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Angiogenesis blockade for the treatment of gastrointestinal cancer

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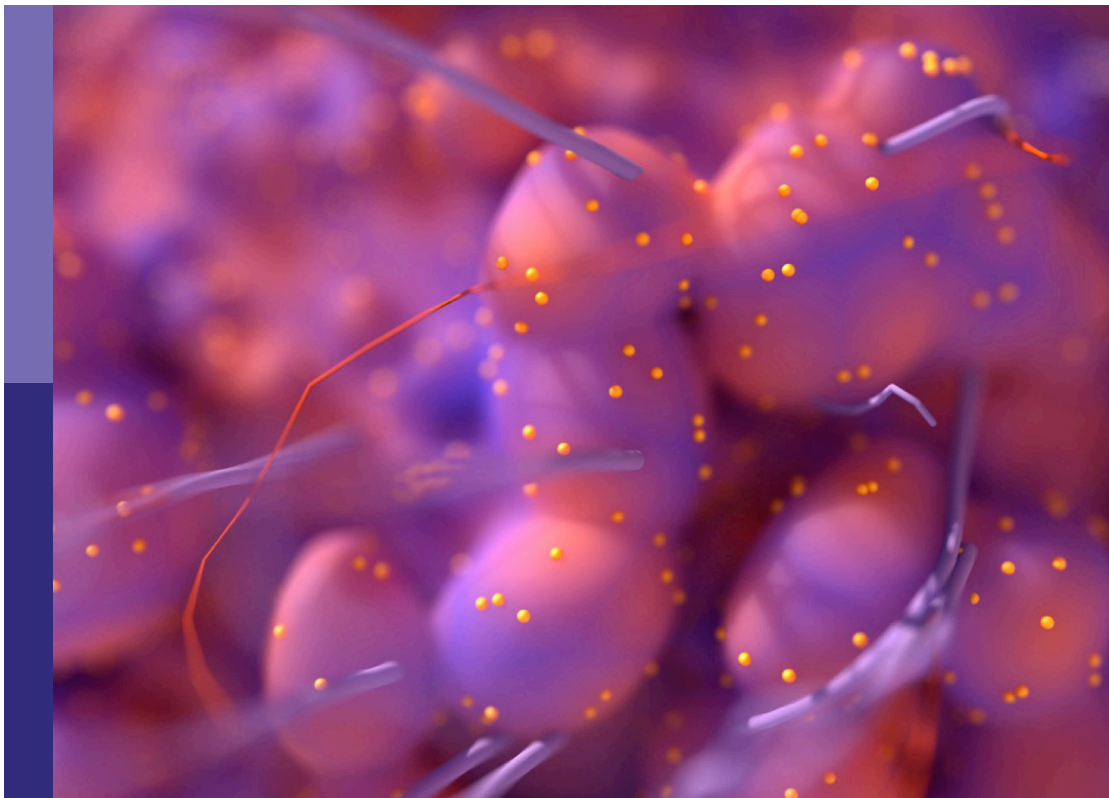
Published in
Frontiers in Oncology
Frontiers in Pharmacology



ISSN 1664-8714
ISBN 978-2-83251-689-8
DOI 10.3389/978-2-83251-689-8

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Angiogenesis blockade for the treatment of gastrointestinal cancer

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Citation

Bai, Z., Solimando, A. G., Passardi, A., Zhang, Z., Sier, C. F. M., He, Y., eds. (2023). *Angiogenesis blockade for the treatment of gastrointestinal cancer*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-689-8

Table of contents

frontiers | Frontiers in Oncology

TYPE Editorial
PUBLISHED 01 February 2023
DOI 10.3389/fonc.2023.1147849

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

RECEIVED 19 January 2023
ACCEPTED 23 January 2023
PUBLISHED 01 February 2023

CITATION
Passardi A, Bittoni A, Bai Z, Zhang Z, Sier C, He Y, Shahini E and Solimando AG (2023) Editorial: Angiogenesis blockade for the treatment of gastrointestinal cancer. *Front. Oncol.* 13:1147849. doi: 10.3389/fonc.2023.1147849

Editorial: Angiogenesis blockade for the treatment of gastrointestinal cancer

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KEYWORDS

angiogenesis, gastrointestinal cancer, ramucirumab, anti-angiogenic therapies, apatinib

Editorial on the Research Topic
Angiogenesis blockade for the treatment of gastrointestinal cancer

Angiogenesis is defined as a process of new blood vessel formation from pre-existing vessels. Since the concept of anti-angiogenesis for the treatment of cancer was proposed in the 1970's, tremendous effort has been invested in the field of vascular research. This has led to the development of numerous agents targeting angiogenesis, with over a dozen of anti-angiogenic drugs approved in clinic application and more in the pipeline of clinical trials (1).

The role of angiogenesis in gastrointestinal tumours is well known and anti-angiogenic agents are widely used in combination with chemotherapy with improved survival outcomes, most notably in colorectal cancer and hepatocellular carcinoma. The review edited by Gonzalez and colleagues focuses on the clinical evidence of efficacy, the ongoing clinical trials and the preclinical rationale underlying new combinations, especially with immunotherapy (Gonzalez et al.). Despite strong preclinical rationale and promising preliminary results in early clinical trials, anti-angiogenic therapies failed to revolutionize anti-cancer treatment in these tumour types. In this context, a greater knowledge of the mechanisms underlying primary and acquired resistance is an essential premise to improve treatment efficacy (Schiffmann et al.). A promising approach to overcome resistance is the use of nanomedicine. In fact, nanoparticles have shown significant advantages as anti-angiogenic drugs favouring targeted delivery, controlled release, prolonged half-life, and increased bioavailability (Yang et al.).

Angiogenesis inhibition is expected to be a promising therapeutic strategy in advanced gastric cancer (AGC). Several trials have been conducted to evaluate the efficacy of anti-angiogenic agents in metastatic disease, but with conflicting results. The most critical efficacy data were reported with ramucirumab, a fully humanized monoclonal antibody directed against the vascular endothelial growth factor receptor-2 (VEGFR2). Ramucirumab in

combination with paclitaxel significantly improved overall survival compared to placebo plus paclitaxel in patients with advanced gastric or gastro-oesophageal junction (GEJ) adenocarcinoma in the global phase 3 RAINBOW study (2). Similarly in the RAINBOW-Asia, a study with a similar design conducted in Asian patients, the median progression-free survival was higher in the ramucirumab plus paclitaxel group than placebo plus paclitaxel group. However the median overall survival was similar (3). On the other hand, trials testing other anti-angiogenic agents and early phase randomized trials (in both neoadjuvant and first-line settings) have shown negative results. Moreover, the lack of predictive biomarkers does not permit to select patients more likely to benefit from an anti-angiogenic approach (Salati et al.). A prospective study investigated the circulating angiogenic biomarkers' predictive role in thirty-five advanced AGC patients receiving ramucirumab and paclitaxel as second-line therapy (D'Alessandro et al.). Results showed that a greater decrease in VEGFC and Ang2 levels measured at the beginning of the third cycle of therapy compared to baseline corresponded to a lower risk of progression and therefore a longer progression-free survival. Interestingly, the study also showed an increase in VEGFC and Ang2 at the progression time, suggesting the activation of alternative pathways such as VEGFC/VEGFR3 and Ang2/Tie2 and supporting the rationale for dual inhibition of Ang2 and VEGRs.

Recent data suggest that inhibition of angiogenesis may also be helpful in preventing the occurrence and progression of gastric cancer precursor lesions (GPL). GPL refers to pathological changes of the gastric mucosa, including atrophic gastritis, intestinal metaplasia and dysplasia associated with the development of gastric cancer. In a preclinical study, Gao et al. investigated the activity of Atractylenolide III (AT-III), the main bioactive component of the traditional Chinese medicinal herb Atractylodes macrocephala, on GPL angiogenesis and expression of angiogenesis related factors. The authors found that AT-III reduced microvessels density and attenuated early angiogenesis in GPL rat models. Moreover, they showed a reduction of HIF-1α and VEGF-A, two important angiogenic markers, in GPL tissues after AT-III treatment and downregulation of DLL4, a component of the Notch signalling pathway involved in angiogenesis. These exciting results suggest a possible role for inhibition of angiogenesis with AT-III in treating gastric cancer precursor lesions, reducing the incidence and mortality of gastric cancer.

Apatinib is the first anti-angiogenic drug approved for treating advanced or metastatic gastric adenocarcinoma in China, where ramucirumab is unavailable. It is recommended in the third line setting, and despite small evidence of efficacy also as second line (Fu et al.). A recent trial explored a new scoring system calculated by combining systemic immune-inflammation index (SII) and prognostic nutritional index (PNI) as a predictor of efficacy in patients treated with intraperitoneal and systemic paclitaxel combined with Apatinib conversion therapy for gastric cancer with positive peritoneal cytology (Ding et al.). The prognosis of patients with high SII-PNI score was significantly worse and multivariate analyses confirmed the score as an independent prognostic factor for both overall survival and progression-free survival.

The phosphatidylinositol-3 kinase (PI3K) signalling pathway plays an essential role in cancer cell survival, angiogenesis and metastasis in several types of tumours, including colorectal cancer (CRC) (4). Recently, inhibition of the PI3k/Akt/mTOR pathway has become a promising therapeutic strategy in CRC patients with some encouraging preliminary results (5). An interesting study by Qin et al. sheds some light on the role of targeting PI3K in colorectal cancer and offers insights into PI3K inhibition biological effects. The authors evaluated ZDQ-0620, a novel pan-PI3K inhibitor, on human CRC cell lines demonstrating a significant activity in terms of inhibition of proliferation, migration and invasion. In addition, it was shown that ZDQ-0620 can significantly suppress angiogenesis through the inhibition of endothelial cell tube formation and vasculogenic mimicry. These data reinforce the evidence of an association between the PI3k/Akt/mTOR pathway and the VEGF-induced endothelial signalling, supporting the rationale for combinatorial PI3K and VEGF inhibition strategies in colorectal cancer, as already studied in other malignancies (6).

Inhibition of angiogenesis is a cornerstone of the treatment of neuroendocrine neoplasms (NENs). In their paper, Lauricella et al. provide an overview of the main molecular events driving angiogenesis in NENs and molecular mechanisms of resistance to anti-angiogenic drugs in these malignancies. In addition, authors discuss the results of clinical trials of several anti-angiogenic agents, including novel compounds such as the HIF-2a inhibitor belzutifan, and different combinatorial treatment, including association of anti-angiogenic agent to immunotherapy or mTOR inhibitors, offering a perspective about present and future treatment of NENs.

Author contributions

AP, AB and AS wrote the first draft of the manuscript, all the authors contributed to manuscript revision, read, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers in Oncology

REVIEW
published: 10 January 2022
doi: 10.3389/fonc.2021.789330

Opportunities and Challenges of Nanoparticles in Digestive Tumours as Anti-Angiogenic Therapies

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Digestive tumours, a common kind of malignancy worldwide, have recently led to the most tumour-related deaths. Angiogenesis, the process of forming novel blood vessels from pre-existing vessels, is involved in various physiological and pathological processes in the body. Many studies suggest that abnormal angiogenesis plays an important role in the growth, progression, and metastasis of digestive tumours. Therefore, anti-angiogenic therapy is considered a promising target for improving therapeutic efficacy. Traditional strategies such as bevacizumab and regorafenib can target and block the activity of proangiogenic factors to treat digestive tumours. However, due to resistance and some limitations, such as poor pharmacokinetics, their efficacy is not always satisfactory. In recent years, nanotechnology-based anti-angiogenic therapies have emerged as a new way to treat digestive tumours. Compared with commonly used drugs, nanoparticles show great potential in tumour targeted delivery, controlled drug release, prolonged cycle time, and increased drug bioavailability. Therefore, anti-angiogenic nanoparticles may be an effective complementary therapy to treat digestive tumours. In this review, we outline the different mechanisms of angiogenesis, the effects of nanoparticles on angiogenesis, and their biomedical applications in various kinds of digestive tumours. In addition, the opportunities and challenges are briefly discussed.

Keywords: digestive tumours, angiogenesis, anti-angiogenesis, nanoparticles, therapy

OPEN ACCESS

Edited by:
Cyril Corbet, Fonds National de la Recherche Scientifique (FNRS), Belgium

Reviewed by:
Francesco Pezzella, University of Oxford, United Kingdom
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *04 October 2021*
Accepted: *10 December 2021*
Published: *10 January 2022*

Citation:
Yang Z, Deng W, Zhang X, An Y, Liu Y, Yao H and Zhang Z (2022) Opportunities and Challenges of Nanoparticles in Digestive Tumours as Anti-Angiogenic Therapies. Front. Oncol. 11:789330. doi: 10.3389/fonc.2021.789330

INTRODUCTION

The human digestive system consists of digestive gland organs (salivary glands, liver, and pancreas) and digestive tubes (oral cavity, pharynx, oesophagus, stomach, small intestine, large intestine, and rectum). Digestive tumours, principally hepatocellular carcinoma, pancreatic cancer, oesophageal cancer, gastric cancer, and colorectal cancer, lead to the greatest number of tumour-related deaths worldwide (1, 2). Moreover, digestive tumours accounted for 43.3% of the cancer incidence from 2000 to 2015 in China (3). The current therapeutic strategies for digestive tumours mainly consist of surgical resection, chemotherapy, radiotherapy, molecular targeting therapy, and immunotherapy. Because of indefinite clinical symptoms, deficient imaging features, and sensitive biomarkers, most patients are diagnosed at an advanced stage with an unsatisfactory 5-year survival rate (4, 5). Chemotherapy, including neoadjuvant and postoperative therapy, which is currently the primary

approach to treat such patients, cannot achieve gratifying curative effects because of the multidrug resistance mechanisms in tumours (6, 7). Therefore, novel therapeutic strategies are required to better treat patients with digestive tumours.

Angiogenesis is the formation of novel blood vessels from pre-existing vessels and is a highly regulated process (8–10). Judah Folkman, considered the father of angiogenesis research, advanced the notion in 1971 that tumour growth depends on angiogenesis, which is essential for removing metabolites, supplying oxygen and nutrients, and promoting the metastatic ability of cancer cells (11, 12). Additionally, Folkman proposed that the tumour size would be limited to less than 2 mm^3 in the absence of angiogenesis and would then enter a dormant state, thus raising the possibility of using anti-angiogenic antibodies for the treatment of cancers (13, 14). Although a variety of anti-angiogenic drugs, such as bevacizumab and sunitinib, were approved worldwide in the following half-century and have certain effectiveness, adaptive resistance and some adverse events associated with poor pharmacokinetics have limited the further application of this therapy (15, 16). The mechanisms for anti-angiogenic therapeutic resistance have been widely reported mainly including direct effects of hypoxia (co-option of normal vessels in paracancerous tissues, vascular mimicry, and induction of tumour invasion and metastasis), the influence of tumour stromal cells (recruitment of tumour-associated macrophages, endothelial progenitor cells, and pro-angiogenic myeloid cells), and upregulating alternative pro-angiogenic factors (17–19). Additionally, some tumour cells have been reported that could continuously grow without angiogenesis, which might result in the resistance of anti-angiogenic therapies (20, 21). Thus, exploring novel anti-angiogenic tactics to surmount the resistance and side effects to achieve better therapeutic effects is urgent.

The rapid advancement of nanotechnology has brought about more opportunities for anti-angiogenic therapies to treat digestive tumours. Due to the highly leaky blood vasculature and absence of functional lymphatic vessels in solid tumours, nanoparticles (20–200 nm in diameter) could avoid immune clearance, further prolonging their half-life and specifically accumulating in tumour tissues, called the enhanced permeability and retention (EPR) effect (22–24). Thus, nanotechnology-based medicine, also called nanomedicine, has made many advances in cancer treatment, especially in the areas of targeted delivery of drugs and medical imaging (25, 26). Moreover, nanoparticles could also solve the aforementioned shortcomings of current conventional anti-angiogenic therapies. In fact, nanoparticles have demonstrated great advantages as anti-angiogenic drugs through targeted delivery, controlled release, prolonged half-life, and increased bioavailability. However, due to their dissimilar physicochemical properties, different nanoparticles possess corresponding features of biodistribution properties and half-lives (27, 28). Therefore, this article aimed to summarize the different mechanisms of angiogenesis and the actual applications of anti-angiogenic nanoparticles in digestive tumours and discuss the current opportunities and challenges.

MECHANISMS OF ANGIOGENESIS

Angiogenesis primarily consists of four sequential steps: I) dissolution of extracellular matrix components surrounding blood vessels like basement membrane glycoproteins by proteolytic enzymes; II) activation and migration of endothelial cell; III) proliferation of endothelial cell; and IV) formation of capillary tubes (29). However, when the balance between anti-angiogenic factors and pro-angiogenic factors is broken in some pathological conditions (like asthma, atherosclerosis, myocardial ischaemia, hypertension, and tumour progression), angiogenic activators will be upregulated, further resulting in aberrant angiogenesis (30).

