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Review

Role of the Angiogenic Factors in Cholangiocarcinoma

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Abstract: Angiogenesis plays a fundamental role in tumor growth and progression. It is regulated by several growth factors, including vascular endothelial growth factor protein family (VEGF) and its receptors, which are probably the most important factors responsible for the development of new vessels. The VEGF family includes several members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF), and their receptors VEGFR-1, VEGFR-2 and VEGFR-3. Other relevant factors are represented by angiopoietins, thrombospondin-1, and endothelins. However, since the therapeutic benefit associated with VEGF-targeted therapy is really complex, a better understanding of these pathways will lead to future advances in the use of these agents for clinic management of tumors. Here we present a review regarding the role of angiogenic factors in cholangiocarcinoma, which arise from cholangiocytes, the epithelial cells of bile ducts. They are rare and aggressive neoplasms with a poor prognosis and limited treatment options, classified as intrahepatic, perihilar, and distal cholangiocarcinoma based on their anatomical location. Therefore, the identification of specific signaling pathways or new tumor biomarkers is crucial in order to develop more effective anti-angiogenic therapies.

Keywords: biliary epithelium; cholangiocarcinoma; angiogenic factors; VEGF

1. Introduction: The Biliary Epithelium in Normal and Pathological Conditions

The human biliary epithelium, which is formed by cholangiocytes, originates at the level of the biliary pole. Cholangiocytes represent the second cellular hepatic population after hepatocytes, corresponding to 3–5% of the total epithelial cells, whose morphology depends on their position in the biliary tree [1]. Cholangiocytes play an active role in both homeostatic and pathologic conditions and they modify the composition of canalicular bile through their several secretory activities [2–4]. These cells are able to activate a number of intracellular cascades: they respond to inflammatory insults through a large variety of proinflammatory mediators that, via autocrine or paracrine mechanisms, determine cell proliferation, and mediate interaction with other liver cells, like hepatocytes, stem/progenitor cells, hepatic stellate cells (HCS), and endothelial and inflammatory cells [5].

The biliary network consists of intra- and extrahepatic bile ducts: IHBDs and EHBDs. IHBDs includes, progressively, ductular-canalicular junctions, Hering's canals, bile ductules, interlobular, septal, and zonal and segmental ducts, whereas EHBDs consists of hepatic ducts. The

ductular-canalicular junction is characterized by the resident progenitor cells compartment of the liver. Hering's canals and ductules are lined by cubic cholangiocytes. Interlobular, septal, and bile ductules are considered small bile ducts, while zonal and segmental ducts are considered large bile ducts. In fact, within small and progressively larger bile ducts, cholangiocytes increase their height and width, showing a morphological, as well as a functional, heterogeneity [6,7]. Cholangiopathies are diseases in which the biliary epithelium represents the main target and they are characterized by an alteration of the correct balance between proliferation and loss of cholangiocytes that is physiologically/normally regulated by several factors such as somatostatin, secretin, gastrin, serotonin, and melatonin [8–12]. Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are diffused cholangiopathies. In PBC patients there is no substantial increase in the risk of liver cancer development [13]. In contrast, PSC is strongly associated with liver cancer development risk: classical PSC of large ducts is the most important risk factor for biliary cancer in developed countries [14,15]. Therefore, in response to different injury, cholangiocytes adapt their phenotype, reacting, and secreting proinflammatory mediators and contributing actively to disease progression or resolution [3]. Moreover, the complexity of the intrahepatic biliary tree represents nowadays an open challenge for liver tissue engineering. Progress in both bioengineered scaffolds and in vitro cholangiocytes culture has led to the employment of cellular constructs to replace or repair the biliary tree [16–18]. Several studies support the concept that biliary development and homeostasis is coordinately regulated by different autocrine factors, including angiogenic factors. In fact, angiogenesis represents the biological process that leads to the formation of new vessels from pre-existing blood and lymphatic vessels through a well-planned cascade of events. In malignant tumors and in the carcinogenesis of biliary tumors, it is dysregulated and neoplastic cells secrete or induce the microenvironment to produce pro-angiogenic signals which are able to expand endothelial cells and to sustain tumor growth. In biliary cancers, many angiogenic factors, such as the vascular endothelial growth factor (VEGF)-A is expressed in more than half of patients and it is correlated with stage, metastasis, and survival rate [19,20]. Therefore, it is important to focus on the link between angiogenic factors and biliary tumor to better detect the possible therapeutic strategies in the prevention of tumor progression, metastasis, and invasion.

2. Classification of Cholangiocarcinoma

Primary liver cancer represents the second most important cause of worldwide mortality for malignancies. Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) are the two most common liver cancers [21]. CCA represents a heterogeneous group of malignant neoplasms originating from damaged cholangiocytes, with a multifaceted molecular pathogenesis. It accounts, at the moment, for approximately 15% of cases with a wide geographical variability correlated to the plurality of risk factors [1]. CCA is currently classified according to the World Health Organization (WHO) and Union for International Cancer Control (UICC) in two major categories, according to their anatomical origin in the biliary tree: *Intrahepatic cholangiocarcinoma* (*iCCA*), which accounts for about 20% of tumors developing within the liver, from the bile duct of the second order and from the most proximal intrahepatic bile duct, and *extrahepatic bile duct carcinoma* (*eBDC*), which represents 80% of CCAs and it can be classified into perihilar (*pCCA*) (60–70%) and distal (*dCCA*) (20–30%). *pCCA* spreads between the second-order bile ducts and the attachment of the cystic duct into the common bile duct, whereas *dCCA* grows in areas between the cystic duct and the ampulla of Vater [22,23] (Figure 1).

