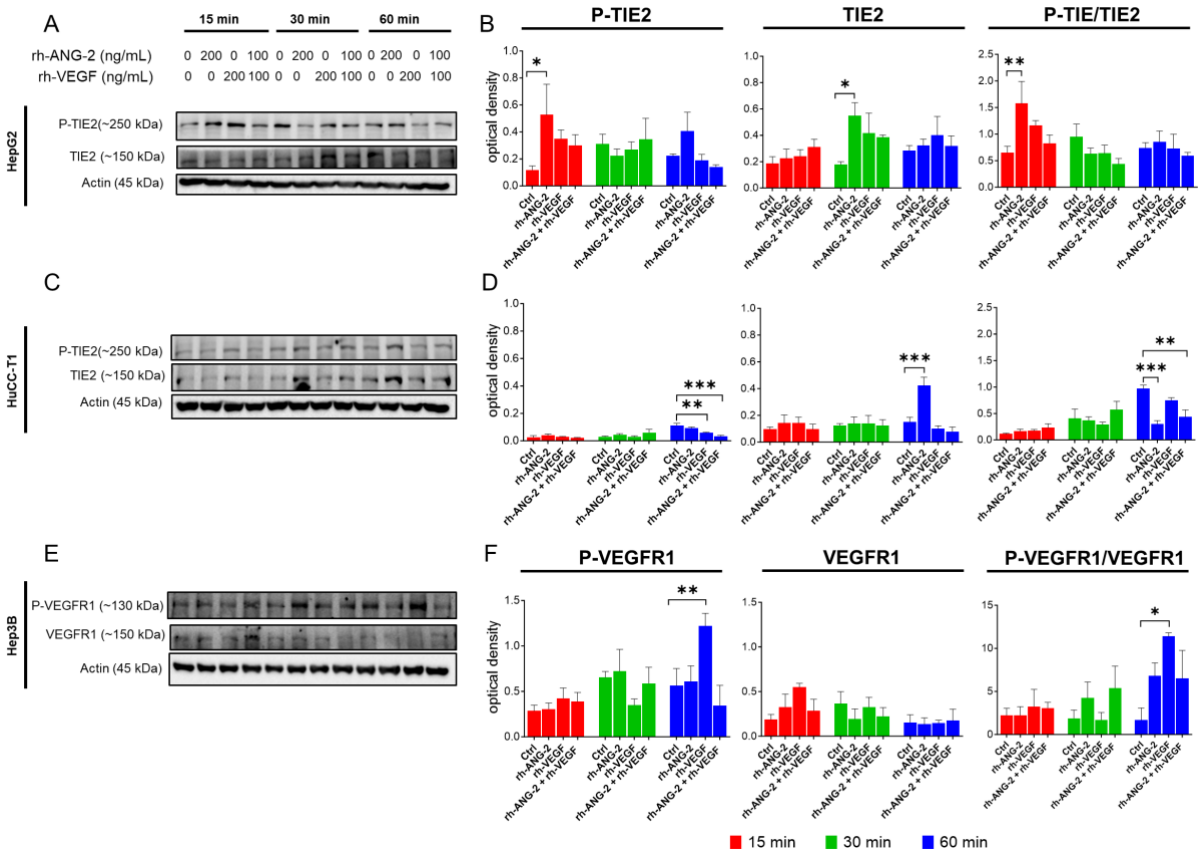

Figure 11 - Western Blot analysis of TIE2 and VEGFR1 receptor expression and activation by phosphorylation. We assessed TIE2 and P-TIE2 expression in HepG2 and HuCC-T1 spheroids (A-D), and VEGFR1 and P-VEGFR1 expression in Hep3B spheroids (E, F). Treatments consisted of 200 ng/mL of rh-ANG-2, 200 ng/mL of rh-VEGF, and a combination of 100 ng/mL each of rh-ANG-2 and rh-VEGF. Protein samples were collected at 15, 30, and 60 minutes post-stimulation. Band intensities were quantified using ImageLab software, and densitometry data were presented as optical density values normalized to β-actin. Comparative analyses were conducted between treated spheroids and controls at corresponding time points. This experiment was replicated a minimum of three times. Statistical differences were determined using Two-way ANOVA, followed by post-hoc tests for multiple comparisons. Thresholds for significance were established as follows: * $p<0.05$; ** $p<0.001$; *** $p<0.001$.

6. Evaluation of ANG-2 and VEGFA protein expressions.

Concurrently with the receptor analysis, our investigation encompassed the assessment of their respective ligands in protein samples. ANG-2 and VEGFA expressions were detected in HepG2, HuCC-T1, and Hep3B spheroids, respectively, at 15, 30, and 60-minute intervals post-stimulation, as illustrated in Figure 12. Notably, the ANG-2 analysis revealed the presence of two distinct bands in samples stimulated with rh-ANG-2. The band exhibiting a higher molecular weight likely corresponds to the added, highly glycosylated recombinant protein. Conversely, control and rh-VEGF-stimulated samples displayed a single band, potentially indicating the presence of endogenously produced protein (Figure 12 A-D).

In both HepG2 and HuCC-T1 cell lines, ANG-2 detection was notably higher in treated cells than in untreated cells following rh-ANG-2 stimulation. Quantitatively, HepG2 spheroids exhibited significantly elevated rh-ANG-2 levels in the rh-ANG-2 treated groups compared to untreated groups at 30 and 60 minutes (Figure 12 A, B). A similar trend was observed in HuCC-T1 spheroids at 15, 30, and 60 minutes post-stimulation (Figure 12 C, D). In contrast, the expression of ANG-2 did not change significantly between different treatments over time in HepG2. However, in the HuCC-T1 cell line, there was a slight decrease in ANG-2 expression after 60 minutes from the stimulation by rh-ANG-2 and rh-VEGF compared to the control group.