Mechanisms of Tumour Angiogenesis

In 1971, Folkman proposed that tumours could not grow more than 2 mm^3 without vascular supply because of insufficient oxygen and nutrition supply and poor clearance of metabolic waste, which would further cause hypoxia or acidosis (31). The physiological angiogenic process is maintained under the dynamically relative homeostasis, which is being referred to as "angiogenic switch" (32). Once this homeostasis is disrupted in tumours, the "angiogenic switch" will be active, and the vascular endothelial cells will be affected to upregulate the secretion of angiogenic promoters and downregulate the secretion of angiogenic inhibitors (33, 34). Over the past 50 years, the complex mechanisms of tumour angiogenesis have been exposed with more intensified researches.

Different types of angiogenic regulators could be released from tumour cells, blood, endothelial cells, and extracellular matrix (35, 36). Currently reported angiogenic promoters include vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factor (TGF), fibroblast growth factor (FGF), angiopoietin-1 and -2 (ANG-1 and -2), and platelet-derived growth factor (PDGF), while angiogenic inhibitors include angiostatin; endostatin; platelet factor-4; tissue inhibitors of metalloproteinases (TIMPs); thrombospondin-1 (TSP-1); interferon (IFN)-α, -β, and -γ; and interleukin (IL)-12 (37). Some biological pathways like metabolic stress (hypoxia, hypoglycaemia, and lower pH), gene mutation (activation of oncogenes and inactivation of anti-oncogene), inflammatory response (tissue inflammatory infiltration), and mechanical stress (interactions by proliferating cells) can turn on the "angiogenic switch", further resulting in tumourigenesis (38, 39). Among these pathways, hypoxia plays an important role in driving tumour angiogenesis, which can stimulate the expression of angiogenic stimulating factors in cancer cells. The transcriptional programs mediated by hypoxia-inducible factor (HIF) can activate hypoxia, which acts as a central regulator of detection and adaptive oxygen levels. HIF promotes the overexpression of VEGF A (VEGFA) and its receptors VEGF receptor-1 and -2 (VEGFR-1 and -2) (16). Moreover, HIF can promote the expression of ANG-2, which helps the proliferation of endothelial cells in hypoxic tumour areas, further destroying the integrity of the vascular wall (40). Tumour hypoxic conditions can also upregulate the expression of PDGF, which

conducts as the mitogen of fibroblast and mesenchymal cells and induces different angiogenic actions (41). Additionally, matrix metalloproteinases (MMPs) can degrade the extracellular matrix, further mediating various changes in tumour microenvironment to advance the angiogenic process (42).

Conventional Anti-Angiogenic Therapy and Limitations

In the past 20 years, various anti-angiogenic agents were developed and prolonged the survival time of patients to some extent. Among them, more than 10 anti-angiogenic agents have been approved for the treatment of different digestive malignancies (**Table S1** of the Supplementary Material) (43, 44). The mechanism of such agents is to prevent tumour cells from obtaining nutrition by restricting available blood vessels and blocking the formation of novel blood vessels in tumour sites. Briefly, the mechanisms of most existing anti-angiogenic strategies include blocking the interactions of VEGF and VEGFR to their respective receptors (**Figure 1**) (43). It was also reported that metronomic chemotherapy, which was defined as using small doses of the high-frequency chemotherapeutic drug to achieve a lower but effective range of drug concentrations over long periods without significant toxicity, could downregulate VEGF, further upregulating the expression of TSP-1 to play an important role in inhibiting tumour angiogenic dormancy (45–47)[a-c]. However, these therapies often have adverse reactions like drug resistance, toxicity, and even thrombotic and haemorrhagic diseases (48).

Despite that anti-angiogenic therapies sometimes stabilize diseases and prolong survival, such treatment might lead to more drug-resistant tumours and a higher patient recurrence rate. Such clinical harm might be related to the compensatory upregulation of angiogenic factors, further promoting tumour angiogenesis and tumour escape mechanism, which lead to acquired drug resistance. Among them, hypoxia plays an important role in tumour resistance to anti-angiogenic therapies and leads to more aggressive metastatic diseases with worse prognosis. Hypoxia-related HIF-1 pathway plays an important role in the resistance to anti-angiogenic therapies and is the main survival factor for cancer cells to overcome the hypoxic environment. Hypoxia is reported to regulate hepatocyte growth factor/mesenchymal–epithelial transition factor (HGF/c-MET) signal pathway, further activating mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) cascades, phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway, and so on to promote tumourigenesis, progression, and drug resistance (49–52)[y3-y6]. In the phase III METEOR trial, advanced renal cell carcinoma (RCC) patients after previous VEGFR-targeted therapy were given cabozantinib (tyrosine kinase inhibitor), which could raise the survival rate (53)[y7].

For example, the anti-angiogenic efficacy of bevacizumab might be greatly weakened by the alternative pro-angiogenic signals generated during tumour proliferation and metastasis (46). The hypoxic microenvironment produced in the anti-angiogenic process could induce HIF-1 α and stimulate the expression of β1 integrin, which had been upregulated in bevacizumab-resistant tumours. Meanwhile, targeting β integrin could enhance anti-angiogenic therapies and inhibit the growth of bevacizumab-resistant tumours in xenograft models (54)[y8]. Additionally, many preclinical and phase I/II clinical trials have shown that a single anti-angiogenic strategy cannot effectively inhibit tumour growth, which promoted the development of multi-drug combination therapies (55). However, in addition to causing serious side effects, combination therapies usually performed poor biodistribution and pharmacokinetic characteristics (56).

With the development of science and technology, the field of nanobiology has attracted increasing attention in recent years. Nanotechnology-based medicine, also known as nanomedicine, has promoted tremendous advances in cancer treatment,

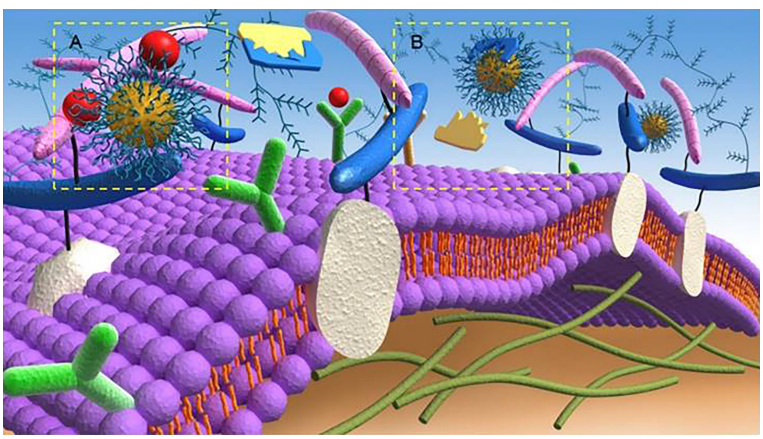

FIGURE 1 | Anti-angiogenic mechanisms of targeting **(A)** angiogenic growth factors and **(B)** proteolytic enzymes of the extracellular matrix by anti-angiogenic strategies. Reproduced with permission (42). Copyright 2018, Ivyspring International Publisher.

especially in the areas of targeted drug delivery and medical imaging. Meanwhile, nanomedicine has made remarkable achievements in the research and development of drug development for clinical tumour treatment. Among them, many nanobased drugs have been used in the clinical chemotherapy of multiple gastrointestinal tumours, such as colorectal cancer, including doxorubicin liposomes, paclitaxel liposomes, and albumin-bound paclitaxel (27). The drugs mentioned above not only can improve the local treatment concentration but also can significantly reduce the non-specific toxicity of organs. Moreover, they can solve the problem of allergies to solvents (such as castor oil) caused by the poor water solubility of some common chemotherapy drugs (such as paclitaxel), further improving the quality of life of patients (57). These nanoparticles take different easily modified and highly biocompatible materials as their main body (such as organic compounds, proteins, lipids, and polymers), which are rationally modified and designed to be multifunctional drugs to achieve specific imaging and precise treatment of tumours (58).

MECHANISM AND SUPERIORITY OF NANOPARTICLES

Generally, nanomedicine is defined as a technology used to study the properties and potential applications of materials sized 20–200 nm (59). Compared with traditional drugs, nanoparticles have unique properties and advantages, such as a large surface area, to achieve a high drug loading rate, easy surface modification to add new functions, protection of drugs from degradation or metabolism, controlled release of drugs, and passive accumulation in tumours (60). They have the potential to modulate the pharmacokinetic and pharmacodynamic characteristics of drugs to increase their therapeutic concentration. Compared with traditional drug delivery methods, intravenous administration of nanoparticles can passively accumulate drugs in tumour tissues through the EPR effect, further improving the drug concentration in the tumour site, which is realized based on the special histopathological characteristics of tumour tissue (61). Briefly, normal blood vessels are composed of dense endothelial cells, which can prevent the escape and extravasation of nanoparticles. However, the blood vessels of tumours are leaky and highly permeable, resulting in the preferential accumulation of nanoparticles in tumour tissues (**Figure 2**) (43). In addition, targeting molecules can be modified on the surface of nanoparticles to bind to highly expressed receptors on the surface of tumour cells to play an active targeting role of tumour tissue (62). Nanoparticles are increasingly widely considered for the diagnosis and treatment of tumours because of their important properties of passive and active targeting.

Passive Targeting of Nanoparticles

Passive-targeting nanomaterials, which can be transported into tumour tissues through the EPR effect, are also the most widely studied nanoparticle drug delivery systems at present. To ensure that the tumour tissues receive sufficient nutrients and oxygen to facilitate rapid tumour growth, the blood vessels of most solid tumours have structural defects and produce a large number of vascular permeability factors so that most tumours exhibit high vascular permeability (63). The phenomenon produced by this special pathological anatomy that can promote the accumulation

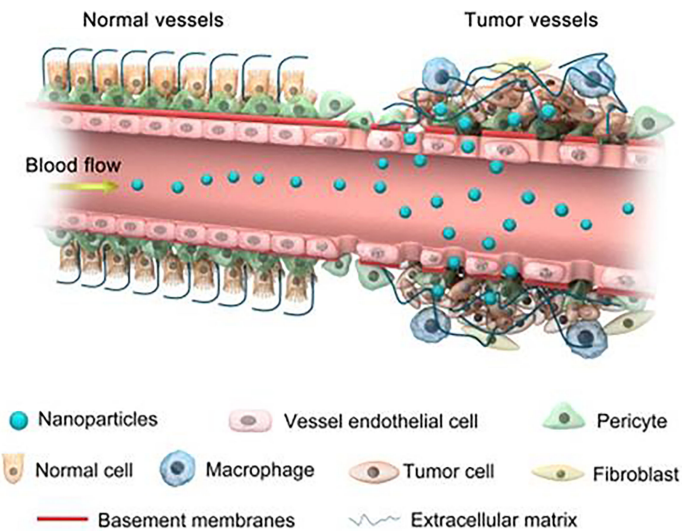

FIGURE 2 | Schematic diagram of nanoparticles targeting tumour tissues through enhanced permeability and retention (EPR) effect. Normal blood vessels are composed of dense endothelial cells, which can prevent the escape and extravasation of nanoparticles. Blood vessels of tumour tissue are leaky and highly permeable, allowing the preferential accumulation of nanoparticles in the interstitial space of the tumour. Reproduced with permission (42). Copyright 2018, Ivyspring International Publisher.

of nanomaterial substances in tumour tissues is called the EPR effect, which mainly manifests as macromolecules with molecular weights greater than 40 kDa selectively leaking out of tumour tissues and accumulating in solid tumours but not in normal tissues (64). The EPR effect is mainly related to the size of nanoparticles. Briefly, when the diameter is less than 4 nm, nanoparticles are not only likely to be filtered through the glomerulus in the systemic circulation and then discharged through the kidney but also diffuse back into the blood circulation after entering the tumour tissue because of the large pores between the vascular endothelium, which reduces the passive-targeting effect.

Nanoparticles greater than 400 nm in diameter are easily swallowed up by the reticuloendothelial system as foreign matter during blood circulation. Even if they reach the blood vessels near the tumour, they cannot permeate into tumour tissues because they are larger than the blood vessels. That is why the EPR effect is best when the size of the nanoparticles is from 20 to 200 nm (65–67). The EPR effect based on the characteristics of solid tumours is a milestone for tumour-targeted drug delivery and has been widely used in the development of nanobased antitumour drugs.

Stimulus-Responsive Nanoparticles

Stimulus-responsive nanoparticles achieve targeted delivery and controlled release of drugs by responding to the stimulation of physics, chemistry, and biomolecules (68). Their advantage is that they can realize the controlled release of drugs through the stimulation of trigger conditions, further reduce the loss of drugs during the process of blood circulation and reduce the toxicity of drugs (69).

It is well known that tumour tissue has a lower pH than normal tissue because cells in normal tissue are powered by oxidative phosphorylation, while in tumour tissue, cells are powered by glycolysis, resulting in the production of large amounts of lactic acid, which is known as the Warburg effect (70). The pH value in normal tissues is close to neutral (7.4), while in most tumour cells, it is slightly acidic (≤6.5) (70). Nanoparticles are prepared using pH-dependent chemical bonds to make them stable under physiological conditions. In a weakly acidic tumour environment, the chemical bonds can break, release the drugs, and improve the local accumulation rate of drugs in the tumour site (71).

According to the literature, cancer stromal cells actively secrete glutathione (GSH), resulting in a concentration of GSH in tumour cells (2~10 mmol/L) 100~1,000 times that in normal cells (2~20 μmol/L) and 100 times that in normal tissue, resulting in a strongly reducing environment in colorectal cancer (72, 73). Due to the existence of the mercaptan group in GSH, it can act as a reducing agent and become an important antioxidant, further decomposing some essential chemical bonds such as disulfide bonds and diselenide bonds (74, 75). Therefore, GSH stimulation-responsive nanoparticles are widely used for targeted delivery and controlled release of antitumour drugs. In addition, temperature, magnetic force, light, electric field, force, ATP, DNA, RNA, and enzymes can also be used as factors to stimulate drug release in nanoparticles, further improving the tumour treatment efficiency (76, 77).

Active Targeting of Nanoparticles

Active-targeting nanomaterials, a novel approach to antitumour nanotechnology, can specifically bind to receptors overexpressed on the surface of tumours and tumour vascular endothelial cells by modifying the corresponding ligands on the surface of nanoparticles. Active targeting mainly depends on the interaction between ligand molecules and the surface receptors of tumour cells. Therefore, the ideal ligands used for active targeted delivery of antitumour drugs should be able to bind to tumour cells as much as possible but not to normal cells. A variety of ligands have been used for active antitumour drug targeted delivery, including folic acid, glucose, peptides, proteins, antibodies, and small interfering RNAs (siRNAs) (78, 79). The advantage of active targeting nanocarriers is that off-target effects can be avoided as much as possible (80). In summary, the aim of active targeting nanomedicine is to achieve a high affinity between receptors and ligands. Compared with passive targeting, the active targeted nanomaterial delivery system can enhance the binding of nanomaterials to tumour cells, reduce the non-specific uptake of nanomaterials, avoid the generation of drug resistance, and increase the distribution of drugs at the tumour site (81). In addition, nanocarriers have a variety of drug delivery capabilities, such as timely administration of chemotherapeutic drugs, targeted drugs, prodrugs, and drug kinases (82).

Imaging Diagnosis

Because different tumour stages require corresponding treatment methods, the treatment of digestive tumours largely depends on accurate imaging diagnostic technology. CT, MRI, and PET are the most commonly used imaging techniques for diagnosing digestive tumours in the clinic. Nevertheless, these techniques are mainly based on the histomorphology and metabolic changes of tumours, which exhibit poor sensitivity in some cases, such as micrometastasis and small tumours (83). Nanoparticles can wrap high concentrations of imaging agents such as iodine, magnetic materials, and radioactive substances inside themselves to amplify the signals generated by tumours. In addition, nanocarriers can weaken the signal intensity of normal tissue, further reducing any interference with the diagnosis (84, 85).

In addition, new technologies such as near-infrared (NIR) fluorescence imaging can be used to observe the tissue morphology and metabolism, providing additional possibilities to detect important anatomical structures and tumour lymph node metastasis in real-time during surgery (86). The wavelength of NIR fluorescence is from 700 to 900 nm, with high tissue penetration (at the centimetre level) (87). After packaging NIR agents into nanoparticles and injection, the fluorescence signal can be captured by a laparoscopic fluorescent imaging system in real time, while human eyes are not sensitive to the NIR wavelength, and their presence will not change the surgical field.

Synergistic Therapy

Phototherapy, a light-mediated therapy, has gradually attracted increasing attention recently because of its advantages of minimal invasiveness, spatiotemporal controllability, and low toxicity in tumour treatment. Phototherapy includes photodynamic therapy (PDT) and photothermal therapy (PTT). PDT mainly relies on photosensitizers to absorb energy under light conditions, causing a series of photochemical and photobiological reactions and producing cytotoxic substances such as reactive oxygen species (ROS), which selectively damage tumour tissues (88). Indocyanine green (ICG), which has been approved by the Food and Drug Administration (FDA) for intraoperative fluorescence imaging in the clinic, is currently widely studied as a PDT agent due to its antitumour effects (89). The principle of PTT is that the photothermal medium converts light energy into heat energy after being irradiated by a laser, causing an increase in the local tissue temperature to achieve the killing effect (90). PTT has a wide antitumour spectrum because the process does not need oxygen. Additionally, most PTT agents are excited by NIR lasers, which can penetrate deeply into tissues and kill more tumour cells (91).