Histologically, the majority of pCCAs and dCCAs are mucinous adenocarcinoma [24]. Conversely, iCCA is characterized by two histological subtypes: one originating from small intrahepatic bile ducts, the other from large intrahepatic bile ducts. The first type is a mixed iCCA, whereas the second type is a mucinous iCCA [25]. The previous histological division is really important and recent data pointed out a noteworthy degree of heterogeneity regarding their different cell of origin, epidemiology and risk factors, pathological and molecular profile, etiology, and pathogenesis, clinical outcome and response to treatment [22,26].

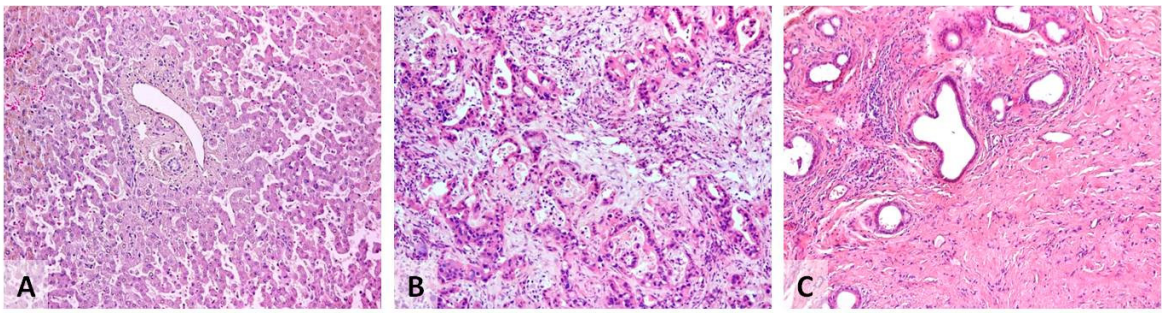

Figure 1. Hematoxylin and eosin (H and E) staining. Representative pictures of human liver biopsies from peritumoral hepatic tissue (**A**); intrahepatic CCA (iCCA) (**B**); and extrahepatic bile duct carcinoma (eBDC) (**C**). They show the dramatic morphological changes in the histological aspect from a non-malignant tissue, with the physiologic hepatic cords, regular sinusoid and portal space containing the typical branches of the hepatic artery, portal vein and biliary ducts (**A**); and the tumoral tissues, where the hyperplastic cellular growth is matched with a large deposition of connective tissue and inflammatory infiltrate (**B**,**C**). Original Magnification (OM) 10×.

However, the extreme heterogeneity of CCA makes it difficult to provide a definitive classification. Cardinale et al. had already emphasized the "increasing burden of classifications". They have considered a CCA classification based on: (i) anatomical location, largely used in the medical literature; (ii) macroscopic pattern of growth, (iii) microscopic features; and (iv) cells of origin. The latter is supported by emerging data regarding radiological and pathological findings in CCAs that originate from different cells inside the biliary tree. Thus, the CCAs can be reclassified as follows: CCAs derived from human hepatic progenitor/stem cell (hHpSC) lineages in canals of Hering, that include histological subtypes of iCCA; CCAs derived from human biliary tree stem/progenitor cell (hBTSC) lineages in peribiliary glands (PBGs) or from epithelium of intra- or extrahepatic large bile ducts [24,27].

2.1. Neoplastic Alterations and Risk Factors

Cholangiocarcinoma occurs as a massive lesion in the liver (iCCA) or as an obstruction of the biliary tract. Furthermore, these two distinct tumors differ in their etiology, risk factors, natural history, clinical behavior, and response to therapies. iCCA presumably originates from small ducts within the liver and can reach large dimensions without any clinical symptoms. In contrast, ductal CCA originates from large ducts, up to the second branching order, and patients experience biliary tract obstruction. Carcinogenesis is characterized by a fibrogenic process, an immune response, angiogenesis and the presence of desmoplastic stroma. Although the major etiologies and risk factors for the development of liver cancer (such as previous hepatitis virus infection, alcohol intake, non-alcoholic fatty liver disease, inflammatory, and proliferative tissue microenvironment) are well known, numerous cases of HCC develop in the absence of any known etiology [15,28,29]. Regarding CCA, PSC confers a high risk of CCA, with PSC-CCA representing the leading cause of PSC-associated mortality. CCA arising in patients with PSC is characterized by extensive PBG involvement and by the activation of the BTSC niche. The presence of duct lesions at different stages suggests a progressive tumorigenesis [30,31]. A peculiar characteristic of the CCA is represented by the presence of a "reactive stroma" responsible for the accentuated and early invasiveness of the neoplasm. Malignant cells establish a two-way network of interactions with the components of the matrix: macrophages, myofibroblasts, and lymphatic endothelial cells are activated by malignant cells and they form a "tumor reactive stroma" (TRS) that, in turn, releases growth factors, proteinases, cytokines/chemokines acting by a paracrine way [32–37].