The analysis of VEGFA expression revealed three bands in the presence of stimulation with rh-VEGF. Among these bands, the two higher molecular weight bands can be attributed to the added recombinant protein. Conversely, there was only one band observed in the control and rh-ANG-2-stimulated samples, which indicates the presence of endogenously produced VEGFA. Regarding Hep3B spheroids, when treated with rh-VEGF (either alone or in combination with rh-ANG-2), there was a significant increase observed in the expression levels of rh-VEGF over time. This increase was notably higher compared to the untreated group, or the group treated solely with rh-ANG-2 (Figure 12 E, F). On the other hand, the expression of VEGFA remained consistent between treatments over time.

7. rh-ANG-2 and rh-VEGF modulated differentially the EMT genes within HCC and CCA spheroids.

Due to the pro-migratory and invasive influence induced by stimulating our cell models with proangiogenic factors, we investigated whether rh-ANG-2 and/or rh-VEGF stimulation could lead to alterations in the expression of EMT markers at the gene and protein levels. Firstly,

gene analyses were conducted for E-cadherin (*CDH1*), N-cadherin (*CDH2*), and Vimentin (*VIM*) in RNA samples obtained from spheroids. We chose this set of EMT markers because they are the most involved in the migration and invasion phenomena of HCC and CCA (Saentaweesuk et al., 2018; L. Hu et al., 2004; S. Lee et al., 2002; Matsumura et al., 2001). Droplet PCR was employed for transcriptomic evaluations due to its capability to detect and analyze even very small quantities of genetic material within the sample.

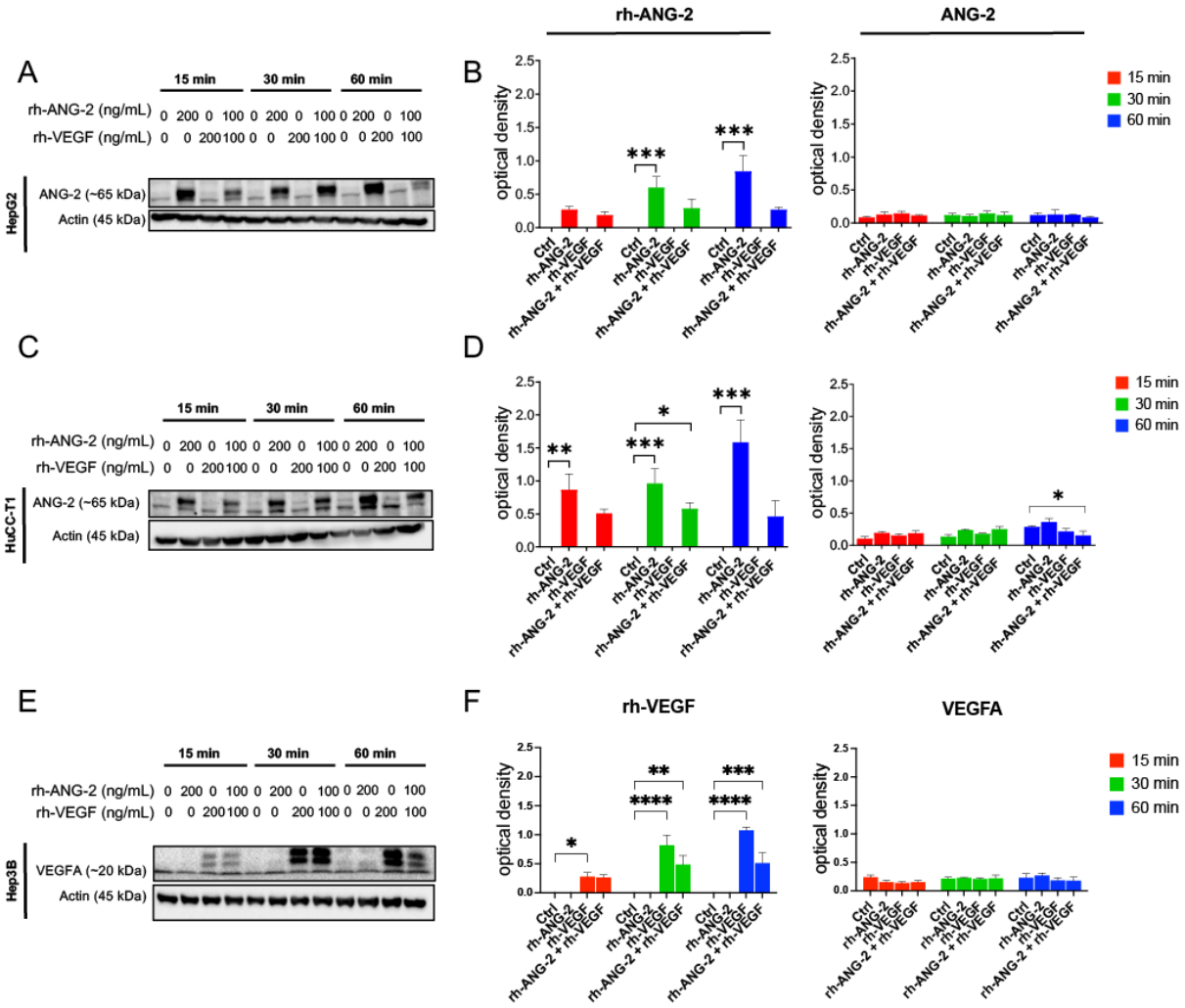

Figure 12 - Western Blot analysis of ANG-2 and VEGFA. We evaluated ANG-2 expression in HepG2 and HuCC-T1 spheroids (A-D), and the expression of VEGFA in Hep3B spheroids (E, F). Treatments included 200 ng/mL of rh-ANG-2, 200 ng/mL of rh-VEGF, and a combination of 100 ng/mL each of rh-ANG-2 and rh-VEGF. Protein samples were harvested at 15, 30, and 60 minutes post-stimulation. Band intensities were quantified using ImageLab software, and densitometry data were presented as optical density values normalized to β-actin. Comparative analyses were performed