A synergistic system of phototherapy and chemotherapy was constructed through nanotechnology, in which the improvement of vascular permeability caused by phototherapy could increase the accumulation of nanoparticles in tumours, further enhancing the effect of chemotherapy (92). The thermal effect induced by PTT not only promotes the release of drugs by the nanoparticles but also changes the permeability of the cell membrane, further increasing the endocytosis of cancer cells to chemotherapeutic drugs (93). Nanotechnology can integrate different therapeutic functions into single nanoparticles, achieving a more thorough treatment mode and bringing new ideas and hope for tumour treatment. Moreover, nanoparticles can achieve tumour theranostics, which means that effective treatment is performed at the same time as tumour diagnosis, while the curative effect is monitored by diagnostic methods at the same time (94).

ANTI-ANGIOGENIC NANOPARTICLES IN DIGESTIVE TUMOURS

Based on the advantages mentioned above, researchers had developed many novel nanoparticles to overcome the drug resistance of anti-angiogenic therapies in digestive tumours. The fundamental mechanisms of enhanced anti-angiogenic treatment through nanoparticles are shown in **Figure 3** (95). Nanoparticles loaded with anti-angiogenic drugs had high drug release efficiency and bioavailability, which could actively target tumour tissues. As for some drugs with poor solubility, nanocarriers could provide better delivery characteristics through liposome coating. In addition, nanoparticles can specifically control drug release through surface modification, effectively reduce the therapeutic dose and administration frequency, and further reduce the cytotoxicity and adverse reactions of chemotherapeutic drugs. For example, some nanoparticles could be transferred to tumour tissues *in vivo* through a magnetic field and then respond to acidic tumour environment for releasing loaded drugs (96). More importantly, combining anti-angiogenic therapies with other targeted therapeutic drugs and/or immunotherapy could effectively reduce resistances by blocking their occurrence mechanism. Co-delivering anti-angiogenic agents and hypoxia-specific siRNA through nanoparticles, the most critical step of tumour resistance (hypoxia) was inhibited to defeat drug resistance and acquire a better therapeutic effect (97, 98). Additionally, tumour-targeted nanoparticles co-delivered by oxygen-generating MnO_2 and sorafenib could

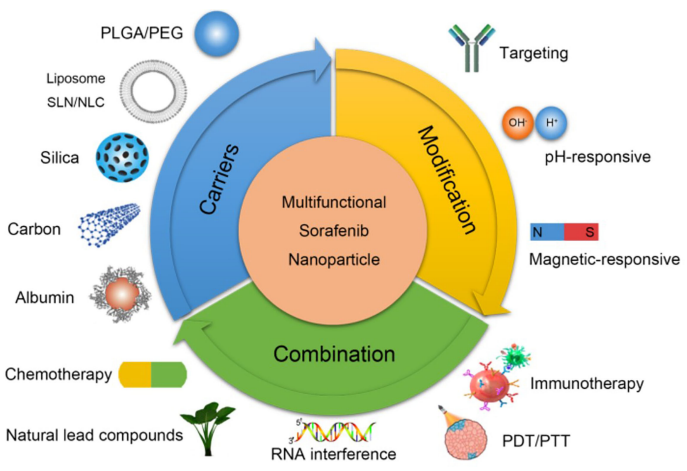

FIGURE 3 | Mechanisms of anti-angiogenic nanoparticles in hepatocellular carcinoma. Improving the biocompatibility of anti-angiogenic agents through different nanocarriers (top left). Increasing the targeting and responsiveness of anti-angiogenic agents through modification methods (top right). Enhancing curative effect and overcoming resistance through combined with other therapies (bottom) (95). Copyright 2018, Ivyspring International Publisher.

decompose H_2O_2 to oxygen to alleviate hypoxia-driven drug resistance further enhance anti-angiogenic effect and provide benefits to digestive tumours treatment (99). The reported anti-angiogenic nanotherapeutics are described below, which we are looking forward to overcoming the limitations of the current strategies, further improving their antitumour therapeutic outcomes in digestive tumours.

Metal and Metallic Compounds in Nanoparticles

Metal nanoparticles have been widely considered with various applications in digestive tumours for anti-angiogenic treatment. Among them, gold (Au) nanoparticles are considered one of the most appropriate therapeutic options against tumours, and they have the advantages of chemical stability, small dimensions, low cytotoxicity, and inherent biocompatibility (100). Additionally, some studies have indicated that Au nanoparticles (AuNPs) possess anti-angiogenic properties. Mukherjee et al. first reported in 2005 that Au nanoparticles could specifically bind to VEGF-165 and basic FGF, further resulting in inhibition of endothelial and fibroblast cell proliferation *in vitro* as well as VEGF-induced permeability and angiogenesis *in vivo*. In addition, such nanoparticles exhibited no significant hepatic or renal toxicity in tumour-bearing mice (101). Based on the superior PTT characteristics of Au nanoparticles, CD44v6-GNSs (Au nanoparticle-conjugated CD44v6 monoclonal antibodies) were constructed. Such CD44v6-GNSs could inhibit the growth of gastric cancer and remarkably extend survival in tumour-bearing mice. Moreover, photoacoustic imaging indicated that CD44v6-GNSs could specifically target the gastric cancer vascular system after intravenous injection *in vivo* (102).

Furthermore, Au nanoparticles possess considerable advantages as carriers for targeted drug delivery. 5-Fluorouracil (5-FU), a thymidylate synthase inhibitor, is a commonly used chemotherapeutic drug against colorectal cancer with various side effects, such as bone marrow suppression, anorexia, and vomiting (103). Liszbinski et al. loaded 5-FU on Au nanoparticles coated with anti-EGFR (EGF receptor) antibodies to treat colorectal cancer. Such AuNP-5FU-EGFR nanoparticles showed superior efficiency in apoptosis induction over single 5-FU with no significant cytotoxic effects in human colorectal cancer cells (104). Delivering siRNA to tumour tissues has always been a great challenge. Because of their higher molecular weight and polyanionic properties, naked siRNAs would be degraded swiftly by serum ribonucleases, causing difficulties in crossing cellular membranes (105). It was reported that Au nanoparticles might be an appropriate and safe choice to deliver siRNA. A novel sequence of siRNA that targeted the oncogene c-Myc was designed and bound to branched polyethylenimine (bPEI)-modified Au nanoparticles. Such siRNA/bPEI/AuNPs could effectively deliver siRNA into human hepatoma cells and successfully silence the c-Myc gene with no significant cytotoxicity (106). Interestingly, the expression of the c-Myc gene was positively correlated with the expression of proangiogenic-related genes in many digestive tumours, including hepatocellular carcinoma, pancreatic cancer, and colorectal cancer (107–109).

Radiation therapy is a common clinical treatment for digestive tumours and could be used as a supplement to neoadjuvant therapy or as an auxiliary mean to prevent postoperative tumour recurrence. As a high atomic number element, Au could cause tumour tissue to have a mass energy coefficient and a higher atomic number than normal tissue while targeting the tumour region, further improving the treatment rate of radiotherapy. Au nanoparticles could enhance the efficacy of radiotherapy by regulating the cell cycle, inducing DNA damage, producing oxidative stress, and potentially interfering with bystander effects (110). Alhussan et al. functionalized Au nanoparticles with polyethylene glycol (PEG) and arginine-glycine-aspartate (RGD), the ligand for integrins, to acquire the $GNP_{PEG\text{-}RGD}$ complex. $GNP_{PEG\text{-}RGD}$ could not only target pancreatic cancer cells but also be used as a drug carrier and radiosensitizing agent. Moreover, the uptake of $GNP_{PEG\text{-}RGD}$ by cancer-associated fibroblasts (CAFs) could be 10% higher than that of pancreatic cancer cells, causing the targeted killing of CAFs and achieving an antitumour effect (111). As a significant stromal cell component, CAFs could promote angiogenesis in digestive tumours by upregulating proangiogenic factors and controlling the biomechanical properties of the tumour matrix, such as elasticity, stiffness, and interstitial fluid pressure (112, 113). There are also studies reporting that biodegradable honeycomb-like gold nanoparticles (HGNs) could act as both radiosensitizing agents and photothermal agents to achieve synergistic photothermal radiotherapy. Such an approach could help nanoparticles accumulate more efficiently to improve the oxygen supply and damage double-stranded DNA in the tumour tissues of xenograft pancreatic cancer mice (114).

As a trace element, copper (Cu) plays an important role in multiple biological processes, such as oxidative metabolism, angiogenesis, tumourigenesis, metastasis, and relapse, while its imbalance can cause various diseases (115). A retrospective study demonstrated that a higher serum copper level was associated with relapse or disease progression in haematological malignancies. In addition, it was positively related to some adverse prognostic markers in chronic lymphocytic leukaemia, such as an increased percentage of unmutated IgVH and higher expression of ZAP70 and CD38 (116). Bai et al. reported hollow copper sulfide (CuS) nanoparticles encapsulating sorafenib and surface modified with anti-VEGFR antibodies. While CuS-SF@CMV nanoparticles kill hepatoma cells by CuS-mediated PTT, sorafenib and anti-VEGFR antibodies inhibit tumour angiogenesis through the PI3K/AKT and Ras/Raf/MEK/ERK pathways to achieve continuous inhibition against tumour metastasis (117). Cui et al. modified the surface of CuS with PEG and cyclic RGDfK peptide [c(RGDfK)] to acquire CuS-PEG-c(RGDfK) nanoparticles, which not only possessed the property of selective tumour uptake but also significantly killed hepatoma cells through thermal ablation (118). There are also studies reporting that cetuximab was modified on CuS to acquire active targeting of CuS nanoparticles (CuS-Ab NPs) with

excellent PTT efficacy and superior biocompatibility in xenograft models (119).

Silver (Ag) nanoparticles are another important therapeutic noble metal widely used in medical applications. Gurunathan et al. first reported that Ag nanoparticles could have anti-angiogenic potential by inhibiting the VEGF-induced PI3K/Akt cell survival signal in bovine retinal endothelial cells (120). It was also reported that Ag nanoparticles could inhibit the process of angiogenesis by exhibiting dose-dependent cytotoxic effects on endothelial cells (121). Ag nanoparticles also had effective antitumour activities in lung cancer, melanoma, cervical cancer, breast cancer, and lymphoma cell lines (122–124). Given the lack of relevant research, additional animal experiments and preclinical studies are expected to validate the effectiveness of Ag nanoparticles in treating digestive tumours.

Superparamagnetic iron oxide nanoparticles (SPIONs) are widely used as targeted drug carriers with superior biocompatibility, good chemical stability, and low toxicity (125). Wang et al. loaded gambogic acid onto magnetic Fe_3O_4 nanoparticles (MNP-Fe_3O_4) called GA-MNP-Fe_3O_4, which inhibited the migration and proliferation of pancreatic cancer cells and downregulated the downstream target gene of angiogenesis, VEGF (126). It has also been reported that using hyaluronate (HA)- and trimethyl chitosan (TMC)-recoated SPIONs could significantly prevent the angiogenesis of colorectal cancer cells (127). Additionally, SPIONs modified with vasculature-specific binding peptides could potentially be used to observe the angiogenic status of gastric cancer *in vivo* (128).

Non-Metallic Nanoparticles

With the advantages of a large relative surface area, adjustable pore size, higher drug loading efficiency, easy functionalization, and good biocompatibility, silica-based nanoparticles have been widely used as drug delivery systems in nanomedicine (129). In addition, silicate nanoparticles have been found to have potential anti-angiogenic effects against retinal neovascularization. Jo et al. demonstrated that intravitreal injection of silicate nanoparticles could effectively reduce anomalous retinal angiogenesis in retinopathy mice without direct toxicity. The specific mechanism might be that such nanoparticles could inhibit VEGF-related angiogenesis by suppressing VEGFR-2 phosphorylation and blocking the activation of ERK (130). Additionally, Setyawati et al. confirmed that silica nanoparticles could inhibit the proliferation, migration, invasion, and viability of endothelial cells, further restraining angiogenesis by triggering the production of intracellular ROS and activating the p53 gene-related pathway. This study also reported that compared with 40- and 100-nm nanoparticles, nanoparticles with a diameter of 60 nm exhibited the most effective inhibitory effect against angiogenesis (131). In another study, mesoporous silica nanoparticles (MSNs) were used to encapsulate evodiamine (EVO) and berberine (BBR) to acquire a delivery platform with temperature and pH responsiveness. This dual drug delivery platform exhibited excellent synergistic therapeutic effects against angiogenesis, cell migration, and invasion in hepatoma and colon cancer cells (132). Fluorescent silica nanoparticles marked by endoglin aptamers have been demonstrated to interfere with the TGF-β pathway by binding to tumour vascular endothelial cell membrane proteins, further inhibiting angiogenesis and reducing vascular density in xenograft hepatocellular carcinoma mice (133). Silica-based nanoparticles have also been reported to have anti-angiogenic potential in pancreatic and colorectal cancers (134–136).

Carbon is the second most abundant element in the body, and the application of carbon-based nanoparticles such as graphene, nanodiamonds, carbon nanotubes, and carbon nanodots in the antitumour, especially anti-angiogenic, field has been widely studied recently (137). Murugesan et al. reported first that carbon-based nanoparticles such as graphite, multiwalled carbon nanotubes, and fullerenes exhibited remarkable anti-angiogenic activity against both FGF- and VEGF-induced angiogeneses in a chick chorioallantoic membrane model (138). Lai et al. found that bovine serum albumin-capped graphene oxide (BSA-GO) could strongly bind to VEGF-A_{165} and act as an effective angiogenesis inhibitor. Such nanoparticles could thereby block the interaction of VEGF-A_{165} with the VEGF receptor and stop the downstream signalling pathway of angiogenesis in hepatoma cells (139).

Recently, Ding et al. developed PEI-modified single-walled carbon nanotubes (SWNTs) to deliver VEGF-targeted siRNA (siVEGF) for synergistic targeted treatment against angiogenesis. The observations in xenograft pancreatic adenocarcinoma mice indicated that such nanoparticles could significantly accumulate in tumour tissues and inhibit the growth and angiogenesis of the tumours. Moreover, low cytotoxicity, good biocompatibility, and negligible organ toxicity were observed in this study (140). However, the renal clearance, toxicology, and biocompatibility of carbon-based nanoparticles are still controversial and limit their further application. Some studies have suggested that they might penetrate the cell membranes of healthy tissue, resulting in harmful inflammatory and fibrotic responses and cell death (141, 142).

Polymeric Nanoparticles and Liposomes

Synthetic and naturally derived polymeric nanoparticles have also received great attention in various biomedical fields, especially in drug delivery systems for cancer treatment and other diseases (143). As a naturally alkaline polysaccharide, chitosan has been widely used as a candidate material for drug carriers, taking advantage of its biodegradability, lower immunogenicity, better biocompatibility, and non-toxicity (144). Chitosan-based nanoparticles have been widely used in the treatment of digestive tumours, including anti-angiogenic therapies. Zhang et al. designed *N*-deoxycholic acid-glycol chitosan (DGC) as a carrier loaded with the commonly used chemotherapeutic agent docetaxel (DCT) and the angiogenic marker peptide for gastric cancer (GX1) to obtain multifunctional vascular targeting nanoparticles (GX1-DGC-DCT). GX1 could effectively promote the uptake of nanoparticles by cells, as observed by confocal laser scanning microscopy. After intravenous injection of GX1-DGC-DCT, tumour growth in xenograft gastric cancer models showed a tumour inhibition rate of 67.05% compared with the single DCT group (145). Some similar studies in gastric cancer reported that chitosan oligosaccharide (COS)-conjugated selenium (Se) and carboxymethyl chitosan (CMCS)-conjugated norcantharidin (NCTD) could remarkably enhance its antitumour efficacy

TABLE 1 | Representative nanoparticles mentioned in review for anti-angiogenic therapies in different digestive tumours.