2.2. Late Diagnosis: Poor Prognosis

pCCA and dCCA account for approximately 80% of all CCAs diagnosed in the USA [38]. Diagnosis is based on a combination of clinical, radiological, biochemical, and histological approaches.

The silent evolution of CCA and the non-specificity of symptoms, only represented by obstructive manifestations of the biliary tract, are the main cause of the late diagnosis of the neoplasia. In general, the advanced stage of the CCA makes the prognosis unfavorable and burdens the mortality rate in the short term [39,40]. Emerging technologies for the diagnosis of malignant biliary disorders consists of an improved view of the biliary tree by cholangioscopy, intraductal ultrasound, and confocal laser endomicroscopy. In addition to conventional cytology, complementary and advanced cytologic techniques such as fluorescent in situ hybridization (FISH) can be applied. Additionally, the latest generation technologies in the field of proteomics and sequencing can find a valid application [38]. The development of innovative technologies has allowed the identification of several important genetic, epigenetic, proteomic, and metabolomic aspects of the CCA, as well as the application of these acquisitions for an early diagnosis. In particular, for diagnosis and prediction of treatment response, biomarkers detected in fluids (serum, bile and urine) or in biopsy samples are currently available but there is no technique sufficiently specific to select CCA from diseases or sensitive enough to identify early CCA. CEA (carcinoembryonic antigen), CA19-9 (carbohydrate antigens 19-9) and CA125 (carbohydrate antigens 125) are the most used markers for diagnosis of CCA and they are also useful to differentiate between HCC and CCA. On the contrary, CYFRA 21-1 (cytokeratin-19 fragment) and CA-242 (monoclonal antibody C 242) have been studied but not used clinically [38,41]. At the moment, the identification of biomarkers is a great challenge in the fight against CCA due to the marked heterogeneity of these tumors [25,42,43] (Table 1).

Table 1. List of the most important diagnostic biomarkers in Hepatocarcinoma (HCC) and Cholangiocarcinoma (CCA).

	Diagnostic Markers	Prognostic Markers
HCC	Endothelial marker (CD34)	Vascular endothelium growth factor (VEGF)
	Smooth muscle marker [32]	Hypoxia-inducible transcription factors (HIF)
	Cell surface heparin sulphate proteoglycan (GPC3)	Endothelium specific markers (CD31, CD34 and VWF)
	Stress protein implicated in cell cycle progression, apoptosis and tumorigenesis (HSP70)	Matrix metalloproteinase (MMPs)
	Catalysts of glutamine synthesis (GS)	CDK inhibitors (p16, p18, p27 and p57)
	Alpha-fetoprotein (AFP)	Cell proliferation markers (Ki-67 and PCNA)
	Zinc-dependent metallopeptidase (CD10)	Tumor suppressor gene/protein (P53)
CCA	Carbohydrate 19-9 antigen (CA19-9)	Epidermal growth factor receptor (EGFR)
	Carcinoembryonic antigen (CEA)	Cytokeratin-19 fragments in serum (CYFRA21-1)
	Interleukin- 6 (IL-6)	
	Cytokeratin-19 fragments in serum (CYFRA21-1)	
	Matrix metallopeptidase 7 (MMP-7)	
	Spermatogenesis associated 20 (SSP411 or SPATA20)	
	Circulating tumor cells (CTC)	

2.3. MDR: Multidrug Resistance; MOC: Mechanisms of Chemoresistance

Cholangiocarcinoma is characterized by a considerable resistance to the common chemotherapeutics and there is no treatment able to improve the prognosis. The use of chemotherapy is currently clearly indicated in the management of inoperable patients due to unresectable, metastatic disease, or recurrent CCA and it includes gemcitabine and cisplatin as the first line treatment [15]. The chemoresistance (MOC) is the result of synergistic mechanisms that make cancer cells refractory to cytostatic drugs [44,45]. It is possible to schematize these different mechanisms: MOC-1 reduces drug uptake and enhances drug efflux; MOC-2 is involved in drug metabolism; MOC3 changes the drug targets; MOC-4 enhances DNA repair; MOC-5 decreases apoptosis and enhances survival; MOC-6 affects the tumor microenvironment; and MOC-7 enhances phenotype transition [40]. MOC