between the treated spheroids and controls at identical time points. This experiment was replicated a minimum of three times. Statistical differences were assessed using Two-way ANOVA, followed by subsequent post-hoc tests for multiple comparisons. Significance thresholds were established as follows: *p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.

RNA samples were obtained from spheroids adhered and subjected to stimulation for durations of 3 and 48 hours. Following collection and reverse transcription, droplets were formed and analyzed using droplet PCR, strictly following the workflow outlined in the manufacturer's protocol. Transcriptomic assessments unveiled distinct expression profiles of EMT markers across all four cell lines. We observed an augmented expression of *CDH2* in HepG2 cells upon stimulation with rh-ANG-2. Notably, in samples treated with both rh-ANG-2 and rh-VEGF after 48 hours, an increase in *VIM* expression was detected. Furthermore, independent increases in *VIM* expression were noted in Hep3B cells under rh-ANG-2 and rh-VEGF stimulation, as well as in HuCC-T1 cells irrespective of any treatment. Conversely, a decline in *CDH1* expression was exclusively noted in Hep3B cells, although these changes were unrelated to treatment (Figure 13). It's important to highlight the sporadic and variable nature of treatment-related alterations observed across different conditions and cell lines. Notably, EGI-1 cells exhibited consistently low expression levels of *CDH2* and *VIM* genes.

8. Proangiogenic stimulation had different impacts on EMT protein expression in HCC and CCA models.

Following the gene expression analysis, protein expression analysis was performed to validate the obtained gene expression profiles. Proteins were extracted from both control samples and treated samples at intervals of 3 and 48 hours post-spheroid stimulation in adherence.

HepG2 cells exhibited a significant reduction in E-cadherin expression as early as 3 hours post-treatment across all conditions compared to controls. This decrease was accompanied by an increase in N-cadherin expression at 3 hours, except in spheroids stimulated with rh-ANG-2 alone. These alterations in protein expression remained consistent at 48 hours, showing no significant fluctuations among the treated groups. Vimentin levels significantly increased in spheroids treated with rh-VEGF and the combination of rh-ANG-2 and rh-VEGF at 48 hours, compared to both controls and the same treatments at 3 hours (Figure 14).