Tumour Category	Design of Nanoparticles	Anti-Angiogenic Mechanism	Antitumour Outcome	Reference
Gastric cancer	Au nanoparticles conjugated CD44v6 monoclonal antibodies (CD44v6-GNS)	Specifically target gastric cancer neovascularization system for achieving photothermal therapy	Inhibit the growth of gastric cancer cells and extend the survivability remarkably of mice	(102)
Colorectal cancer	5-FU loaded on Au nanoparticles which were coated with anti-EGFR antibodies (AuNP-5FU-EGFR)	Specifically target EGFR positive tumour cells for enhancing the delivery of 5-FU antineoplastic agents	Superior efficiency on apoptosis induction than single 5-FU with no significant cytotoxic effects in colorectal cancer cells	(104)
Hepatocellular carcinoma	Polyethylenimine-modified Au nanoparticles were bound to siRNA, which targeted oncogene c-Myc (siRNA/bPEI/AuNPs)	Successfully silence c-Myc gene, which positively correlated with the expression of pro-angiogenic-related genes with no significant cytotoxicity	Enhance the cellular uptake of siRNA without significant cytotoxicity	(106)
Pancreatic cancer	Functionalize Au nanoparticles with polyethylene glycol and arginine-glycine-aspartate ($GNP_{PEG-RGD}$)	Inhibit the cancer-associated fibroblasts related to angiogenesis	Increase the nanoparticles uptake by cancer-associated fibroblasts to kill such cells	(111)
Pancreatic cancer	Honeycomb-like gold nanoparticles mediated interventional photothermal therapy combined with brachytherapy (HGN-mediated IPT-BT)	Improve oxygen supply to overcome hypoxia-related resistance to anti-angiogenic therapies	Improve oxygen supply and damage double-stranded DNA in tumour tissues of xenograft pancreatic cancer mice	(114)
Hepatocellular carcinoma	Hollow copper sulfide nanoparticles encapsulating sorafenib and surface modified with anti-VEGFR antibodies (CuS-SF@CMV)	Inhibit tumour angiogenesis through PI3K/AKT and Ras/Raf/MEK/ERK pathways	Enhance synergistic PTT and chemotherapy against hepatoma cells through homotypic cell targeting and immune escape	(117)
Hepatocellular carcinoma	Modify the surface of CuS with PEG and cyclic RGDfK peptide (CuS-PEG-c(RGDfK))	Promote selective angiogenic tumour cells uptake of nanoparticles and kill such cells	Target nanoparticles to tumour vasculature and $\alpha v\beta 3$ integrin-expressing tumour cells mediated efficient photothermal ablation of tumours	(118)
Pancreatic cancer	Load gambogic acid onto magnetic Fe_3O_4 nanoparticles (GA-MNP- Fe_3O_4)	Downregulate the downstream target gene of angiogenesis	Inhibit the migration and proliferation of cancer cells	(126)
Colorectal cancer	Recoat superparamagnetic iron oxide nanoparticles using hyaluronate and trimethyl chitosan (SPION-TMC-HA)	Block the initiator (HIF-1α) and end (EP4) of HIF-1α/COX2/PGE2/EP4 signalling pathways	Prevent proliferation, migration, invasion, angiogenesis, and colony formation of the cancer cells	(127)
Gastric cancer	Couple GEBP11 peptide to meso-2,3-dimercaptosuccinic acid-coated Fe_3O_4 magnetic nanoparticles and Cy5.5 fluorescent dye (Cy5.5-GEBP11-DMSA-MNPs, CGD-MNPs)	Target to tumour angiogenesis by coating novel vasculature-specific binding peptide, GEBP11	Could observe the angiogenic status of gastric cancer in xenograft cancer mice	(128)
Hepatocellular carcinoma and colorectal cancer	Encapsulate evodiamine and berberine through mesoporous silica nanoparticles	Response to tumour microenvironment and release the drugs for improving local drug concentration and biocompatibility	Exhibit excellent synergistic therapeutic effect against angiogenesis, cell migration and invasion in hepatoma and colon cancer cells	(132)
Hepatocellular carcinoma	Mark mouse endoglin aptamer, YQ26 to fluorescent silica nanoparticles (YQ26-FSiNPs)	Interfere with TGF-β pathway by binding to tumour vascular endothelial cell membrane protein, further inhibiting angiogenesis to reduce vascular density	Achieve prominently high targeting efficiency and therapeutic effects both *in vitro* experiments and *in vivo* animal studies	(133)
Pancreatic cancer	Polyethylenimine modified single-walled carbon nanotubes linked with candesartan to deliver VEGF targeted siRNA (SWNT–PEI–CD/siVEGF)	Deliver VEGF-targeted siRNA (siVEGF) for the synergistic and targeted treatment of tumour angiogenesis	Nanoparticles accumulate in tumour tissues and inhibit the growth and angiogenesis of tumour with low cytotoxicity and negligible organ toxicity	(140)
Gastric cancer	Load docetaxel and gastric cancer angiogenic marker peptide, GX1 through *N*-deoxycholic acid-glycol chitosan (GX1-DGC-DCT)	Decorated with GX1, which exhibited high affinity and specificity with the gastric cancer vasculature for targeted delivery hydrophobic docetaxel	Promote the uptake of nanoparticles in cells and inhibit tumour growth in xenograft gastric cancer models	(145)
Gastric cancer	Chitosan oligosaccharide conjugated selenium (COS–Se)	Reduce the expressions of CD34 and VEGF in treated tumour tissues	Inhibit proliferation and metastasis both *in vitro* and *in vivo*	(146)
Gastric cancer	Carboxymethyl chitosan conjugate norcantharidin (CNC)	Downregulate expressions of VEGF	Enhance the antitumour efficacy *in vivo*	(147)
Colorectal cancer	Carboxymethyl dextran-conjugated trimethyl chitosan (TMC-CMD)	Decrease angiogenesis-related genes expression including TGF, VEGF, and FGF	Reduce both *in vitro* and *in vivo* tumour growth and angiogenesis	(148)
Colorectal cancer	Polyethylene glycol chitosan lactate conjugated with hyaluronate and co-delivered anti-IL-6 siRNA (H-PCL-siRNA IL-6)	Co-delivery IAPs inhibitor (BV6) and anti-IL-6 siRNA by nanoparticles to achieve simultaneous therapy	Decrease cell migration, proliferation, colony formation, and angiogenesis in cancer cells and	(149)

(Continued)

TABLE 1 | Continued

Tumour Category	Design of Nanoparticles	Anti-Angiogenic Mechanism	Antitumour Outcome	Reference
			suppress cancer progression in xenograft colorectal cancer mice	
Colorectal cancer	Carboxylated graphene oxide conjugated with trimethyl chitosan and hyaluronate to load HIF-1α-siRNA (siRNA loaded CGO-TMC-HA)	Suppress the CDKs/HIF-1α pathway-related resistance to anti-angiogenic therapies	Decrease the proliferation, migration, angiogenesis and colony formation in cancer cells	(150)
Hepatocellular carcinoma	Load epirubicin in chitosan nanoparticles (EPI-NPs)	Actively target tumour cells and release the drugs for superior efficacy and higher safety	Reduce angiogenesis, overcome resistance and enhance therapeutic efficacy with lower cardiotoxicity	(152)
Colorectal cancer	Polyethylene glycol-polycaprolactone liposome to deliver apatinib and docetaxel (Lipo-Apa and DOC)	Construct drug delivery system for the delivery of apatinib and docetaxel for synergistic therapy	Decrease angiogenesis, promote apoptosis and inhibit proliferation in xenograft colorectal cancer mice	(159)
Pancreatic cancer	Polyethylene glycol- polylactic acid micelle to coencapsulate paclitaxel and itraconazole (PIM)	Demonstrate optimized systemic pharmacokinetics and increase tumour drug accumulation due to serum stability	Increase drug accumulation, normalize blood vessels and inhibit tumour growth in a human orthotopic pancreatic cancer model	(160)

5-FU, 5-fluorouracil; EGFR, endothelial growth factor receptor; AuNP, Au nanoparticle; bPEI, branched polyethylenimine; HGN, honeycomb-like gold nanoparticle; IPT-BT, interventional photothermal–brachytherapy; VEGFR, vascular endothelial growth factor receptor; PTT, photothermal therapy; PEG, polyethylene glycol; SWNT, single-walled carbon nanotube.

through regulating the VEGF-related pathway to repress angiogenesis with non-toxic effects (146, 147). In addition, the delivery of siRNA by chitosan-based nanoparticles was also studied extensively. Nikkhoo et al. reported carboxymethyl dextran-conjugated TMC (TMC-CMD) nanoparticles loaded with signal transducer and activator of transcription 3 (STAT3)-specific siRNA and BV6, a well-known inhibitor of apoptosis (IAP) inhibitor. The results showed that such nanoparticles could reduce both *in vitro* and *in vivo* tumour growth and angiogenesis by decreasing the expression of related genes, including TGF, VEGF, and FGF, in colorectal cancer (148). Some similar studies reported that chitosan-based nanoparticles loaded with IL-6-specific siRNA and HIF-1α-specific siRNA could inhibit colorectal cancer progression and angiogenesis (149, 150). Epirubicin (EPI), as an anthracycline derivative, is a first-line chemotherapy drug against various digestive tumours (151). Nasr et al. loaded EPI in chitosan nanoparticles to treat hepatocellular carcinoma, and it exhibited lower cardiotoxicity and superior results in reducing angiogenesis, overcoming resistance, and enhancing the therapeutic efficacy (152). Chitosan-based nanoparticles have also been reported as potential candidates for anti-angiogenic treatment in other digestive tumours, such as cholangiocarcinoma and pancreatic cancer (153–156).

PEG, polylactic acid (PLA), and polycaprolactone (PCL) are FDA-approved commercially available biodegradable copolymers that are widely used to prepare nanoparticles for drug delivery (157). Apatinib (a selective VEGFR-2 inhibitor) and DCT (Taxotere) are widely used for combined treatment in digestive tumours, but their curative effects appear to be impaired due to the disadvantages of their poor pharmacometabolic characteristics (158). Yu et al. constructed PEG-PCL liposomes as a drug delivery system for apatinib and DCT. They could achieve locally higher drug concentrations and prolong the release time, further decreasing angiogenesis, promoting apoptosis, and inhibiting proliferation in xenograft colorectal cancer mice (159). Liu et al. prepared PEG-PLA micelles to coencapsulate paclitaxel (PTX) and itraconazole (ITA) to produce PTX-ITA micelles (PIM) nanoparticles. PIM showed excellent systemic pharmacokinetics and increased drug accumulation in the tumour site. Additionally, PIM normalized blood vessels and inhibited tumour growth in both a human orthotopic pancreatic cancer model and genetically engineered spontaneous pancreatic ductal adenocarcinoma mice (160). Liposomal nanodelivery systems have also been widely studied in various digestive tumours, such as hepatocellular carcinoma and gastric cancer (161–163).

FUTURE PERSPECTIVES AND SUMMARY

Digestive tumours account for nearly half of the cancer incidence in China and cause the most tumour-related deaths worldwide. As a common molecular targeted therapy in the clinic, angiogenesis plays an important role in the development of digestive tumours. Anti-angiogenic therapies have been identified as an effective direction in digestive tumour treatment, but they are associated with some limitations, such

as potential resistance and adverse reactions. Nanomaterials are considered superior tools to solve the above problems and achieve individual therapies. However, the successful translation of nanoparticles from the laboratory to the clinic could improve cancer treatment, but many challenges remain. In recent years, nanoparticles have been shown to possess stronger biocompatibility and more efficient tumour targeting capability through reasonable functionalization and modification. By adjusting their surface properties and the size and shape of nanoparticles, their drug toxicity and pharmacokinetics can be changed both *in vitro* and *in vivo* (164). In this article, we reviewed the biomedical applications of different nanoparticles, including metal/metallic compounds, non-metallic nanoparticles, polymeric nanoparticles, and liposomes, in various digestive tumours (**Table 1**). These nanoparticles can be used in various ways, such as delivering siRNA, antihypoxia, molecular targeting peptide phototherapy, and photothermal anti-angiogenic therapy.

Although nanoparticles have potential therapeutic effects, their application still has some limitations. The complexity and diversity of tumours and the different properties of nanoparticles would lead to different uptake *in vivo*. A better understanding of their intracellular transport and cellular uptake mechanisms might effectively reveal the potential therapeutic benefits of nanoparticles. Moreover, careful evaluation of their toxicity is required before the clinical applications of nanoparticles. Although various studies have proven that nanoparticles exhibit better biocompatibility and scarce cytotoxicity in preclinical models, their potential toxicity and an uncertain fate in the human body are still worrying. Therefore, developing more appropriate models to further evaluate the toxicity of nanoparticles is of great importance. In addition, nanoparticles also need to solve the phenomenon of drug resistance against anti-angiogenic therapies. Although some scientific and technical considerations are required before translational clinical applications, we have adequate reason to believe that combined with existing treatment strategies, anti-angiogenic therapies could become part of the treatment approach to digestive tumours.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to this review. ZZ and HY conceived and designed the review. ZY, WD, XZ, YA, and YL retrieved and reviewed the literatures. ZY, WD, and XZ wrote the manuscript. ZZ and HY reviewed and edited the manuscript. All authors read and approved the manuscript.

FUNDING

This work was supported by grants from the National Key Technologies R&D Program (No. 2015BAI13B09), National Key Technologies R&D Program of China (No. 2017YFC0110904), and Clinical Center for Colorectal Cancer, Capital Medical University (No. 1192070313).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.789330/full#supplementary-material

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frontiers in Oncology

ORIGINAL RESEARCH
published: 19 January 2022
doi: 10.3389/fonc.2021.791912

Predictive Effect of Systemic Immune-Inflammation Index Combined With Prognostic Nutrition Index Score on Efficacy and Prognosis of Neoadjuvant Intraperitoneal and Systemic Paclitaxel Combined With Apatinib Conversion Therapy in Gastric Cancer Patients With Positive Peritoneal Lavage Cytology: A Prospective Study

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OPEN ACCESS

Edited by:
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Beijing Friendship Hospital, China

Reviewed by:
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Jing Wu,
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *09 October 2021*
Accepted: *21 December 2021*
Published: *19 January 2022*

Citation:
Ding P, Yang P, Sun C, Tian Y, Guo H, Liu Y, Li Y and Zhao Q (2022) Predictive Effect of Systemic Immune-Inflammation Index Combined With Prognostic Nutrition Index Score on Efficacy and Prognosis of Neoadjuvant Intraperitoneal and Systemic Paclitaxel Combined With Apatinib Conversion Therapy in Gastric Cancer Patients With Positive Peritoneal Lavage Cytology: A Prospective Study. Front. Oncol. 11:791912. doi: 10.3389/fonc.2021.791912

Background: Gastric cancer with only peritoneal lavage cytology (GC-CY_1) is a special type of gastric cancer, which is defined as stage IV. The pre-treatment systemic immune-inflammation index (SII) and prognostic nutritional index (PNI) are representative blood indexes of systemic inflammatory response and nutritional status. However, the clinical significance of combined detection of these two indexes is still unclear. This study aims to evaluate the clinical value of the new score system by combining SII and PNI (SII-PNI score) as a predictor of efficacy and prognosis after neoadjuvant intraperitoneal and systemic (NIPS) paclitaxel combined with Apatinib conversion therapy for GC-CY_1 patients.

Methods: We registered a prospective clinical study involving 36 GC-CY_1 patients from April 2018 to August 2019 (NCT03718624). All patients underwent re-laparoscopic exploration after treatment. According to free cancer cells (FCCs) status, these patients were divided into FCCs group and non-FCCs group. The SII-PNI score ranged from 0 to 2 as follows: score of 2, high SII (≥512.1) and low PNI (≤52.9); score of 1, either high SII or low PNI; score of 0, no high SII nor low PNI.

Results: All patients underwent re-laparoscopic exploration after 3 cycles of NIPS paclitaxel and Apatinib conversion therapy. Among them, 28 cases (77.78%) were in non-FCCs group, and 8 cases (22.22%) were in FCCs group. The SII-PNI score of non-FCCs patients was significantly lower than that of FCCs patients (p=0.041). The prognosis of patients with high SII-PNI score was significantly worse than that of patients with low SII-PNI score (p<0.001). Multivariate analysis showed that SII-PNI score was an independent prognostic factor for predicting overall survival and progression-free survival (p=0.001, 0.002).

Conclusion: Pretreatment SII-PNI score is an important predictor for the efficacy of GC-CY_1 patients after NIPS paclitaxel combined with Apatinib conversion therapy, which can help to identify high-risk groups and predict prognosis.

Keywords: apatinib, abdominal exfoliation cytology positive, gastric cancer, systemic immune-inflammation index (SII), prognostic nutrition index (PNI)

INTRODUCTION

Gastric cancer ranks fifth in morbidity and fourth in mortality worldwide (1). Distant metastasis of gastric cancer mainly occurs through blood, lymphatic and direct invasion of adjacent organs (2). However, the most common type of recurrence after treatment in advanced gastric cancer patients is peritoneal metastasis (3, 4). Gastric cancer patients with only positive peritoneal lavage cytology (GC-CY_1) is defined as the presence of free cancer cells in the abdominal cavity without peritoneal implantation or distant metastasis (5). In recent years, GC-CY_1 is defined as stage IV in the 15^{th} edition of the Japanese Classification of Gastric Cancer (6). Moreover, the eighth edition of the International Union Against Cancer (AJCC) TNM staging system considers GC-CY_1 as an independent diagnostic criterion for distant metastasis(M1) (7).