characterizes the multidrug resistance (MDR) phenotype in CCA and is represented by the so-called *resistoma*, which in turn corresponds to the complete series of proteins associated with MOC and expressed in every moment of cancer evolution. MOCs result in reduced drug intake, increase in drug efflux, a lower percentage of intracellular active agent, and changes in molecular targets. MDR can be counteracted using gene therapy, pharmacological targeting, such as VEGF, which are secreted from tumors that up-regulate MDR through the activation of VEGFR2 and Akt. In fact, recent data indicate the inhibition of angiogenesis as an interesting therapeutic target against the tumor microenvironment [46]. Although data are still lacking and related aspects to the drug resistance mechanisms need to be clarified, effective anti-angiogenic therapy could be based on the identification of markers of reactivity or development, essential for an individual treatment. In addition, MDR can be also counteracted using nanotechnology to increase the absorption of conventional drugs [45]. Nanotechnologies are opening new therapeutic horizons in order to counteract MOCs through different methods: (i) antitumor drugs are encapsulated in micelles formed by surfactants, as polyethoxylated castor oil and polysorbate 80, in aqueous solution: these micelles are able to chemosensitize MDR tumor cells improving the accumulation of antiblastic drugs; (ii) two groups of poly(ethylene oxide)-poly(propylene oxide), amphiphilic copolymers, which are the linear poloxamers and the branched poloxamines: these PEO-PPOs inhibit efflux transporters in different types of MDR cells and cause energy depletion in tumor cells resulting in altered ABC pumps-mediated ATP-dependent drug efflux; (iii) some natural polymers such as anionic polysaccharides and in particular the thiolated derivatives of chitosan, improve the oral bioavailability [47]. One of the mechanisms of action involved would be related to the overload of the efflux capacity of ABC transporters; and (iv) nanoparticle systems achieve simultaneous administration of anticancer drugs and chemosensitizer. For example, vincristine and verapamil, or paclitaxel and tariquidar, doxorubicin, and elacridar, which are encapsulated in a nanocarrier, overcome the resistance more effectively than when they are individually administrated. Research has yet to confirm that nanocarrier systems are safe to use: liposomal encapsulation of doxorubicin, for example, is able to reduce doxorubicin-related cardiotoxicity, but it has also unexpected side effects [44]. Regarding the anti-angiogenic inhibitors, despite advances in the clinical development, the appropriate dosing pattern and duration of the VEGF receptor for the treatment of cancer is yet to be established. For this reason, in a possible perspective therapeutic approach to anti-angiogenesis activity, the main mechanism involved could be the prevention of binding pro-angiogenic factors to their corresponding receptors. These bindings stimulate gene expression and intracellular signaling involved in cell growth, apoptosis, survival, metastasis and basement membrane degradation. The development of nanosystems that include superior stability, better pharmacokinetic, limited side effects, the binding of these antigens/receptors to functionalized nanoparticles (NPs) offer potent opportunities for tumor targeting and attack [48]. Many natural compounds could be applied as an alternative therapy against cancer, sustaining the conventional treatments to block several signaling pathways involved in cell survival, proliferation, differentiation, and apoptosis. The association of conventional phytotherapics and chemotherapeutics can reduce their toxicity through a reduction of pharmacologically useful dosages. The potential ability of the natural sesquiterpenes β-caryophyllene (CRY) and β-caryophyllene oxide (CRYO) to modulate the IL-6/STAT3 pathway in human HCC and CCA cells has been recently investigated. They interfere with the development and progression of liver cancer [49].

3. Molecular Pathways in Cholangiocarcinoma

A large spectrum of malignancies, such as CCA, originates in the background of chronic inflammation. In fact, inflammatory pathways are not only crucial in carcinogenesis but also in tumor invasion and migration, for example, through the promotion of oxidative stress [50]. Oxysterols from biliary cholesterol are oxidation products involved in tumor development and progression through the activation of the Hedgehog signaling pathway, which promotes the desmoplastic response and it is strictly related to CCA cell proliferation and migration [51]. Furthermore, inflammatory cytokines

activate inducible nitric oxide synthase (iNOS) contributing to nitrosative stress and generating an excess of nitric oxide, which leads to the inhibition of DNA repair proteins and oxidative DNA lesions [52]. Moreover, different proinflammatory cytokines induce cyclooxygenase-2 (COX-2), which is implicated in the carcinogenesis of CCA [53]. In particular, interleukin-6 (IL-6) is produced by cholangiocytes upon inflammatory stimuli and secreted by CCA cells [54]. IL-6 upregulates the antiapoptotic protein Bcl-2 and the myeloid cell leukemia sequence (Mcl-1) by an AKT-dependent mechanism. It also acts via a signal transducer and activator of transcription- (STAT) dependent mechanism to increase the presence of Mcl-1 [55]. Similarly, inhibition of the JAK kinases, which activates STAT3 downstream of IL-6 signaling, has been studied in models of CCA to confirm the important role of neutralizing antibodies for the use of IL-6 in the management of CCA (Figure 2). It is important to underline the key role of PI3K-AKT-mTOR signaling, where AKT activation leads to phosphorylation of the mammalian target of rapamycin (mTOR) pathway and its deregulation promotes tumor development, cell survival, and angiogenesis [56]. For this reason, several PI3K pathway inhibitors are under evaluation in a large group of human cancer.