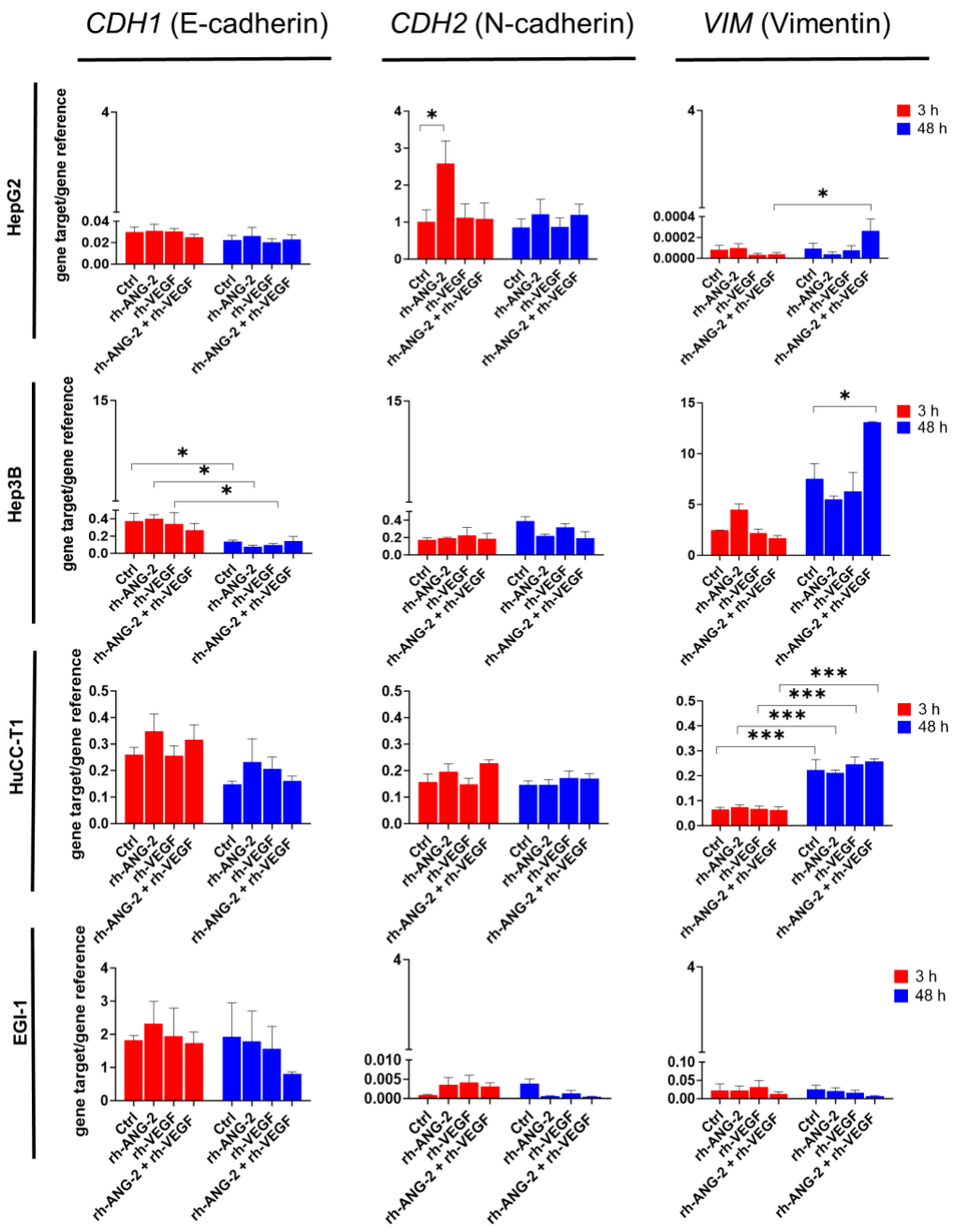

Figure 13 - Assessment of gene expression levels of EMT markers *CDH1*, *CDH2*, and *VIM*. Spheroids were either untreated (Ctrl) or treated with 200 ng/mL of rh-ANG-2 alone, 200 ng/mL of rh-VEGF alone, or a combination of 100 ng/mL each of rh-ANG-2 and rh-VEGF. RNA extraction was performed at 3 and 48 hours post-treatment. The gene target copies were normalized to reference gene copies (β actin). Comparative analyses were conducted between the treated spheroids and controls at the respective time points, as well as between spheroid gene expression levels at 48 hours and 3 hours within the same treatment condition. These assays were performed in triplicate for reliability.

Statistical differences between groups were evaluated using Two-way ANOVA, followed by post-hoc tests for multiple comparisons. Significance levels are established as follows: * $p<0.05$; *** $p<0.001$.

In Hep3B spheroids, a significant decrease in E-cadherin was observed at 48 hours in spheroids treated with both stimuli compared to the 3 hours, correlating with increases in both N-cadherin and Vimentin. These markers also exhibited a significant rise with rh-VEGF treatment alone (Figure 14). In HuCC-T1 cells, only Vimentin showed a significant increase at 48 hours when stimulated with rh-ANG-2 compared to the 3-hour measurement (Figure 14).

Conversely, in EGI-1 spheroids, E-cadherin levels remained constant regardless of treatment or time point, and both N-cadherin and Vimentin were undetected, possibly due to the noted low gene expression levels (Figure 13, Figure 14).

9. The E-cadherin to N-cadherin switch was observed in the outermost and migrating cells of the spheroids.

To examine how E-cadherin and N-cadherin behaved within spheroids after stimulation, we conducted an immunofluorescence assay. We opted for immunofluorescence over Western blotting because it enables distinguishing between non-migrating and migrating cells within the 3D spheroid structure. The formed spheroids were transferred into adherence plates that included a glass slide at the base, facilitating the adherence of the spheres. Post-stimulation for periods of 3 and 48 hours, the attached spheroids were immobilized, and the slides were labeled using specific antibodies. Although we observed no noticeable differences among the three treatments, we combined the results, treating the 'treated' condition as representative of all three treatment types.

In HepG2 spheroids, at 3 hours post-treatment, untreated cells showed uniform E-cadherin expression, while treated spheroids displayed reduced expression at the periphery. By 48 hours, both treated and untreated spheroids had even lower E-cadherin expression, particularly in migrating cells (indicated by red arrows in Figure 15 A). Similarly, Hep3B spheroids showed decreased E-cadherin expression at 3 hours post-treatment, more prominently at 48 hours in treated spheroids compared to controls, especially in the outer and migrating cells (Figure 15 B).

N-cadherin expression was initially low in both HCC cell lines at 3 hours post-stimulation and remained unaffected by treatment. However, by 48 hours, there was a widespread increase in N-cadherin intensity, with treated spheroids showing stronger expression compared to controls (Figure 15 A for HepG2 and Figure 15 B for Hep3B).

In HuCC-T1 spheroids, E-cadherin expression significantly reduced at 48 hours, irrespective of treatment, particularly at the migrating front (highlighted by red arrows in Figure 15 C).