Currently, the prognosis of GC-CY_1 patients is poor, and there is no universal consensus on the most suitable treatment for these patients (5, 8). Systemic chemotherapy has been widely accepted as the standard treatment for patients with stage IV and has been proved to improve the prognosis (9). However, due to the existence of peritoneal-plasma barrier, chemotherapeutic drugs cannot directly act on the abdominal cavity (10). Therefore, systemic chemotherapy alone is less effective in treating GC-CY_1 patients. The results of PHOENIX-GC study carried out by Ishigami H eal scholars provided a new treatment idea for GC-CY_1 patients (11). The combination of systemic chemotherapy and intraperitoneal chemotherapy is considered to be a promising conversion therapy. Meanwhile, Apatinib is an orally active Tyrosine Kinase Inhibitor (TKI), which can effectively inhibit the formation of tumor blood vessels, thus playing an anti-tumor effective and well tolerated for various malignant tumors (12–15). Our previous study found that neoadjuvant intraperitoneal and systemic (NIPS) paclitaxel combined with Apatinib has achieved good results in the conversion treatment of GC-CY_1 patients, and the R0 resection rate is 77.78% (16). Unfortunately, some patients are still unable to benefit from NIPS paclitaxel combined with Apatinib because of the heterogeneity of gastric cancer or tumor insensitivity to its uniformity (17). However, there is still a lack of reliable indicators to predict efficacy and prognosis of patients before conversion treatment, which might help to optimize the treatment strategies.

Growing evidence show that the occurrence and development of gastric cancer are closely related to the systemic inflammatory response (17, 18). Systemic immune-inflammatory index (SII) is a new inflammatory indicator based on the counts of peripheral blood neutrophils, lymphocytes, and platelets, which can comprehensively reflect the inflammatory response of the body (19). Many studies have confirmed that SII is closely related to the prognosis of various malignant tumors (20, 21). Meanwhile, a study have showed that nutritional status during treatment was also a key factor affecting chemotherapy response (22). As a simple and feasible nutritional detection index, prognostic nutritional index (PNI) is confirmed to be related to the prognosis of various malignant tumors, and is widely used to evaluate the occurrence of perioperative complications and predict the prognosis (23). Previous studies generally used inflammatory markers such as neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) to evaluate the prognosis of patients with gastric cancer (24, 25). However, there are few studies on the efficacy and prognosis of NIPS paclitaxel combined with Apatinib in GC-CY_1 patients using SII combined with PNI.

In this study, we evaluated the predictive effect of pre-treatment SII-PNI score on efficacy and prognosis in GC-CY_1 patients after NIPS paclitaxel combined with Apatinib conversion treatment to determine the optimal parameters for predicting survival and clinical response to this combined regimen.

MATERIALS AND METHODS

Study Design and Participants

This is a prospective clinical study of NIPS paclitaxel combined with Apatinib for GC-CY_1 patients in the Fourth Hospital of

Hebei Medical University from April 2018 to August 2019. This trial was registered at Clinical Trials. gov: NCT03718624, and approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (approval number: 2018088). All patients and/or the legal guardians/surrogates/power of attorneys were informed about the potential adverse effects and signed informed consents.

The following inclusion criteria were applied: (I) gastric adenocarcinoma confirmed by histopathology and free cancer cells (FCCs) positivity confirmed by exfoliated cells in the abdominal cavity; (II) preoperative computed tomography (CT) imaging showed no distant organ metastasis and no distant lymph node metastasis above the third station; (III) patients aged ≤75 years; (IV) the Eastern Cooperative Oncology Group (ECOG) activity status score was ≤2 points; (V) patients had good bone marrow function(before treatment in patients with peripheral blood examination if there is no bone marrow suppression, or bone biopsy exclude blood system diseases show good bone marrow function), liver function(the peripheral blood test showed that ALT, AST ≤ 2.5*ULN and TBIL< 1.5*ULN), heart function(no atrial fibrillation, angina pectoris, cardiac insufficiency, ejection fraction less than 50% and poor hypertension control), and kidney function(the peripheral blood test showed that serum creatinine ≤ 1.5*ULN before treatment), and were able to tolerate chemotherapy; (VI) there were no other serious immunosuppressive diseases or simultaneous malignant tumors; (VII) and pathological human epidermal growth factor receptor 2 (HER2) tests were negative prior to the operation. Patients were excluded if they presented with the following: (I) difficulty taking oral medications (such as dysphagia, chronic diarrhea, and gastrointestinal obstruction, etc.); (II) high blood pressure that could not be controlled by a single antihypertensive drug treatment; (III) 24 hour urine protein quantification >1.0 g; (IV) imaging results showing the tumor had invaded important blood vessels or the investigator judged that the tumor was highly likely to invade important blood vessels during treatment and cause fatal bleeding; (V) abnormal blood coagulation; and (VI) other comorbidities that may seriously endanger the safety of the patient or affect the completion of the study as determined by the investigator.

Chemotherapy Regimen

The treatment regimens of all patients in this study were consistent with our previous study (16). Treatment commenced on the day after the laparoscopic exploration, and each cycle of treatment lasted for 3 weeks. On the 1st and 8th day of the treatment cycle, paclitaxel was infused *via* an intraperitoneal (IP) chemotherapy pump (IP route 20 mg/m^2, dissolved in 1,000 mL of normal saline, infusion for more than 1 hour) and intravenously (IV) (IV route 50 mg/m^2, dissolved in 500 mL of saline, infusion for more than 1 hour). Dexamethasone and cimetidine were administered before paclitaxel treatment. Oral S-1 (a contemporary oral fluoropyrimidine) 80 mg/(m^2·d) was given 30 minutes after breakfast and 30 minutes after dinner for 14 consecutive days. At the same time, Apatinib 500 mg/d was administered orally for 21 consecutive days. The dose of S-1 was determined according to the body surface area (BSA) as follows: for BSA <1.25 m^2, 80 mg/(m^2·d) S-1 was administered; for BSA 1.25-1.50 m^2, 100 mg/(m^2·d) S-1 was administered; and for BSA >1.50 m^2, 120 mg/(m^2·d) S-1 was given. After one month of rest, radical D2 operation was arranged, and then another six cycles of NIPS paclitaxel combined with Apatinib conversion treatment were repeated 1 month postoperatively.

Assessments

Four weeks after the completion of three cycles of NIPS paclitaxel combined with Apatinib conversion therapy, the objective efficiency and resectability of the tumor were evaluated by computed tomography(CT). Tumor response was assessed based on the rules established by the Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 (3), which was divided into complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD).

And laparoscopic exploration was performed again. If free cancer cells (FCCs) negative confirmed by exfoliated cells in the abdominal cavity and are defined as non-FCCs, then standard D2 lymph node dissection is performed. However, if FCCs were still detected in the abdominal cavity, then the original chemotherapy regimen would be continued. And in this study, all patients were divided into FCCs group and non-FCCs group according to FCCs status after NIPS paclitaxel combined with Apatinib conversion treatment by re-laparoscopy and peritoneal cytology.

Definitions and Follow-up

The peripheral venous blood was collected in fasting state within one week before chemotherapy in all patients. The counts of peripheral neutrophils, lymphocytes, and platelets were measured and analyzed by an automatic blood analyzer (Beckman Coulter LH750), and the levels of peripheral albumin were measured and analyzed by an automatic blood analyzer (Beckman Coulter AU5800). The definitions of PNI and SII were shown as follows: PNI= albumin (g/L) + 5×total lymphocyte counts(10^9/L) (26); SII= platelet × neutrophil/lymphocyte counts (27).

All patients were recommended to have a follow-up visit every 3 months in the first 2 years, and every 6 months after 2 years. Follow-up methods mainly include telephone encounter, outpatient visits, and hospitalization. The hospital examination items included CT of chest, abdomen, and pelvis, as well as esophagogastroduodenoscopy (EGD) and tumor markers. In this study, the deadline for follow-up was September, 1st, 2021. Overall survival (OS) was defined as the time interval from treatment to cancer-related death or final contact, and OS was the preferred destination. And progression-free survival (PFS) was measured from the time of treatment initiation to clinical or radiographic progression or death from any cause.

Statistical Analyses

SPSS version 21.0 and GraphPad Prism 5.01 were used for statistical analyses. The receiver operating characteristic curve (ROC) and the area under the ROC Curve (AUC) was used to evaluate the predictive ability of SII and PNI in distinguishing FCCs patients and non-FCCs patients, and the optimal cut-off values of SII and PNI with the highest Youden index were

obtained. Survival analysis was performed using Kaplan-Meier method. Cox proportional hazards regression model was used for univariate and multivariate analysis. Relative risk was assessed using hazard ratio (HR) and 95% confidence interval (CI). Spearman correlation analysis was used to evaluate the relationship between PNI and SII. $p< 0.05$ indicated that the difference was statistically significant.

RESULTS

Patients' Demographic Information and Tumor Characteristics

This study prospectively included 36 GC-CY_1 patients according to the inclusion and exclusion criteria (**Figure 1**). The demographic information and tumor characteristics of the patients are summarized in **Table 1**. There were 25 males (69.44%) and 11 females (30.56%). The median age of the patient was 54 years old, ranging from 32 to 66. The tumor lesions was ≥5 cm in diameter (75.00%) in 27 cases, and less than 5 cm in 9 cases (25.00%). The median values of pre-treatment SII and PNI were 328.4 and 53.3, respectively, while the median values after three cycles of conversion treatment were 328.4 and 46.9, respectively. Meanwhile, before conversion therapy(r=-0.431, p=0.009; **Figure 2A**) and after 3 cycles of conversion therapy(r=-0.580, p=0.001; **Figure 2B**), there is a close negative correlation between the two systemic indicators of SII and PNI.

Optimal Cut-Off Values of SII and PNI Before and After Conversion Treatment

36 GC-CY_1 patients underwent laparoscopic exploration combined with peritoneal cytology after 3 cycles of NIPS paclitaxel and Apatinib conversion therapy. Among them, 28 cases (77.78%) were FCCs negative (non-FCCs), all negative patients underwent R0 resection. The remaining 8 patients (22.22%) were found to have free cancer cells (FCCs) in the abdominal cavity, and continued the original chemotherapy regimen of Apatinib conversion therapy. After 3 cycles of conversion therapy, 3 patients were evaluated by CT for local lesion progression, and 5 patients underwent laparoscopic exploration and peritoneal cytology again. Of the 5 patients, only 1 was negative, 2 were still positive and 2 had peritoneal metastasis.

The mean SII and PNI in the 28 patients with non-FCCs were 408.9 ± 179.1 and 54.8 ± 4.7, respectively. Meanwhile, the mean pre-treatment SII and PNI in the 8 patients with FCCs were 677.8 ± 277.6 and 48.5 ± 4.8, respectively. The pre-treatment SII in FCCs patients was significantly higher than that in non-FCCs patients (p=0.006) (**Figure 3A**), while the PNI in FCCs patients was lower than that in non-FCCs patients (p=0.002) (**Figure 3B**). Furthermore, we found that after three cycles of conversion therapy, the average levels of SII and PNI in 28 patients with no-

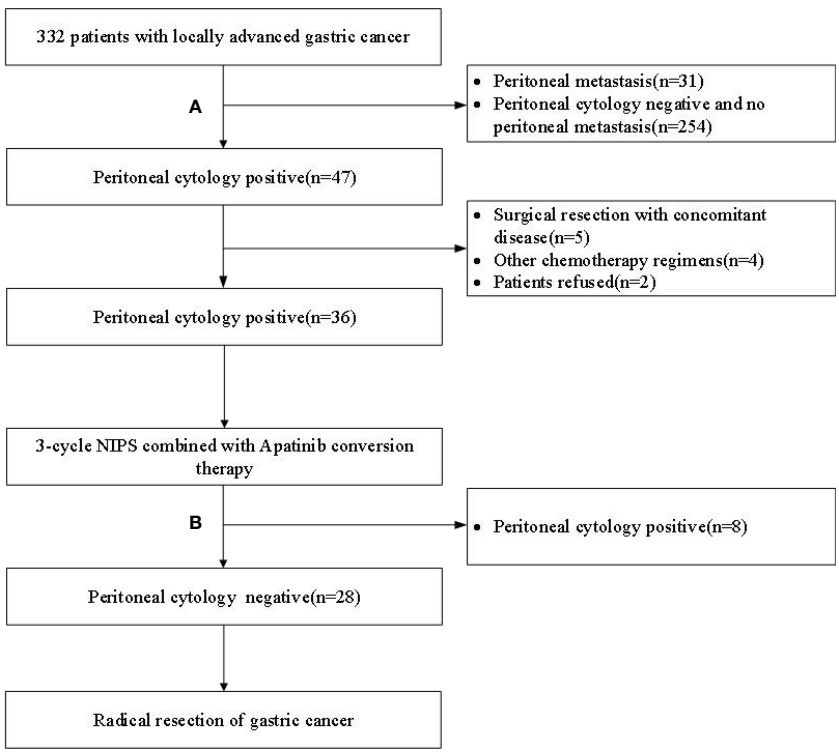

FIGURE 1 | Flow chart of patient enrollment and exclusion. **(A)** Laparoscopic exploration + peritoneal cytology; **(B)** Re-laparoscopic exploration + peritoneal cytology; NIPS, Neoadjuvant intraperitoneal and systemic.

TABLE 1 | Patient demographic information and tumor characteristics.

Characteristics	Case (%)	Mean (SD)	Range
Sex			
Male	25 (69.44)		
Female	11 (30.56)		
Age (years)		54 (10.4)	32-66
ECOG performance status			
0	30 (83.33)		
1	6 (16.67)		
Tumor size (cm)		7.8 (3.2)	3.4-10.6
<5.0	9 (25.00)		
≥5.0	27 (75.00)		
Differentiation			
Poor	30 (83.33)		
Moderately or well	6 (16.67)		
Lesion site			
Cardia	13 (36.11)		
Stomach	4 (11.11)		
Gastric antrum	14 (38.89)		
Whole stomach	5 (13.89)		
Pathological T stage			
T3	6 (16.67)		
T4	30 (83.33)		
Pre-treatment SII		553.6 (372.5)	77.5-1311.2
Pre-treatment PNI		54.3 (6.3)	41.0-68.5
Posttreatment SII		402.0 (247.5)	72.6-1048.0
Posttreatment PNI		47.3 (4.8)	38.0-58.2

ECOG, Eastern Cooperative Oncology Group; SII, Systemic immune-inflammatory index; PNI, prognostic nutritional index.

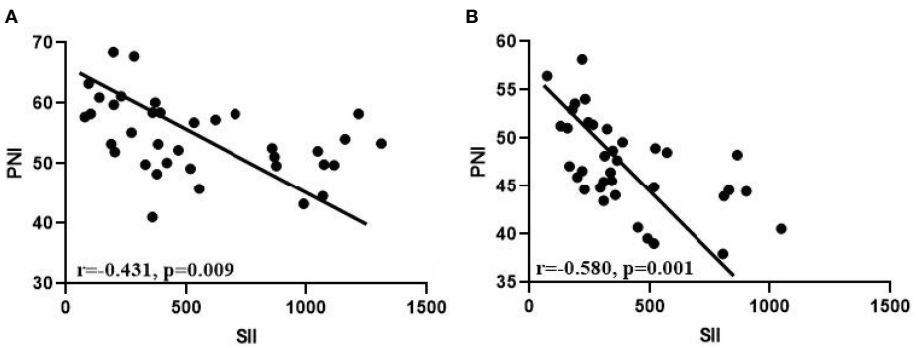

FIGURE 2 | Correlation analysis between SII and PNI. **(A)** Before conversion therapy; **(B)** After 3 cycles of conversion therapy.

FCCs were not significantly different from those in 8 patients with FCCs (374.5 vs. 498.3, p=0.299; 47.8 vs. 45.3, p=0.193) (**Figures 3C, D**).

In order to determine the cut-off value of continuous variables, we constructed ROC curves and calculated AUC to evaluate the changes of SII and PNI before and after conversion therapy to distinguish FCCs and non-FCCs patients. The optimal cut-off value of SII before conversion therapy was 512.1 (AUC=0.817, 95%CI: 0.619-1.000, p=0.007), and the corresponding sensitivity was 0.875 and specificity was 0.821 (**Figure 4A**). The optimal cut-off value of PNI was 52.9 (AUC=0.884, 95%CI: 0.769-0.999, p=0.001), with the corresponding sensitivity of 0.679 and specificity of 0.863 (**Figure 4B**). However, after the three-cycle translational therapy, the optimal cut-off value of SII was 487.5 (AUC=0.524, 95%CI: 0.321-0.726, p=0.823), and the optimal cutoff value of PNI was 46.9 (AUC=0.578, 95%CI: 0.364-0.792, p=0.460), which failed to accurately distinguish FCCs and non-FCCCs patients (**Figures 4C, D**). According to the optimal cut-off values of SII and PNI before conversion therapy, all patients were divided into three group: score of 2 (n=10), high SII (≥512.1) and low PNI (≤52.9); score of 1(n=13), either high SII or low PNI; score of 0(n=13), no high SII nor low PNI.