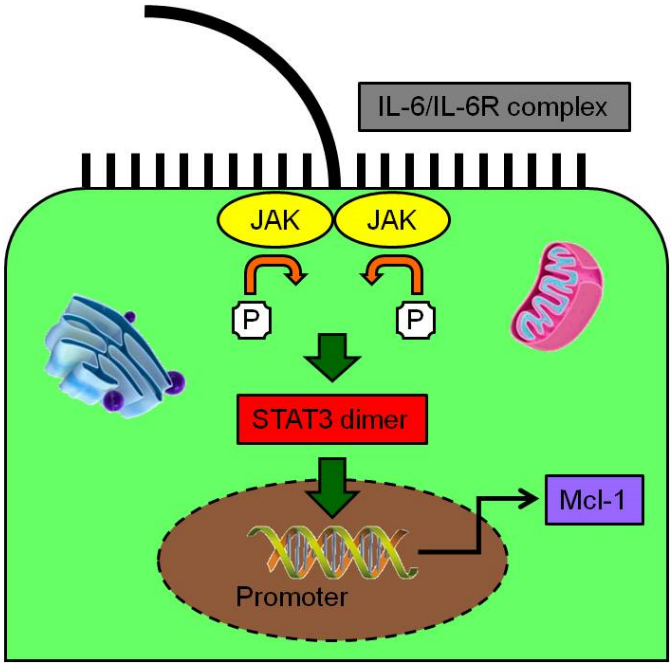

Figure 2. Schematic illustration of Il-6/STAT3 signaling. This pathway can be activated in malignant cholangiocytes and it consists in the binding of IL-6 to its receptor. This process promotes JAK with subsequent phosphorylation, activation, dimerization, and nuclear translocation of STAT3 in the form of STAT3 dimer. STAT3 upregulates Mcl-1 transcription, which play an important role in cellular growth.

Likewise, Notch dysregulation, upregulated in PSC and CCA, is implicated in inflammation and carcinogenesis [57]. The inhibition of Notch signaling can occur via several mechanisms, such as the block of Notch receptor activation with γ-secretase inhibitors, the block of the ligand with monoclonal antibodies or the block of the transcriptional activity of Notch intracellular domain (NICD) by inhibiting peptides [58]. Together with the cytokines and the inflammatory process, various growth factor tyrosine kinases are involved in the carcinogenesis of CCA, such as the ERBB family of receptor tyrosine kinases and the hepatocyte growth factor (HGF) receptor, c-met. The first one leads to the downstream activation of mitogen-activated protein kinase (MAPK); in fact, the upregulation of ERBB2 is related to biliary epithelium tumor in mice [59]. In CCA patients, erlotinib, an EGFR inhibitor, has had limited success in human clinical trials, while lapatinib, a dual inhibitor of EGFR and HER2 (another ERBB receptor) is significantly effective in the inhibition of CCA cell lines compared to trastuzumab, that selectively blocks HER2 [60]. The second abovementioned growth

factor, HGF, activates MET in CCA, thus promoting tumor invasion, and protection from apoptosis and angiogenesis in association with the activation of ERBB family members, such as ERBB2 [61]. MET amplification has been reported in different malignancies, such as gastric, ovarian, lung cancer, and always correlated with a poor clinical outcome [62]. FGFR is also a tyrosine kinase receptor involved in cell transformation and angiogenesis. In solid tumors, fusions of FGFR gene, a class of driver mutations with a crucial role in some cancers, has been studied. It has been reported that FGFR2-BICC1 gene fusion in CCA and its overexpression is linked with an increase in cell proliferation and altered cell morphology [63]. As we previously reported, CCA is characterized by desmoplastic stroma with α-SMA positive cancer-associated fibroblasts (CAFs). The latter induces tumor progression and migration through secretion of several factors like matricellular proteins, growth factors, chemokines, and matrix metalloproteinases [64]. A fibrogenic response anticipates CCA development and induces a malignant growth. Hence, some studies have demonstrated a decrease in fibrosis and carcinogenesis in CCA with 1D11 treatment, a transforming growth factor β (TGF-β) antagonist [65]. In addition, a strong potential of CAFs has been demonstrated. An example is navitoclax, a BH3 mimetic, which increases selective CAF apoptosis and impairs α-SMA expression, contributing to the reduction of metastasis and to the survival of models of CCA [66]. In the end, an increased understanding of CCA carcinogenesis, tumor stroma interactions, angiogenesis, and key molecular signaling pathways would help to define specific therapies for each CCA subtypes with an improvement in patient survival.