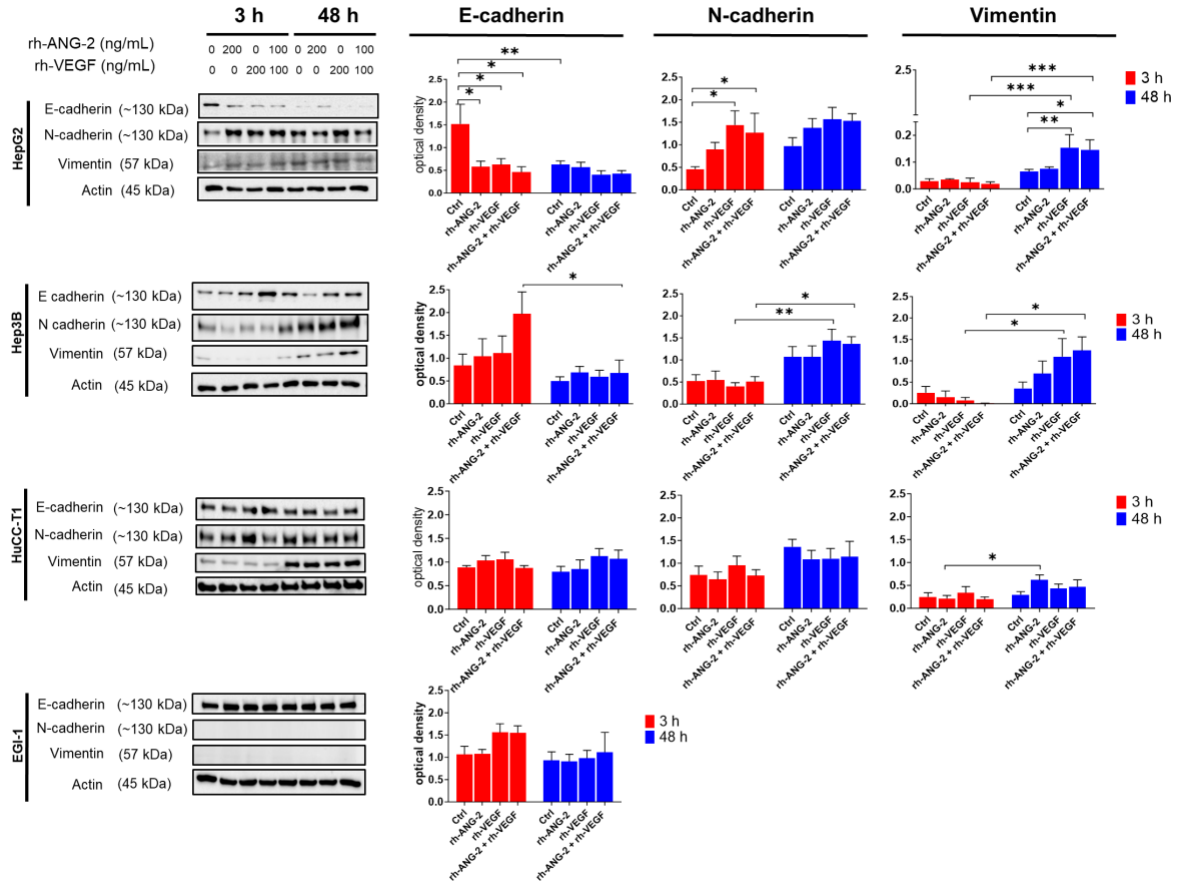

Figure 14 - Assessment of protein expression levels of EMT markers E-cadherin, N-cadherin, and Vimentin. We assessed E-cadherin, N-cadherin, and Vimentin protein expression in HepG2, Hep3B, HuCC-T1, and EGI-1 spheroids treated with 200 ng/mL of rh-ANG-2, 200 ng/mL of rh-VEGF, or a combination of 100 ng/mL each of rh-ANG-2 and rh-VEGF. Protein samples were collected at 3 and 48 hours post-stimulation. Quantitative analyses were performed by ImageLab software, presenting densitometry data as normalized optical density values to β-actin. Comparative analyses were conducted between treated spheroids and controls at corresponding time points, as well as longitudinally within treatment groups from 3 to 48 hours. The experiment was replicated a minimum of three times for result reliability. Statistical significance was determined by Two-way ANOVA with post-hoc tests for multiple comparisons (* $p<0.05$; ** $p<0.001$; *** $p<0.001$).

Meanwhile, there was a slight increase in N-cadherin levels. EGI-1 spheroids consistently showed E-cadherin expression at both 3 and 48 hours post-stimulation, with no visible difference between stimulated and unstimulated cells. However, at 48 hours, the E-cadherin signal varied, being stronger in regions where cells had a compact structure and weaker in

migrating cells (Figure 15 D). EGI-1 spheroids did not exhibit N-cadherin expression under any experimental conditions, consistent with Western blot results.