The Relationship Between SII-PNI Score and Curative Effect of Conversion Therapy

All patients received 3 cycles of NIPS paclitaxel combined with Apatinib conversion therapy and the whole abdominal enhanced

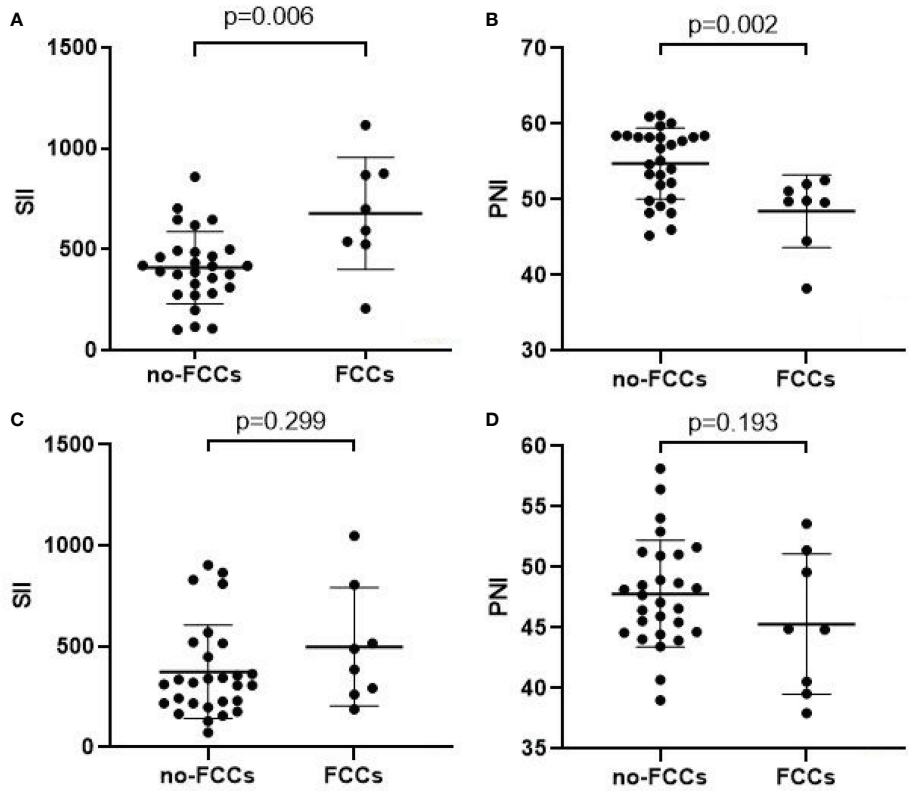

FIGURE 3 | Relationship between tumor response and the SII(A/C)/PNI(B/D). **(A, B)** Before conversion therapy; **(C, D)** After 3 cycles of conversion therapy.

CT scan was evaluated by RECIST 1.1. According to RECIST criteria, there were 5 cases of CR (13.89%), 24 cases of PR (66.67%), 5 cases of SD (13.89%), and 2 cases of PD (5.56%) (**Figure 5**). There was no difference in SII-PNI score between non-PD patients and PD patients (p=0.534) (**Table 2**). However, the SII-PNI score was significantly lower in patients with non-FCCs than in those with FCCs (p=0.041) (**Table 3**).

Relationship Between SII-PNI Score and Prognosis

All patients were followed up with the median follow-up period of 25.5 months (15.6-38.4months). The 2-year OS was 69.44% and the median overall survival (mOS) was 19.9 months (95%CI: 6.9-31.7 months). The 2-year progression-free survival (PFS) was 58.33%, and the median PFS (mPFS) was 17.2 months (95%CI: 5.9-26.5 months). Subgroup analysis showed that the 2-year OS (82.14% vs 25.00%, p=0.000) and PFS (71.43% vs 12.50%, p=0.000) of non-FCCs group were better than those of FCCs group after re-laparoscopic exploration (**Figures 6A, B**). Meanwhile, the 2-year OS of patients with SII-PNI score of 0, 1, and 2 were 92.31%, 69.23%, and 40.00%, respectively, and the difference between the three groups was significant (all p<0.001, **Figure 6C**). And, the 2-year PFS of the three groups was 84.62%, 53.85%, and 30.00%, respectively, and the difference was significant among the three groups (all p<0.001, **Figure 6D**). Multivariate analysis showed that SII-PNI score (p=0.001, p=0.002), tumor differentiation (p=0.031, p=0.029), and the FCCs status after NIPS paclitaxel combined with Apatinib conversion therapy (p=0.001, p=0.003) were all independent risk factors affecting 2-year OS and PFS of GC-CY_1 patients (**Tables 4**).

DISCUSSION

At present, it is believed that the positive peritoneal FCCs are the early stage of peritoneal colonization in gastric cancer, which is called GC-CY_1 (3). These patients have poor prognosis and poor surgical treatment effect, and the median survival time is 12 months (28). There are differences in the treatment strategies for GC-CY_1 patients worldwide. The fifth edition of the Guidelines for the Treatment of Japanese Gastric Cancer Association proposed that if GC-CY_1 patients did not have other distant organ metastasis, they could receive surgical treatment at first and postoperative chemotherapy to further prolong survival (29). However, the National Comprehensive Cancer Network (NCCN) guidelines in the United States recommend that GC-CY_1 patients should be treated according to the principle of advanced gastric cancer (30). Chemotherapy should be carried out first, and then exploration can be carried out again after chemotherapy. Patients with negative intraperitoneal FCCs may benefit from surgical treatment, while patients with persistent positive FCCs are recommended to continue chemotherapy (3).

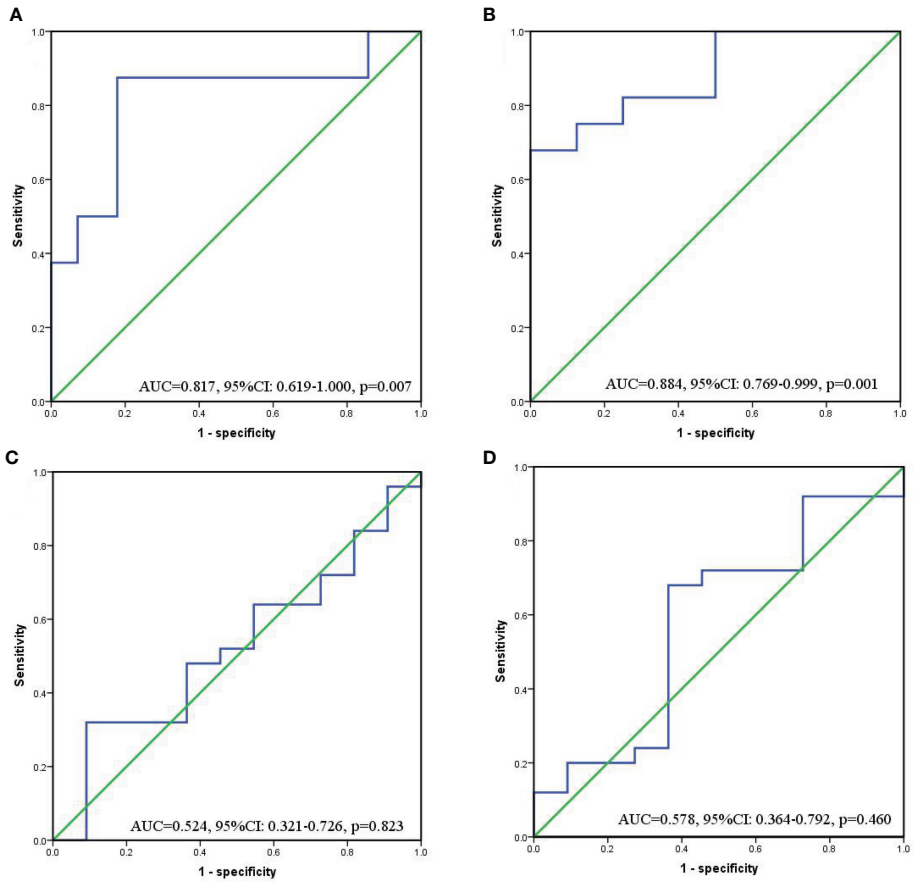

FIGURE 4 | ROC curves for discriminating patients with FCCs and those with non-FCCs according to values of the SII **(a/c)** and PNI **(b/d)**. **(A, B)** Before conversion therapy; **(C, D)** After 3 cycles of conversion therapy.

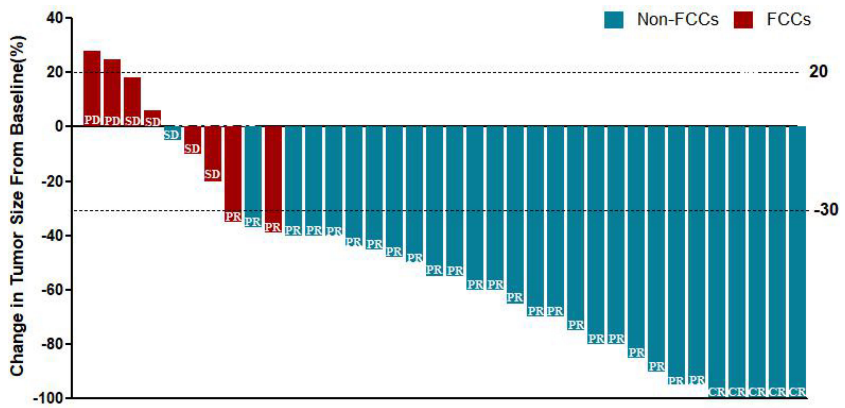

FIGURE 5 | A waterfall plot of ranked best tumor shrinkage.

Meanwhile, our previous study has confirmed that NIPS paclitaxel combined with Apatinib was effective in the treatment of GC-CY$_1$ patients and prolonged their survival time (16). Nevertheless, not all patients can benefit from it, with about 22% of the disease progression after treatment. For these patients, this combination treatment not only increases the relevant medical costs, but also may weaken the immune system and delay the best timing of surgery. Therefore, before NIPS paclitaxel combined with

TABLE 2 | Relationship between tumor response and the SII-PNI score.

Tumor response	SII-PNI score(%)			p value
	0 (n=13)	1 (n=13)	2 (n=10)	
Non-PD (n=34)	13 (100)	12 (92.31)	9 (90.00)	0.534
PD (n=2)	0 (0)	1 (7.69)	1(10.00)	

TABLE 3 | Relationship between FCCs status and the SII-PNI score.

FCCs status	SII-PNI score(%)			p value
	0 (n=13)	1 (n=13)	2 (n=10)	
Non-FCCs (n=28)	12 (92.31)	11 (84.62)	5 (50.00)	0.041
FCCs (n=8)	1 (7.69)	2 (15.38)	5 (50.00)	

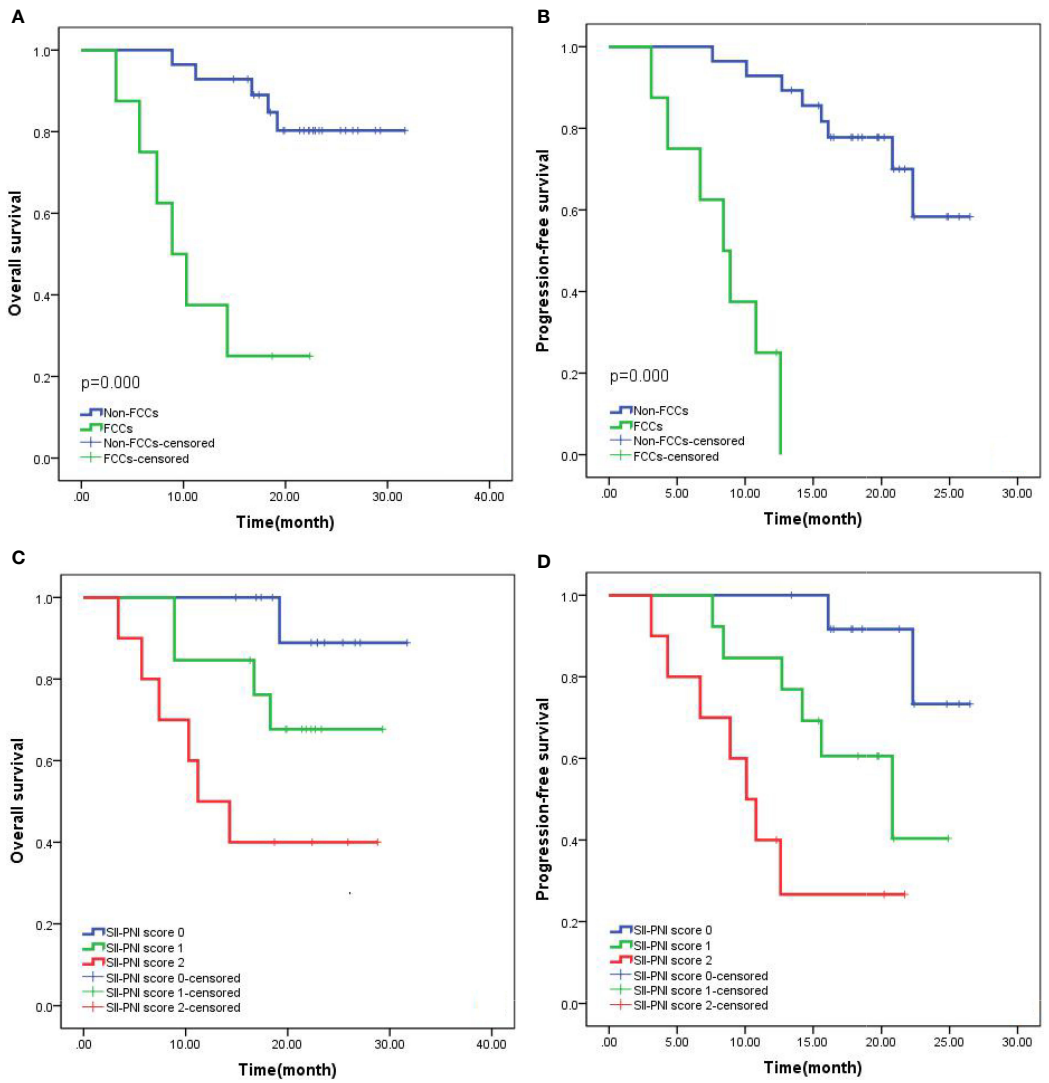

FIGURE 6 | Kaplan-Meier survival curves in patients with GC-CY$_1$. **(A, B)** 2-year overall survival, progression-free survival based on FCCs status; **(C, D)** 2-year overall survival, progression-free survival based on SII-PNI score.

TABLE 4 | Multivariate analysis of the clinicopathological characteristics for the prognosis of GC-CY$_1$ patients.

Independent factor	2-year OS Multivariate analysis			2-year PFS Multivariate analysis		
	Hazard ratio	95% CI	p value	Hazard ratio	95% CI	p value
Sex			0.315			0.143
Female	1.000	reference		1.000	reference	
Male	1.066	0.521-1.412		1.243	0.721-1.772	
Age (years)			0.083			0.251
≤50	1.000	reference		1.000	reference	
>50	1.268	0.897-1.879		1.153	0.790-1.553	
FCCs status			0.001			0.003
Non-FCCs	1.000	reference		1.000	reference	
FCCs	5.578	3.426-10.142		5.114	3.234-9.981	
SII-PNI score			0.001			0.002
0	1.000	reference		1.000	reference	
1	1.748	1.541-3.632		1.927	1.077-2.774	
2	3.576	2.578-6.895		3.152	1.569-5.072	
Tumor size (cm)			0.052			0.061
<5.0	1.000	reference		1.000	reference	
≥5.0	1.920	1.256-3.492		1.759	1.352-3.152	
Differentiation			0.031			0.029
Poor	1.000	reference		1.000	reference	
Well	2.571	1.287-3.379		2.496	1.772-4.218	

SII, Systemic immune-inflammatory index; PNI, Prognostic nutritional index; FCCs, Free cancer cells.

Apatinib is carried out for patients with GC-CY$_1$, a simple indicator to accurately predict the therapeutic effect will be beneficial to the formulation and selection of individualized treatment regimens.

In recent years, an increasing number of studies have confirmed that inflammatory response is closely related to the occurrence and development of tumors (31, 32). As a systemic inflammatory response indicator, SII has been confirmed to be closely related to the prognosis of patients with gastric cancer by many studies, which can be used to predict the prognosis of patients (19, 33). On the other hand, numerous studies have shown that malnutrition not only affects the clinical decision-making of cancer treatment, but also increases the incidence of complications and mortality, and reduces the quality of life of patients (34, 35). As an indicator reflecting the nutritional status of patients, PNI is widely used to evaluate the occurrence of perioperative complications and predict the prognosis (36, 37). To the best of our knowledge, we were the first to combine the SII and PNI of GC-CY$_1$ patients before receiving NIPS paclitaxel combined with Apatinib conversion therapy to establish the SII-PNI score as a new scoring system for predicting the efficacy and prognosis of patients.

Previous studies have shown that FCCs status is one of the most important factors to evaluate the effectiveness of GC-CY$_1$ patients after conversion therapy (5). Unfortunately, the curative effect of treatment is difficult to predict by using clinical pathological information before conversion treatment. Therefore, we focus on pre-treatment SII and PNI to overcome the challenges associated with predicting therapeutic efficacy. Previous studies have shown that SII can be used to predict the pathological complete remission and prognosis of patients after neoadjuvant chemotherapy for breast cancer (38, 39). PNI is also widely used to evaluate the efficacy and prognosis of chemotherapy for advanced non-small cell lung cancer and colorectal cancer (40, 41). However, the application of systemic inflammatory response index combined with nutritional status index, namely the SII-PNI scoring system to predict the efficacy and prognosis of GC-CY$_1$ patients receiving NIPS paclitaxel and Apatinib conversion therapy is rarely reported. This study analyzed the relationship between SII-PNI score and efficacy of NIPS paclitaxel combined with Apatinib in GC-CY$_1$ patients after conversion therapy. The results of this study showed that the SII-PNI score before treatment was closely related to the efficacy of conversion therapy. The lower SII-PNI score before treatment, the more likely the FCCs positive will turn to negative, and the more likely the NIPS paclitaxel combined with Apatinib conversion treatment will be successful. This suggests that the SII-PNI score may be a promising candidate for predicating the efficacy response of GC-CY$_1$ patients after receiving NIPS paclitaxel combined with Apatinib conversion therapy.