4. Angiogenic Factors

The pathogenesis of CCA is closely linked to the formation of a new vascular network that provides more oxygen and nutrients, thus influencing cell survival, tumor growth, and the development of metastasis [67–69]. It involves different mechanisms, whose investigation could be important in the identification of novel potential therapeutic approaches. In particular, cytokines and growth factors assume a main role to sustain the carcinogenesis in the biliary system through the damage of tumor suppressor genes and oncogenes. Normally, blood vessels remain quiescent and they rarely form new branches under physiological conditions. The vascular tree develops during early embryogenesis through a combination of vasculogenesis and angiogenesis. Vasculogenesis represents the de novo formation of new blood vessels from endothelial progenitors, while angiogenesis refers to the formation of new vessels from preexisting ones. However, to support the high proliferative rate of cancer cells, tumors need to quickly develop new vascular networks. However, tumor blood vessels do not have sufficient time to mature and, thus, they are characterized by an immature phenotype, which has a decreased functionality. Their poor functionality has dramatic consequences for the tumor microenvironment and can lead to hypoxia, decreased immune cell infiltration and activity, and increased risks of metastatic dissemination. This improper development of tumor blood vessels is linked to the abnormal levels of growth factors secreted by tumor and stromal cells, including vascular endothelial growth factor (VEGF), angiopoietins (Angs), platelet-derived growth factor (PDGF-B), and transforming growth factor (TGF-β). In addition, the production of VEGF-A and TGFβ cause the polarization of macrophage toward the pro-angiogenic phenotype M2 [69]. The anti-angiogenic therapies could correct the structural and functional defects of tumor blood vessels. The normalization of the tumor vasculature could restore proper blood vessel functionalities and may help in preventing cancer cells from acquiring an aggressive fate associated with the hypoxic microenvironment [70]. Additionally, increased tumor perfusion could improve the beneficial role of chemotherapeutic drugs and radiotherapy. Unfortunately, at the moment complete resection remains the only resolutive treatment of cholangiocarcinoma and novel pharmacological agents are necessary, especially to improve the prognosis, which is now estimated to be around five-year survival in 0–40% of resected cases [71]. Several studies also reported that cancer cells could be able to activate quiescent endothelial cells, especially during an early stage of tumor progression, in a process known as “angiogenic switch”, characterized by a parallel increase of proangiogenic factors and a decrease of antiangiogenic molecules [72–79]. For that reasons it is crucial to study and to deepen the knowledge regarding the

role of angiogenic factors in tumors to better understand how the anti-angiogenic factors can influence and target the growth and progression of cholangiocarcinoma.

4.1. Vascular Endothelial Growth Factors (VEGFs) and Placental Growth Factor (PGF)

It is well known that VEGF is overexpressed in many types of tumors (i.e., colorectal, lung, and breast cancer) other than in CCA and it appears closely correlated with increased microvessel density (MVD) and with tumor stage [64,80–83]. VEGF is a family of molecules which includes different isoforms (VEGF-A, -B, -C, -D, -E, and placenta growth factor) secreted by several cells such as endothelial cells, neutrophils and platelets [84]. The specific function of each subunit of VEGF is mediated by the interaction between the ligand and its corresponding receptor VEGFR (vascular endothelial growth factor receptor). VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1) are two tyrosine kinase receptors mainly involved in angiogenesis and vasculogenesis processes while the third form VEGFR-3 (Flt-4) acts principally during lymphogenesis [72].

Several in vivo studies documented the role of VEGF in CCA and its ability to induce vascular permeability, cell migration and vasodilation, which promote angiogenesis in tumor parenchyma [85–88]. Möbius et al. investigated the expression of VEGF-A and the MVD in patients with extrahepatic cholangiocarcinoma to evaluate neovascularization and its prognostic role in this tumor, demonstrating an improved prognosis in patients with low MVD compared to patients with high MVD [86]. No correlation between VEGF-A levels and survival has been found, in accordance to previously research conduct by Kawahara et al. in which VEGF expression resulted lower in CCA respect to control patients [89]. In contrast with these data, patients affected by extrahepatic biliary tract carcinomas showed an overall significantly worse perspective of survival with a high VEGF positivity compared to patients in which this factor is undetectable [90]. Yoshikawa et al. analyzed the VEGF levels in both intrahepatic and extrahepatic CCA, demonstrating that their expression is higher in both tumors compared to the control and also proved that high VEGF levels clinically correlated with intrahepatic metastasis [87]. Most recently, other authors analyzed the expression of cyclooxygenase 2 (COX2) and VEGF-C in different stages of CCA to elucidate their clinical (based on tumor infiltration) and pathological (based on cell differentiation) involvement in this disease [91]. COX2 and VEGF-C result overexpressed in a late clinical and pathological stage of CCA indicating their contribution in tumor cell differentiation and in metastatic process [91]. Benckert et al. evaluated the expression of VEGF/VEGFR and their interaction with TGF-β/TGF-β receptor pathway on human CCAs after surgical resection and they found that these two growth factors are up-regulated and could thus be responsible for neoangiogenesis [85]. VEGF expression has been detected and correlated with advanced disease stage and poor prognosis of CCA. The addition of an antiangiogenic therapy to the chemotherapy induces a synergistic effect. The upregulation of the angiogenic pathway underlines the potential possible use of an antiangiogenic therapy associated with the chemotherapy. Bevacizumab, a recombinant humanized monoclonal antibody against VEGF, is an important therapeutic agent exploited against cancers. The normalization of the tumor vascularization has been suggested as a new objective to increase survival. In a phase II non-comparative study, the addition of bevacizumab to gemcitabine and oxaliplatin regimen (GEMOX) is associated with a seven-month median progression-free survival (PFS) with tolerable safety in metastatic carcinomas of the biliary tract [92]. Moreover, in vitro experiments show a time-dependent increase of VEGF levels in response to a paracrine/autocrine TGF-β stimulation, suggesting that TGF-β induces VEGF activation via transcription factor Sp1. Therefore, in addition to canonical TGF-β target genes, VEGF represents the first proangiogenic factor regulated by TGF-β1 through Sp1. Molecules such as Bevacizumab show promising results because of their ability to neutralize VEGF-induced angiogenic effect which leads to a decrease of tumor progression [93]. Vandetanib, an inhibitor of both VEGFR and EGFR factors, reveals anti-tumor and anti-metastatic properties [87], while Apatinib, which selectively suppresses VEGFR2 signaling and results in anti-apoptosis action, is proven as one of the candidates in the treatment of CCA [94]. The efficacy of Apatinib has been recently validated demonstrating

another mechanism through which this molecule influences apoptosis in CCA cells: the authors highlighted that Apatinib is able to reverse the VEGF signal by the inhibition of PI3K/Akt and VEGFR2/RAF/MEK/ERK pathways, which are usually upregulated during cell proliferation and differentiation in many organs [46].