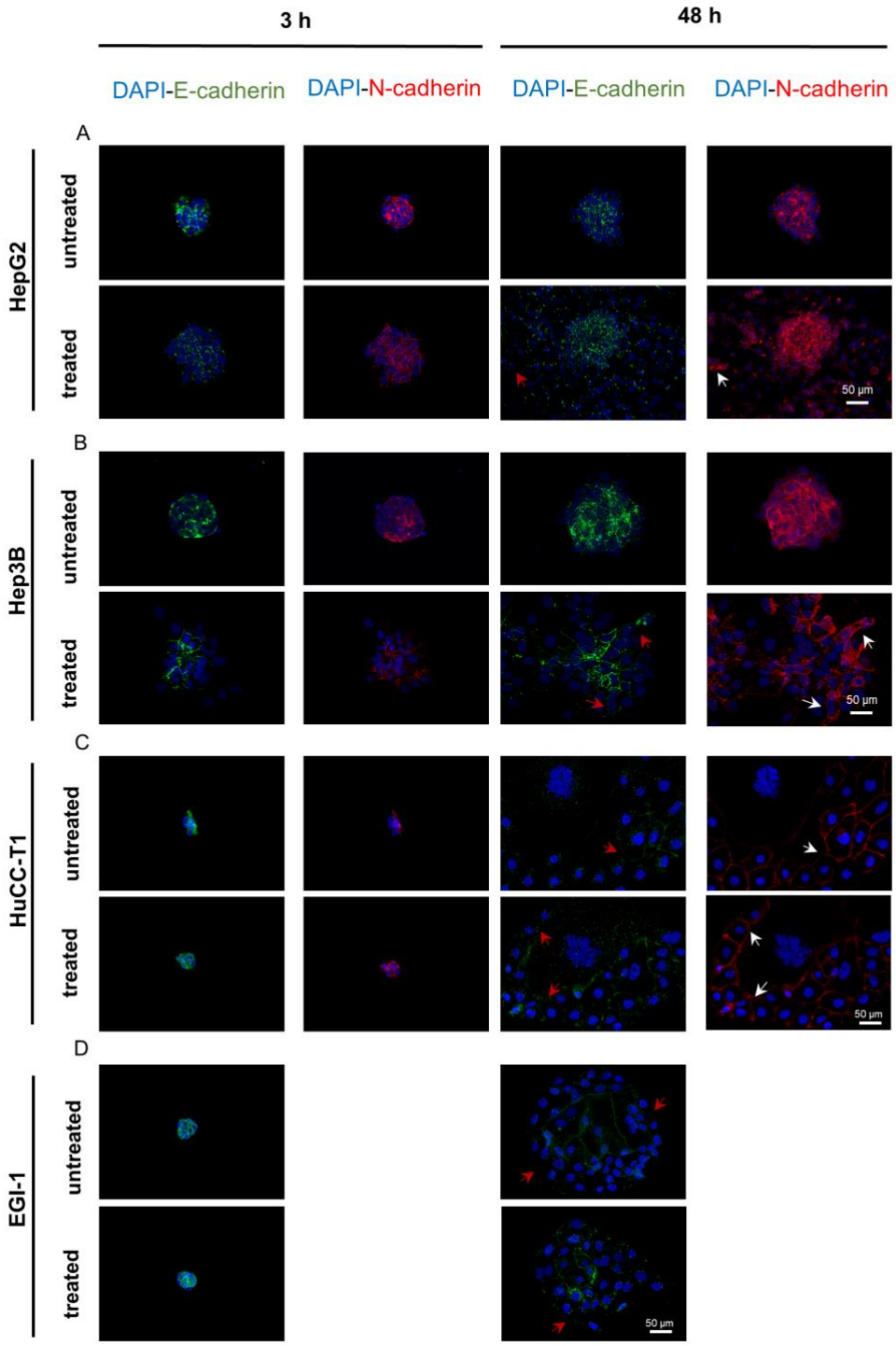

Figure 15 - Immunofluorescence analysis of E-cadherin and N-cadherin expression in HCC and CCA spheroids post-stimulation. Representative images depicting spheroids from (A) HepG2, (B)

Hep3B, (C) HuCC-T1, and (D) EGI-1 cell lines. E-cadherin is highlighted in green, while N-cadherin is shown in red fluorescence. Cell nuclei are counterstained using DAPI. Red and white arrows signify the loss of E-cadherin and the upregulation of N-cadherin expression, respectively, at 3 and 48 hours post-stimulation.

DISCUSSION

Research extensively details the involvement of ANG-2 and VEGF in HCC and, to a lesser extent, iCCA. This insight stems from a comprehensive analysis of patient samples, encompassing serum and tissue biopsies (Cai et al., 2022; Ao et al., 2021; Kimawaha et al., 2021; S. B. Choi et al., 2017). Elevated levels of these proteins and increased gene activity underscore their pivotal roles in driving angiogenesis and neo-angiogenesis, critical processes that fuel cancer growth and contribute to disease severity. Despite the recognized significance of these proteins, there is still a lack of comprehensive research exploring the effects of ANG-2 stimulation on HCC and CCA, either on its own or in combination with VEGF. While prior studies have established VEGF's direct influence on HCC (S. Peng et al., 2014; L. Zhang et al., 2012) and CCA (M. Huang et al., 2018; H. Peng et al., 2016) in controlled laboratory settings, the combined effects of VEGF and ANG-2, known for their synergistic impact on cancerous blood vessel cells, remain insufficiently explored and warrant greater evaluations (Lobov et al., 2007; L. Zhang et al., 2003). Recent investigations by Rawal et al. (Rawal et al., 2022) have underscored the distinctive behavior of liver cancer cells when cultured in 3D setups compared to conventional 2D cultures. This finding implies that 3D models may more accurately mimic the actual conditions of cancer growth and its surrounding microenvironment. Subsequent studies in the field (Blidisel et al., 2021; Pinto et al., 2020) have lent further support to this notion. Building upon these insights, we established a 3D culture system using cell lines derived from HCC - HepG2 and Hep3B - as well as CCA cell lines - HuCC-T1 and EGI-1. Our primary objective was to comprehensively investigate the response of these cells to direct stimuli that foster blood vessel formation, a pivotal process in cancer progression. Within the HCC models, we utilized spheroids from HepG2 and Hep3B cell lines to represent less aggressive and more aggressive tumor phenotypes, respectively. Comparative analyses encompassing gene expression, drug responsiveness, and signaling pathways have consistently indicated that Hep3B demonstrates a notably more aggressive phenotype than HepG2 (Qiu et al., 2015). Regarding CCA, spheroids were derived from HuCC-T1, and EGI-1 cell lines to emulate intrahepatic and extrahepatic tumors, respectively. While global gene expression profiles suggest a greater similarity between HuCC-T1 and