We also evaluated the relationship between SII-PNI score and prognosis. The 2-year OS of patients with SII-PNI score of 0, 1, and 2 were 92.31%, 69.23%, and 40.00%, respectively, and the difference between the three groups was significant. Meanwhile, similar results were also obtained in patients with PFS. Furthermore, this study analyzed the risk factors that may affect the survival of GC-CY$_1$ patients receiving NIPS paclitaxel combined with Apatinib conversion treatment, and found that the SII-PNI score was an independent risk factor affecting the 2-year OS and PFS of patients. The possible mechanism of SII-PNI predicting prognosis are as the followings: The higher SII-PNI score indicates a relative increase of neutrophil and/or platelet counts. Neutrophils release active nitrogen, reactive oxygen species, and elastase, activate the P13K-AKT signaling pathway, and promote the proliferation of tumor cells (36, 42). In addition, platelets may play a certain role in the growth, proliferation, and metastasis of tumors, mainly by secreting related tumor growth factors to promote tumor growth. Platelets are also involved in the escape of tumor cells from the host immune system (43). The increase in SII-PNI score also indicates that

lymphocytes are relatively reduced, leading to a reduced immune regulation function and promoting the progression of tumor deterioration (44). The decrease of serum albumin level in the body reflects the decrease of nutritional status of patients (45). The worse the nutritional status is, the lower the immunity of the body will be, which will lead to the progress of disease.

It is noteworthy that a few limitations of current research also exist. First, this prospective study was conducted in a single center with a small sample size (n=36), which is the main limitation. Second, this study only selected NIPS paclitaxel combined with Apatinib for analysis. Therefore, larger, multi-center prospective studies investigating different treatment regimens are urgently needed to confirm our findings.

CONCLUSIONS

In conclusion, our study suggests that the SII-PNI score is a promising predictor of the efficacy and survival outcomes of GC-CY_1 patients after NIPS paclitaxel combined with Apatinib conversion therapy. These findings may be beneficial to the formulation of therapeutic strategies and clinical risk stratification to avoid unnecessary toxicity/adverse effects in patients who are unlikely to benefit from treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Fourth Hospital of Hebei Medical University (approval number: 2018088). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Contributions: (I) Conception and design: QZ. (II) Administrative support: QZ. (III) Provision of study materials or patients: P'aD, PY, YT, HG, and YaL. (IV) Collection and assembly of data: P'aD, PY, YT, and HG. (V) Data analysis and interpretation: P'aD and CS. (VI) Manuscript writing: All authors. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Cultivating Outstanding Talents Project of Hebei Provincial Government Fund (No.2019012); Hebei public health committee county-level public hospitals suitable health technology promotion and storage project (No.2019024); Hebei Medical University Education and Teaching Research Project (No.2020CGPY-12, No.2020CHYB-23); Hebei University Science and Technology Research Project (No.ZD2019139).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 02 March 2022
doi: 10.3389/fonc.2022.848952

ZDQ-0620, a Novel Phosphatidylinositol 3-Kinase Inhibitor, Inhibits Colorectal Carcinoma Cell Proliferation and Suppresses Angiogenesis by Attenuating PI3K/AKT/mTOR Pathway

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The PI3K/AKT pathway plays a central role in human cancers, aberrant activation of this pathway is associated with tumorigenesis, cancer progression and angiogenesis. Based on the importance of the PI3K/AKT pathway in malignancies, we developed a 4-aminoquinazoline derivative, ZDQ-0620, initially envisioned as a novel pan-PI3K inhibitor. This study aimed to evaluate the potential target of ZDQ-0620 and its anticancer effect in human colorectal carcinoma (CRC). PI3K-kinase activity test showed IC50 of ZDQ-0620 against PI3Ka was 0.5 nM; molecular docking, CETSA assay and western blotting was further performed to predict ZDQ-0620 was a PI3K/AKT pathway inhibitor by targeting PI3K. To identify the effect of ZDQ-0620 on CRC cells, Sulforhodamine B (SRB) assay, flow cytometry, and Cell morphology analysis were conducted. The results showed that ZDQ-0620 inhibited the proliferation, migration and invasion of CRC cells, induced apoptosis through G0/G1 cell cycle arrest and mitochondrial pathway. Additionally, ZDQ-0620 inhibited the migration and tube formation of human umbilical vein endothelial cells (HUVECs). *In vivo*, neovascularization of rat aortic ring and chick chorioallantoic membrane (CAM) induced by VEGF was diminished when treated with ZDQ-0620. These results indicate that ZDQ-0620 induce apoptosis and anti-angiogenesis *via* inhibits the PI3K/AKT pathway. We suggest that the great potential of ZDQ-0620 as an effective treatment candidate against CRC.

Keywords: colorectal cancer, cell cycle arrest, apoptosis, angiogenesis, PI3K

OPEN ACCESS

Edited by:
Zhigang Bai,
Beijing Friendship Hospital, China

Reviewed by:
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *05 January 2022*
Accepted: *10 February 2022*
Published: *02 March 2022*

Citation:
Qin X, Liu M, Xu C, Xing B, Xu X, Wu Y, Ding H and Zhao Q (2022) ZDQ-0620, a Novel Phosphatidylinositol 3-Kinase Inhibitor, Inhibits Colorectal Carcinoma Cell Proliferation and Suppresses Angiogenesis by Attenuating PI3K/AKT/mTOR Pathway. Front. Oncol. 12:848952. doi: 10.3389/fonc.2022.848952

INTRODUCTION

Colorectal cancer, one of the most common cancers worldwide, has been a serious threat to human health (1). There are about 1.36 million new cases of colorectal cancer in the world every year, which is the third most common malignant tumor globally (2). The incidence of colorectal cancer ranks the third in men and the second in women. Every year about 690,000 cases of death, ranking the fourth malignancy (3). Despite advances in diagnosis and treatment over the past few decades, colorectal cancer remains a major health problem and a significant socio-economic burden (4). Only a few cases of CRC are detected early enough, so early accurate diagnosis and targeted treatment plan is particularly important, such as surgery, chemotherapy, and radiotherapy (5, 6). Rapid tumor growth, characterized by rapid progression and poor prognosis, is a major problem affecting the treatment of CRC (7).

With the research on oncogenic signaling pathways that regulate the proliferation, invasion, metastasis and angiogenesis of cancer cells, several possible hot therapeutic targets have been identified recently (8, 9). Among them, the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway is one of the most frequently activated in human cancers (10). PI3K/AKT/mTOR pathway is widely present in cells and is involved in cell growth, proliferation, differentiation regulation and other aspects (11). PI3K phosphorylates PIP2 to produce the second messenger phosphatidylinositol-3,4,5 -trisphosphate (PIP3) (12). Overactivation of PI3K leads to an increase in PIP3 levels, which in turn activates downstream AKT phosphorylation (13). In addition, overexpression of AKT has been proved in many cancers including CRC, which has a variety of biological activities, including inhibition of tumor cell apoptosis, promotion of invasion and metastasis, and regulation of tumor angiogenesis (8, 14). MTOR, a serine/threonine protein kinase, is a downstream molecule of AKT in the PI3K/AKT pathway and is involved in the regulation of protein synthesis, cell apoptosis, angiogenesis, etc (15). Activated PI3K/AKT can further activate mTOR through the TSC1/2 complex. Subsequent activation of mTOR promotes cell growth and cell cycle progression through phosphorylated translation regulator p70S6 kinase (p70S6K) and eukaryotic promoter (EIF) 4E binding protein 1 (4EBP1) (16, 17). Therefore, PI3K/AKT/mTOR pathway has become a key therapeutic target for cancer treatment (10, 11). Inhibiting the overactivation of the PI3K pathway in CRC appears to be a promising therapeutic strategy.

To find a novel structure-like PI3K inhibitor, we initiated a pharmacophore-oriented design. Our previous study designed and synthesized a series of 4-aminoquinazolines derivatives containing hydrophilic group acting on the PI3K/AKT/mTOR pathway (18). Among them, ZDQ-0620 (2,4-difluoro-N-(2-methoxy-5-(4-(3-morpholinopropyl)-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazin-6-yl)pyridin-3-yl)benzenesulfonamide) (**Figure 1A**) exhibited the best *in vitro* anti-cellular activity. In the present study, we intend to characterize biological effect of ZDQ-0620 in CRC cells, including cell proliferation, migration, invasion and angiogenesis, and further clarify the underlying mechanisms through modulating the PI3K/AKT/mTOR pathway, which sheds a light upon the thought of developing PI3K inhibitor and provides a promising direction as an anti-cancer drug against CRC in future.

MATERIALS AND METHODS

Cells and Reagents

All human cell lines used in this study were purchased from ATCC (Manassas, VA) and were cultured in RPMI-1640 medium (Gibco, USA), containing 10% fetal bovine serum (Hyclone, USA), penicillin-streptomycin (100 U/ml, Hyclone) at 37°C in a 5% CO_2 incubator. Propidium iodide (PI), Sulforhodamine B (SRB), and Hoechst 33342 were purchased from Sigma–Aldrich (St. Louis, MO). Primary antibodies aganist p-AKT (Ser473) (#9271), p-AKT (Thr308) (#9275), AKT (#4691), p-mTOR (#2971), mTOR (#2972), 4EBP1 (#9644), p-4EBP1 (#2855), p70S6K (#9202), p-p70S6K (#9205), Cyclin D1 (#2922), Bcl-2 (#4223), Bax (#5023), Caspase-3 (#9662), Cleaved caspase-3 (#9661), Caspase-9 (#9508), Cleaved caspase-9 (#7237), PARP (#9532), and β-actin (#3700) were purchased from Cell Signaling Technology (MA, USA). HRP and FITC-conjugated secondary antibodies were obtained from ZSGB-BIO (Beijing, China). All reagents were stored at -80°C.

Measurement of Cell Proliferation

Sulforhodamine B (SRB) Assay

Cells were inoculated into 96-well microplates at a concentration of 200 μL/well. Different concentrations of ZDQ-0620 were added for 24h, 48h and 72h, respectively, and fixed with 50% trichloroacetic acid solution for 1 hour. After washing with deionized water for 5 times, 0.4% sulforhodamine B (SRB) was used for staining in the darkroom. After 30min, the residual SRB was removed with 1% glacial acetic acid, and dried at room temperature. Finally, the absorbance was measured at 490 nm with a microplate analyzer (Synergy 2, Bio-Tek, USA).

Colony Formation Assay

The cells were seeded in 6-well plates at a density of 2×10^3 cells per well and cultured overnight. Cells were then treated with specified concentrations of ZDQ-0620, GSK2126458 or DMSO for 48h. After replacing the new medium, the cells were cultured for 7 days. The culture medium was discarded, fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet for 15 min, and photographed with digital camera.

LDH Assay

Cells were inoculated in 96-well plates at 3×10^3 and co-cultured with specific concentrations of ZDQ-0620, GSK2126458 or DMSO for 48 h. The supernatant was collected and co-incubated according to kit instructions (Wanlei, China), and the absorbance was determined at 490 nm using an enzyme plate analyzer (Synergy 2, Bio-Tek, USA). The percentage of lactate dehydrogenase release can be used as an indicator of cytotoxicity of compounds.

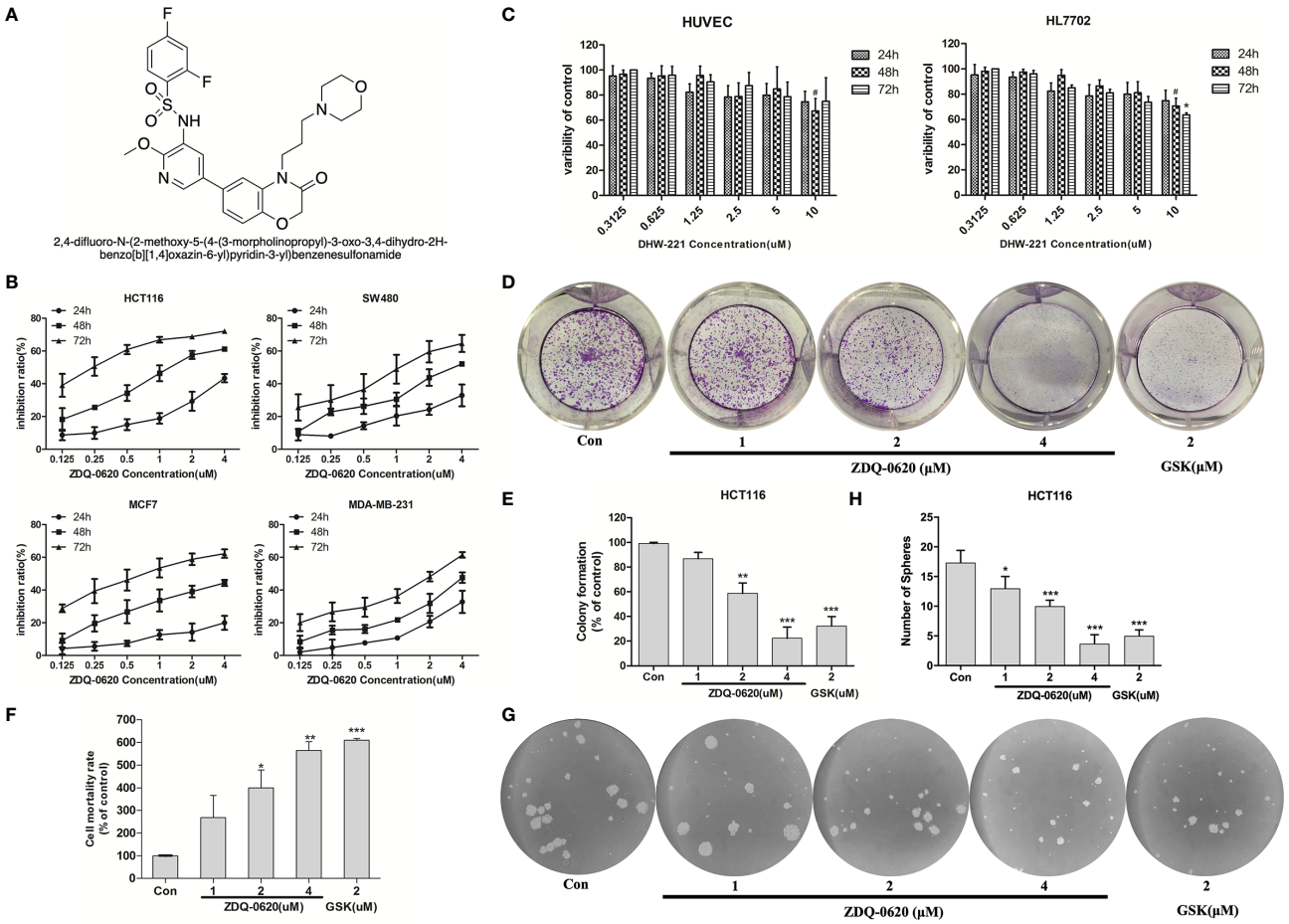

FIGURE 1 | ZDQ-0620 inhibits the viability and proliferative ability of carcinoma cells. **(A)** The structure of compound ZDQ-0620. **(B)** The viability of HCT116, SW-480, MCF-7, and MDA-MB-231 cells treated with various concentrations of ZDQ-0620 at 24, 48, and 72 h was determined by sulforhodamine B (SRB) assay. **(C)** A SRB assay was also used to determine the cytotoxicity of ZDQ-0620 toward normal cell lines (HUVEC and HL7702) at 72). **(D–H)** The inhibitory effect of ZDQ-0620 on the growth of HCT116 cells was measured by a colony formation assay **(D)**, LDH assay **(F)**, and tumor sphere formation assay **(G)**. Panel **(E)** the quantitative results of the colony formation assay. Panel **(H)** the quantitative results of the tumor sphere formation assay. Each value is the mean (± SD) from triplicate samples; $^{\#}p < 0.05$ vs. the control; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control. One-way analysis of variance followed by Tukey's *post hoc* multiple-comparisons test.

Tumor Sphere Formation Assay

Cells were inoculated at 4×10^3 on a low-attached 6-well plate (Corning, USA) and cultured in serum-free DMeM-F12 (Gibco) containing EGF (20ng/ml, Peprotech), B-FGF (20ng/ml, Peprotech) and B27(1:50 dilution, BD Biosciences). After 9 days, the number of tumor globules formed was counted by phase contrast microscope.