A co-administration of pazopanib and trametinib appears to be an efficient treatment in refractory CCA, preventing the angiogenesis through parallel inhibition of RAF/MEK/ERK cascade VEGFR and PDGFR [95]. In addition, several authors highlighted that treatment with PlGF antibodies has shown beneficial effects in several experimental models for chronic liver disease, including cirrhosis, HCC, and CCA, by improving tumor burden [77]. This is probably due to inhibition of the proliferative effect of PlGF on macrophages and endothelial cells [96]. The up-regulation of PIGF is also correlated to liver-intestine cadherin decrease in ICC leading to angiogenesis, an increase of tumor aggression and consequently to poor outcome, while an inhibition of PIGF in experimental mouse model seems able to reduce the pathological angiogenesis and the vascular supply [76]. Since clinical studies using sorafenib (Nexavar) for treatment of CCA have shown minimal therapeutic benefit, the use of monoclonal antibodies targeting PlGF may serve as a potential treatment in patients with aggressive liver tumor. Taken together, this evidence supports the fact that a large number of actual preclinical studies have the VEGF pathway as a target due its ability to orchestrate tumor angiogenesis acting not only alone, but also in cooperation with other molecules and mechanisms (Figure 3).

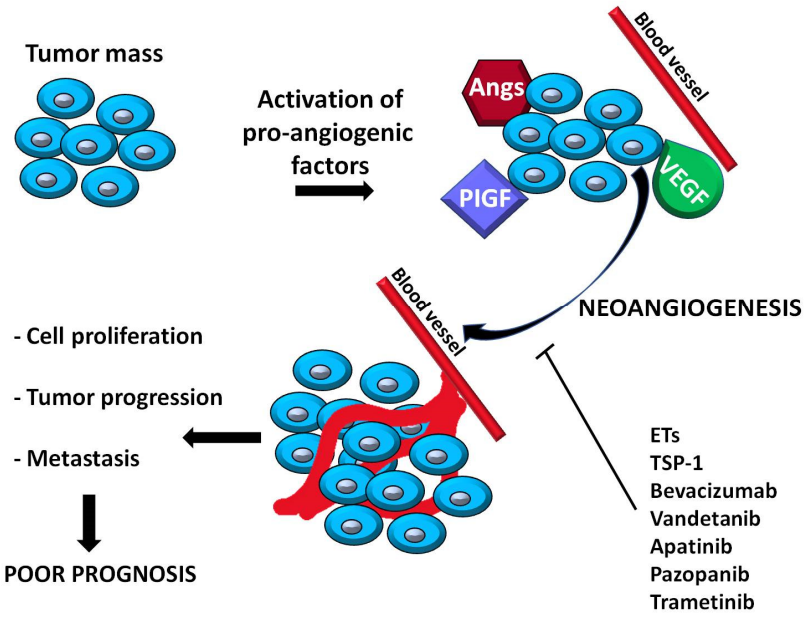

Figure 3. Neovascularization in biliary tract cancer. Cartoon shows the involvement of VEGF and other pro-angiogenic factors (PIGF and Angs) in the exacerbation of tumor-driven neoangiogenesis. New vascularization contributes to cell proliferation, tumor progression, and metastasis leading to poor prognosis. Molecules that interfere with VEGF signaling (ETs, TSP-1, apatinib, pazopanib, bavacizumab, vandetanib, and trametinib) have proved to be able to inhibit tumor evolution.

4.2. Angiopoietins and Thrombospondin 1

The neovascular status in CCA has also been tested through the evaluation of angiopoietins-1 and -2 (Ang) and thrombospondin-1 (TSP-1) [73]. The Ang system consists of the transmembrane endothelial tyrosine kinase Tie2 and its antagonistic circulating ligands Ang-1 and -2. It has been proved that Ang-2 can compromise the vascular integrity by facilitating the permeability to pro-angiogenetic factors as VEGF. Indeed, according with a previous in vitro research [97], Tang et al. detected high levels of VEGF in CCA accompanied to elevated Ang-2 expression in 57.6% of the analyzed samples together with an increase of MVD. It has been also demonstrated that Ang-2 is detectable in bile of

CCA patients. This suggests that tumor cells may produce Ang-2 in order to maintain neoangiogenesis at high levels like the cellular strategy "demand and supply" [78]. No significant differences are found for Ang-1. These data demonstrate that both VEGF and Ang-2 act as pro-angiogenic factors in tumor-associated blood vessels.