HepG2 as reported by Scherer et al. (Scherer et al., 2020), other references have indicated analogies between HuCC-T1 and Hep3B. These similarities include responses to anti-tumoral drugs with inhibitory effects on the cell cycle (C.-Y. Huang et al., 2018). This accumulating evidence designed HuCC-T1 as a promising model for testing our hypotheses. For our investigation into eCCA, we selected the EGI-1 cell line due to its well-documented characterization in the existing literature (Fabris et al., 2011; Huether et al., 2007; Okaro, 2002), despite the limited availability of CCA-derived cell lines. Initially, our focus was on determining whether stimulation with rh-ANG-2 and/or rh-VEGF influenced cell viability, regardless of the concentration used, to ensure that the stimulation did not induce toxic effects. Employing an endpoint assay, we specifically evaluated the number of viable cells producing ATP. It is important to note that assessing viability does not necessarily indicate proliferative capacity, which may be affected by the spheroid structure. Cells at the core of a spheroid can remain viable but not actively proliferating (Weiswald et al., 2015). Subsequent migration assays revealed increased motility in spheroids from all cell lines upon exposure to rh-ANG-2 and rh-VEGF, although the extent varied across the different cell types. Notably, both HepG2 and HuCC-T1 cell lines exhibited similar responses to rh-ANG-2 stimulation. In contrast, Hep3B spheroids displayed no significant response to rh-ANG-2 but demonstrated an increased response to rh-VEGF. Despite the established understanding of the combined impact of ANG-2 and VEGF (Yoshiji, 2005; Y. Zhu et al., 2005), our research revealed that the concurrent application of rh-ANG-2 and rh-VEGF did not amplify migration in either HepG2 or iCCA spheroids. This finding suggests a potential opposing influence of ANG-2 on VEGF's actions, aligning with observations in other cancer models where ANG-2 activation resulted in decreased migratory potential typically induced by VEGF (Ley et al., 2004). Similarly, in Hep3B cells, the migration reliant on rh-VEGF did not exhibit further enhancement upon the introduction of rh-ANG-2, implying that rh-VEGF may be the primary driver of migration in this specific cell line. This observation aligns with the findings reported by Sharma et al. (Sharma et al., 2016), where a decrease in Hep3B migration was demonstrated following VEGF silencing. The hypothesis that ANG-2 or VEGF could stimulate migration gained substantial support with the addition of two specific inhibitors, Trebananib for ANG-2 and Bevacizumab for VEGF, which not only nullified the pro-migratory effect but also led to a reduction in the expression of the respective proteins, as confirmed by Western blot analysis. Remarkably, EGI-1 spheroids consistently displayed increased motility regardless of the stimulation received. Notably, control spheroids exhibited migration and growth in all cell lines, with the most pronounced effects observed in EGI-1.

This evidence suggests an intrinsic link between migratory mechanisms and spheroid culture conditions, particularly influenced by the high migration rate of cells at the spheroid periphery (Costa et al., 2016).

The invasion assay validated the distinct impacts of rh-ANG-2 and rh-VEGF on HuCC-T1 and Hep3B spheroids, respectively, aligning with previous observations from the migration assays. However, a general trend of increased invasive capacity following stimulation over time was noticed across all cell lines, except for EGI-1, which consistently exhibited higher invasiveness irrespective of time and stimulation.

VEGF's role in promoting proliferation and invasiveness in both endothelial and non-endothelial cells is well-established (Duffy et al., 2013). For instance, in ovarian cancer, VEGF has been identified to accelerate tumor cell invasion into Matrigel by facilitating the degradation of the extracellular matrix (A. Zhang et al., 2006). Similarly, in T-47D breast cancer cells, VEGF significantly enhanced cellular invasion through Matrigel and fibronectin-coated transwell membranes (Price et al., 2001). While the role of ANG-2 in this context is less understood, several studies have indicated a correlation between escalated doses of ANG-2 and increased proliferation in various estrogen receptor-positive (ER+) breast cancer cell lines (Han et al., 2016). Likewise, in glioma and extravillous trophoblast cell lines, the addition of exogenous ANG-2 has been associated with heightened invasiveness (Hou et al., 2021; B. Hu et al., 2003). Similar to the migration assay, the simultaneous use of rh-ANG-2 and rh-VEGF yielded no noticeable impact in the invasion assays conducted on Hep3B or HuCC-T1 cells. This absence of influence suggests a potential antagonistic relationship between these factors within our tumor models.

To investigate potential mechanisms underlying the observed effects on migration and invasiveness, we delved into the expression and phosphorylation of key receptors for ANG-2 and VEGF after proangiogenic stimulation. Our analysis focused on TIE2 and its phosphorylated form (P-TIE2) in HepG2 and HuCC-T1 spheroids, as well as VEGFR1 and its phosphorylated form (P-VEGFR1) in Hep3B spheroids.

In HepG2 spheroids, the noticeable increase in the P-TIE2/TIE-2 ratio post-stimulation suggests a direct involvement of rh-ANG-2 on TIE2, contributing to the observed pro-migratory behavior. Similar activation of the TIE2 receptor by ANG-2 has been linked to neovascularization pathways in HCC (Vanderborght et al., 2020), suggesting its potential role in HCC progression (Tanaka et al., 2002). However, in HuCC-T1, despite the rise in TIE2 expression, the lack of augmented phosphorylation by rh-ANG-2 suggests that this axis may not directly contribute to acquiring pro-migratory or pro-invasive capabilities. The decreased

P-TIE2/TIE2 ratio following rh-ANG-2 treatment might indicate that TIE2 activation did not occur. Previous studies have revealed that in tumoral cells, ANG-2 can signal through other receptors like integrins, leading to cell invasion and survival in glioma and breast cancer cells (H. S. Lee et al., 2014; Imanishi et al., 2007; B. Hu et al., 2006). Investigating this pathway in our models could be a promising field for future research. Furthermore, the reduction in TIE2 phosphorylation observed in HuCC-T1 spheroids following treatment with rh-VEGF alone or in combination with rh-ANG-2 could potentially validate an antagonistic interaction between rh-VEGF and rh-ANG-2. This observation might also imply a possible inhibitory impact of rh-VEGF on TIE2 activation. This finding aligns with a study demonstrating that VEGF induces the shedding of TIE2, resulting in a loss of function in endothelial cells (Findley et al., 2007).

For Hep3B, known for its prominent pro-migratory and pro-invasive response to rh-VEGF treatment, we examined the expression and phosphorylation-based activation of VEGFR1. Although not extensively characterized, VEGFR1 is expressed in various cancer cells and has been associated with increased invasive capabilities in numerous studies (Lichtenberger et al., 2010; F. Fan et al., 2005; Wey et al., 2005; H. Zhang, 2002). Our findings confirm that rh-VEGF activates VEGFR1 in our 3D Hep3B model, suggesting that rh-VEGF binds to and activates this receptor, thereby enhancing invasive capabilities. The analysis of ANG-2 and VEGFA proteins revealed that samples stimulated with rh-ANG-2 or rh-VEGF displayed multiple bands, denoting the presence of both exogenously added recombinant protein (higher molecular weight bands) and endogenously produced proteins (lower weight bands). Remarkably, while the levels of endogenously produced ANG-2 and VEGFA remained steady, there was a significant increase observed in the amounts of rh-ANG-2 and rh-VEGF in both HepG2 and HuCC-T1 (for ANG-2) and in Hep3B (for VEGFA) over time, compared to the control samples. This pronounced rise indicates a temporal escalation in the quantity of exogenously added proteins binding to their respective receptors. These data provide the foundation for a deeper exploration of the temporal dynamics and implications of these interactions within our cellular systems.

Based on the encouraging results obtained in the evaluation of migration and invasion, the phenomenon of EMT, acknowledged as a crucial mechanism that aids cell migration (Mittal, 2018), was examined. Ribatti et al. (Ribatti et al., 2020), and Giannelli et al. (Giannelli et al., 2016), among others, have highlighted how EMT significantly contributes to tumor progression by boosting invasion and metastasis across various cancer types, including liver cancer. Additionally, Dong and colleagues (Dong et al., 2018) have established a link

between proangiogenic signals and the promotion of metastasis through EMT in lung cancer. Therefore, this investigation aimed to understand whether the proangiogenic factors ANG-2 and VEGF influence the expression of EMT markers, utilizing gene and protein analysis. HepG2 spheroids exhibited significant alterations in EMT markers within 3 to 48 hours post-treatment, characterized by reduced E-cadherin levels and increased N-cadherin and Vimentin expression. Conversely, Hep3B spheroids primarily showed marker changes at later stages, especially with rh-VEGF treatment at the protein level. HuCC-T1 spheroids consistently displayed heightened Vimentin expression linked to rh-ANG-2 treatment at the protein level, despite its presence across all conditions at the gene level. On the other hand, EGI-1 spheroids showcased a distinct profile, unlike any other cell lines. They exhibited heightened migration and invasiveness, and surprisingly, they displayed a unique expression profile of specific EMT markers. This was distinguished by stable E-cadherin levels and an absence of N-cadherin or Vimentin expression. Our collective findings highlight the propensity of cell line-derived spheroids to undergo EMT, yet with distinct responses to ANG-2, VEGF, or their combination. HuCC-T1 cells showed a gradual reaction to stimuli, particularly evident through significant Vimentin alterations, hinting at a potential 'partial' EMT phenomenon (Liao et al., 2021; Lüönd et al., 2021), associated with increased metastatic potential in various cancer models (Haerinck & Berx, 2021; Saitoh, 2018). Vimentin's significant role in metastasis was supported by its identification as a crucial factor for metastatic potential in aggressive CCA cell lines (Saentaweesuk et al., 2018). This aligns with the established correlation between abnormal Vimentin expression in CCA tissues and unfavorable patient outcomes (Mao et al., 2013; Thiery et al., 2009). The response of HuCC-T1 spheroids to proangiogenic stimuli supports these observations.

Immunofluorescence supported the EMT changes, notably in HepG2 and Hep3B spheroids. In our assessment of E-cadherin and N-cadherin spatial distribution through immunofluorescence, we identified a consistent pattern across all cancer cell models examined. Specifically, we noted a distinct decline in E-cadherin levels and an increase in N-cadherin expression at the outer migrating layer of the spheroids. This observation aligns with the established understanding that EMT predominantly occurs at the invasive front of tumors, where cells typically lose their cell-cell contacts (Pinto et al., 2020; Tevis et al., 2017; Thiery et al., 2009). Surprisingly, even the untreated spheroids gradually displayed changes in EMT marker expression over time, suggesting that the conditions within the spheroids might inherently trigger this mechanism. It appears that Western blot analysis of the entire spheroid lysate may not precisely capture the alterations in EMT markers occurring at the spheroid's

periphery. Although immunofluorescence analysis improved our comprehension of marker expression variations, especially at the spheroid's edge, its qualitative nature lacked the precision to discern subtle differences attributable to diverse stimulations.

In summary, our findings indicate that spheroids derived from HCC and iCCA cell lines exhibit both commonalities and distinct responses to proangiogenic stimuli. While the direct involvement of rh-ANG-2 and rh-VEGF is apparent, the observed differences underscore the intricacies of their effects in these unique cell line models. Future research directions will involve assessing the responsiveness to proangiogenic stimuli by primary cell lines obtained from human HCC and iCCA, essential for further validation in patient-derived xenografts.

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