In addition to VEGF and Angs, TSP-1 may be another important factor involved in CCA neoangiogenesis. TSP1 is a multifunctional matrix protein which plays a controversial role in angiogenesis and tumor progression. Analyses of TSP-1 in CCA revealed that MDV is lower in TSP-1 positive patients compared to negative cases, showing a potential inhibitory role of this factor in CCA angiogenesis [98]. Findings from past years indicated VEGF as the responsible for estrogens (ERs) involvement in neovascular process, both in normal and in tumor tissues of breast cancer cells and mammary gland [99]. In 2009 Mancino et al. showed the impact of estrogens on VEGF and VEGFRs expression on human liver biopsies and cell line (HuH-28) derived from intrahepatic CCA [100]. Furthermore, in *vitro* experiments showed that 17β-estradiol, a VEGF agonist, is able to increase VEGF and VEGFR levels in CCA concomitantly with the stimulation of cell proliferation. On the contrary, ER and VEGF-TRAP (a receptor-based VEGF inhibitor) showed antagonist effects by blocking the proliferative response induced by 17β-estradiol in HuH-28 cells [100]. These data demonstrate the key role of VEGF in arbitrating estrogens-induced proliferation on human CCA. Considering this evidence, in the last decades the attention has moved toward the role of vascular growth factors in light of their crucial involvement in the pathophysiology and in the development of CCA to draw new potential anti-angiogenic therapies, which will be less invasive and dangerous than surgery.

4.3. Endothelins

The endothelin family consists of a group of 21 amino acid peptides produced primarily in the endothelium, epithelium, and smooth muscle cells [101]. Among the three isoforms of endothelins, endothelin-1 (ET-1) is the most abundant and widely expressed. Several studies have described their pleiotropic biological activity. These peptides are involved in the regulation of different processes, such as cell proliferation, angiogenesis and apoptosis. Furthermore, changes in transcriptional activity of endothelin and its receptors may be involved in the process of carcinogenesis and in the pathogenesis of numerous diseases. Their role has been evaluated in the development of breast, prostatic, colorectal, ovarian, lung, kidney, and endometrial cancer. ET-1 works through plasma membrane–localized endothelin receptor A (ETAR) and endothelin receptor B (ETBR), which are two classical G-protein–coupled receptors (GPCR). ETAR has been reported to be an important vasoconstrictor and growth-promoting receptor, it also sustains tumor progression, angiogenesis and metastatic diffusion [102]. In fact, ETAR-specific antagonists, such as BQ123, are utilized as potential cancer therapeutic drugs, while ETBR can block cell growth and vascular constriction [103]. Several studies showed that the overexpression of ET-1 and its receptors in many cancers (prostate, ovary, bladder, bowel, and kidney) are closely related to MVD and to VEGF levels in tumor cells, proving that ET-receptor antagonists could potentiate the therapeutic efficacy of conventional anticancer drugs [104–108]. In disagreement with previous studies in which the authors detected an increase of ET-1 and endothelin receptors (ET-Rs) expression, other investigators found that in CCA the binding of ET-1 to its receptors blocks in vitro the cellular growth [75]. In addition, ET-1 exerts an inhibitory effect on tumor proliferation accompanied to a decrease of VEGF and VEGF-R expression on CCA cells. In line with this observation, the inhibition induced by ET-1 is reversed by two antagonists of ET receptors, confirming their ability to mediate this uncommon effect in CCA [75]. In the end, endothelin receptor antagonists could open the possibility of a therapeutic use as adjuvant treatment of human cancer, but we still need to fully understand the usefulness of endothelin receptor antagonism in the clinical setting and to completely determine the frequency and the role of receptor subtypes in cell proliferation.

5. Conclusions and Future Prospective

Taken together, the studies reported in this review emphasize the potential use of anti-angiogenic factors in the modulation of cholangiocarcinoma in combination with other factors for a final synergic effect. CCA, originating from biliary epithelium, is a malignancy difficult to treat. In fact, CCA heterogeneity and multiplicity of the pathogenic factors can contribute to the: (i) CCA etiology; (ii) interactions between the neoplastic cells and their microenvironment; (iii) difficulty in classifying; (iv) limiting treatment options; and (v) lack of biomarker-driven targeted approaches for managing and/or preventing this aggressive liver malignancy. For this reason, the study of cholangiocarcinoma is markedly complex and it currently represents a great challenge. Angiogenesis is a critical component in the growth and progression of several tumors and it remains one of the main target for their therapeutic approach. At the moment, gemcitabine and platinum-based combination chemotherapy have been defined as the standard first-line chemotherapy for unresectable or metastatic biliary tract tumors. Unfortunately, the benefit of first-line therapy is limited, and for those patients whose tumors progress, no standard second-line chemotherapy has yet been established. Currently, the greatest clinical progress could be represented in the rapid advancements of nanotechnologies. New approaches with biomaterials and nanoparticle constructions are promising in order to further improve the efficacy of delivery of "drugs" to contrast the growth of CCA, so highly heterogeneous not only in initiation and location, but also in progression. Therefore, there is a need to further develop our understanding in the process of angiogenesis to optimize the selection and delivery of material to target tissues to treat angiogenesis-dependent tumors.

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