Determination of Apoptosis

Cell Morphology Analysis

Cells with a density of 2×10^4 were cultured overnight on a 6-well plate. After 48h treatment with ZDQ-0620, GSK2126458 or DMSO, Hoechst 33342 (Beyotime Bio, China) was stained at 37°C for 20 min. After two PBS rinses, fluorescence microscopy (IX71, Olympus, Japan) was used to take pictures. The nuclei of apoptotic cells are densely stained, completely or partially bright blue.

Annexin V-FITC/PI *Assay*

In brief, the cells were inoculated in 6-well plates at a density of 2×10^3 per well. ZDQ-0620, GSK2126458 or DMSO were treated for 48 h, and the collected cells were stained in the dark with Annexin V-FITC and PI (Wanlei, China) for 10-20 min. Finally, the cells were sorted and quantitatively analyzed by flow cytometry (Becton-Dickinson, NJ, USA).

Transmission Electron Microscopy

Cells were seeded into 6-well plates at a density of 5×10^4 cells/well. After treated with ZDQ-0620 for 24 h, the cells were collected and fixed with 3% glutaraldehyde, then fixed with 1% O_SO_4, then dehydrated with ethanol step by step, embedded and sected. It was stained with uranium acetate and lead citrate and observed under transmission electron microscope. (H-7650, Hitachi, Japan).

Mitochondrial Membrane Potential Assay (△ψm)

The cells were inoculated in 6-well plates at a density of 1×10^4 overnight and treated with the specified concentration of ZDQ-0620, GSK2126458 or DMSO for 48 h. The cells were incubated in an incubator at 37°C for 20 min with 1 mL of JC-1 staining solution per well. After washing with PBS, the cells were imaged under a fluorescence microscope (IX71, Olympus, Japan). Red fluorescence indicates normal MMP, while green fluorescence indicates decreased MMP.

PI3K Activity Assay

The effect of ZDQ-0620 on PI3K kinase assay was measured by ADP-GloTM Lipid Kinase Systems (Promega, USA). Treatment for experiments was carried out according to the manufacturer's instructions.

Cell Cycle Assay

Cells were seeded in 6-well plates at a density of 2×10^5 per well. After treated with ZDQ-0620 for 48 hours, cells were collected and fixed overnight in 70% cold ethanol at 20°C. The cells were rinsed with cold PBS buffer and then stained darkly with PI for 15 minutes at room temperature. The samples were analyzed by FACS Calibur flow cytometry (Becton-Dickinson, NJ, USA).

Western Blotting

Cells were exposed to gradient dose of ZDQ-0620 for 48 h. The expression levels of related proteins in cells were determined by standard Western blot.

Molecular Docking

Using the GLIDE module of the Schrodinger software with the Maestro interface to predict the binding modes of PI3K and ZDQ-0620. ZDQ-0620 was drawn by ChemBio3D Ultra 13.0 software and prepared using the Ligprep. Then, the crystal structures of PI3Kα was download from the Protein Data Bank and prepared by the Protein Preparation Wizzard. The active pocket of proteins was generated with the Grid Generation tool. Other parameters are keep as software defaults. The docking poses were analyzed with Pymol.

Immunofluorescence Images

Cells with a density of 1×10^4 were inoculated overnight in 6-well plates and treated with ZDQ-0620 (2μM) for 24 h, fixed with 4% paraformaldehyde, permeated with 0.2%Trion-X for 20min, incubated with immunostain blocking buffer for 1 h, and incubated overnight with p-Akt (Ser473) at 4°C. After washing with 4 °C of PBS, the cells were incubated at room temperature for 1 h with secondary antibody, and then the nuclei were stained with DAPI for 15min. The staining cells were imaged by fluorescence microscopy (IX71, Olympus, Japan).

CETSA Assay

HCT116 cells were treated with a specified concentration of ZDQ-0620 for 3 h, then the cells were collected with PBS containing protease inhibitors and transferred into 200μL EP tubes. Cells were placed in a PCR apparatus (2720, Gene, Singapore) and subjected to heat shock at 37~77°C for 3min. Lysed for 3 times in liquid nitrogen - room temperature cycles. Centrifuge at 13 000 RPM at 4°C for 20 min. Loading buffer was added and standard Western blot was performed.

Wound Healing Scratch Assay

Cells of 1×10^4 density were inoculated in 6-well plates until 90% were full. Cell-free area was formed in the designated area of sterile pipetting tip, and cells were treated with ZDQ-0620, GSK2126458 or DMSO after twice washing with PBS. Images were taken under an inverted microscope (IX71, Olympus, Japan) at 0 and 24 h, and the Image J software analyzed and calculated the proportion of cells migrating to the cleared area (wound healing). Migration rate=(1-width $_{24h}$)/width $_{0h}$.

Migration and Invasion Assay

Cells with a density of 4×10^4 were seeded into the upper cavity of a 24-well transwell plate (Corning, USA) without or coated with matrix gel (Becton Dickinson, USA). The lower cavity was cultured in complete medium containing 10% fetal bovine serum for 48h. The migrated and invaded cells were fixed with 70% acetaldehyde, stained with 0.1% crystal violet and photographed under a microscope (IX71, Olympus, Japan).

Tube Formation Assay

The matrix adhesive was mixed with RPMI-1640 medium in equal volume, and then added into 48-well plate at 100μL/well to cure. 1×10^5 cells/100μL/well HUVEC cells were seeded into 48-well plates with the specified concentration of ZDQ-0620, GSK2126458 or DMSO. After culture for 3 h, the tube formation was observed by inverted microscope (IX71, Olympus, Japan).

Chick Chorioallantoic Membrane (CAM) Assay

The sterilized fertilized eggs (SAIS Poultry Co. Ltd., China) were incubated in an incubator at 37.8°C and 60% humidity for 5 days. The $4cm^2$ pore was opened at the end of the air chamber, and the eggshell membrane was discarded to protect the chorioallantoic membrane of the chicken embryo from damage. VEGF and/or ZDQ-0620 and GSK2126458 were added to allantoic membrane and sealed for further incubation for 48 h. The microvascular growth was observed with digital camera. Ten eggs in each group were incubated.

Rat Aortic Ring Sprouting Assay

The rats were purchased from Vital River Technology Co., Ltd (Beijing, China). The aortas of 6-week-old SD rats was cut into 2mm long rings and randomly divided into groups, then the aortas were embedded in a 96-well plate with 70 μL matrix gel and solidified in an incubator for 30 min. The specified concentrations of VEGF and/or ZDQ-0620 and GSK2126458 were added and incubated for 7 days. Photograph with a microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Unless otherwise stated, all experiments in this study were repeated three times. Statistical analysis was performed using

GraphPad Prism 5 (GraphPad software, CA, USA). Groups were compared with the unpaired Student's t-tests, and multiple groups were compared with the one-way ANOVA. All data were expressed as means values ± SD. $p < 0.05$ indicated statistically significant.

RESULTS

Effect of ZDQ-0620 on the Growth of HCT116 Cells

To verify the function of ZDQ-0620 as a new therapeutic compound, we first measured cell growth inhibition on four cancer cell lines (HCT116, SW480, MCF-7, MDA-MB-231) using SRB assay. ZDQ-0620 significantly inhibited the growth of all tested cells in a dose- and time-dependent manner (**Figure 1B**). Especially, 0.1uM of ZDQ-0620 induced strong reduction in growth rate of HCT116 reached 40% at 72h. To predict side effects of ZDQ-0620, it was exposed to normal cell lines (**Figure 1C**). The cytotoxicity of ZDQ-0620 to HUVEC and HL7702 cells was much lower than that to tumor cells, suggesting the hypo-toxicity of ZDQ-0620 to normal cells.

Moreover, HCT116 cells were selected to investigate the long-term toxic effect of ZDQ-0620 on the proliferation. The colony formation, LDH release and Sphere-forming of HCT116 cells were significantly inhibited in a dose-dependent manner (**Figures 1D–H**), the activity value was stronger than that of GSK2126458, as a positive drug. These results suggested that ZDQ-0620 significantly inhibited the growth of cancer cells and has lower cytotoxicity in normal cell lines.

ZDQ-0620 Induces G0/G1 Cell Cycle Arrest and Induces CRC Cells Apoptosis Through the Mitochondrial Pathway

To examine the mechanism responsible for ZDQ-0620-mediated cell growth inhibition, the cell cycle distribution was performed by PI staining, hence followed by flow cytometry (FACS) detection. As shown in **Figures 2A, B**, ZDQ-0620 induced a dose-dependent accumulation of cells in the G0/G1 phase. Moreover, to confirmed the effects of ZDQ-0620 on cell cycle regulation, we examined the expression of endogenous cyclins after 48 h treatment with ZDQ-0620. As shown in **Figures 2C, D**, ZDQ-0620 played an important role in G0/G1 cell cycle arrest by down-regulating the levels of cyclin D1, but has little effect on cyclin B1, which regulates the G2/M phases.

The PI3K/AKT/mTOR pathway can prevent programmed death of tumor cells and inhibit apoptosis, thus promoting the survival of tumor cells. To assess the pro-apoptotic effect of ZDQ-0620, inverted microscope and Hoechst 33342 staining assay was used. As shown in **Figure 3A**, apoptosis was confirmed by the presence of condensed chromatin and nuclear fragmentation. ZDQ-0620 treatment group marked with brighter fluorescence in Hoechst staining assay. In addition, cell apoptosis was detected by Annexin V/PI staining. In **Figures 3B, C**, compared with the control, the proportion of apoptotic cells (Annexin V positive) in ZDQ-0620 increased significantly and showed a concentration-dependent manner. The ultrastructure of cells observed by transmission electron microscopy was <0.2 μm, which is the gold standard for the determination of apoptosis. As shown in **Figure 3D**, cells treated with ZDQ-0620 showed typical apoptotic characteristics, such as

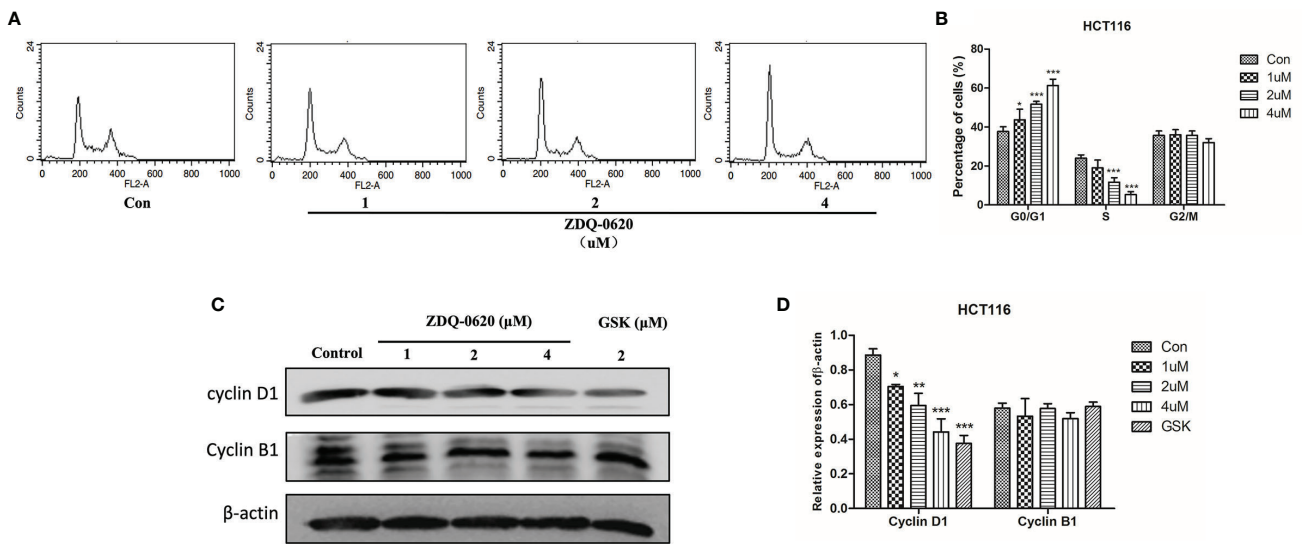

FIGURE 2 | ZDQ-0620 induces HCT116 cell cycle arrest. **(A)** ZDQ-0620 were performed by PI staining, hence followed by flow cytometry (FACS) detection. **(B)** The raw histogram is shown on the left and the quantification of individual cell cycle stages on the right. **(C)** The expression levels of cell cycle-related proteins in HCT116 cells after 48 h of treatment with the specified concentrations of ZDQ-0620 and GSK2126458 were assessed by western blotting. **(D)** The histogram shows average protein expression levels. Each value is the mean (± SD) from triplicate samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control. One-way analysis of variance followed by Tukey's *post hoc* multiple-comparisons test.

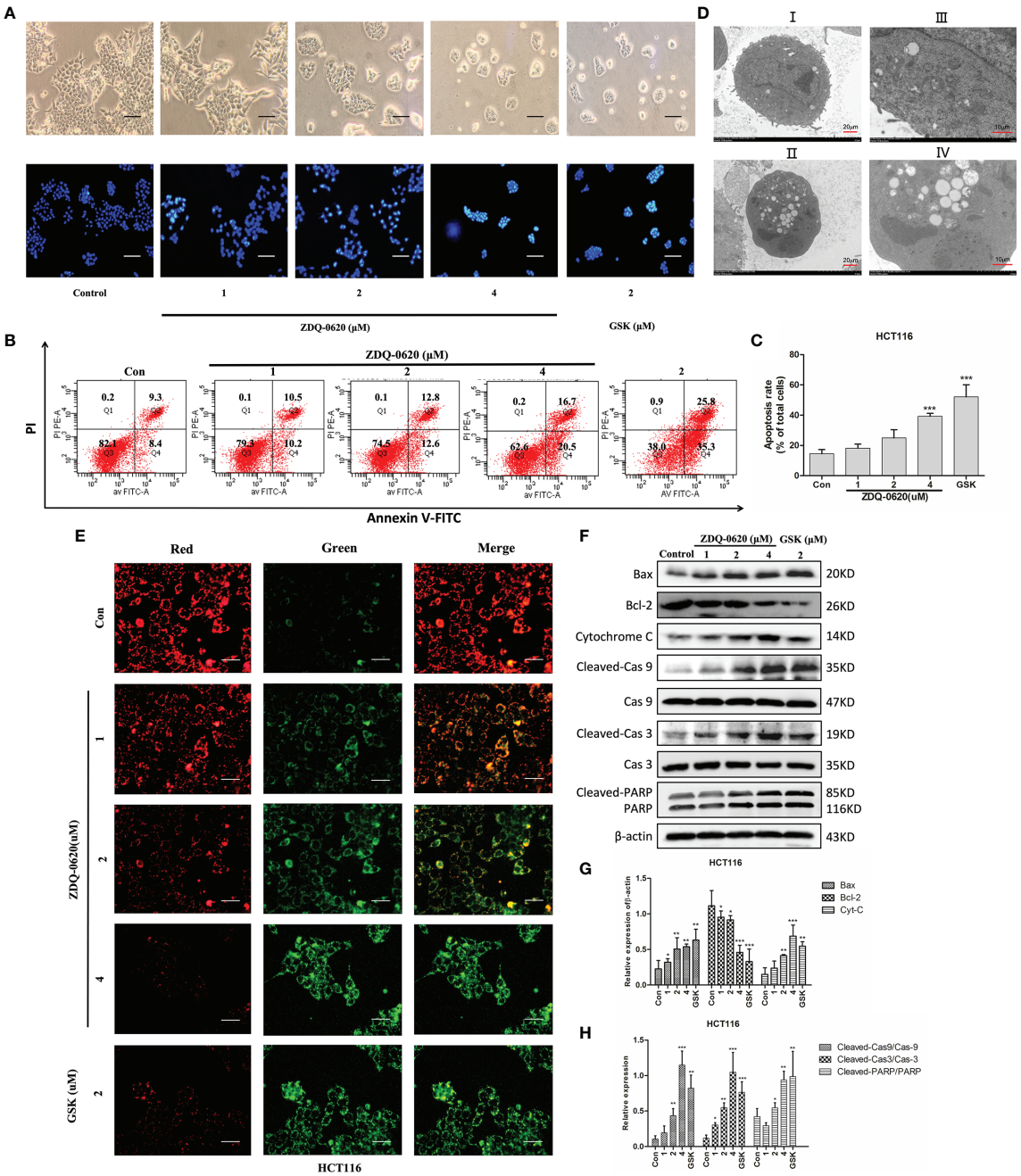

FIGURE 3 | ZDQ-0620 induces HCT116 cells apoptosis. **(A)** Morphology of the cells (magnification, ×40) and Hoechst 33342 staining results. Apoptotic cells are bright blue; scale bar, 50 μm. **(B)** Analysis of the apoptotic effects of ZDQ-0620 and GSK2126458 by Annexin V/propidium iodide (PI) staining. **(C)** The histograms show the percentages of apoptotic HCT116 cells following treatment with ZDQ-0620 and GSK2126458 for 48 h (right). **(D)** Transmission electron microscopic analysis of the morphological changes occurring in cells after 48 h treatment (I and II: scale bar = 20 μm [left]; III and IV: scale bar = 10 μm [right]). **(E)** Effect of mitochondrial membrane potential detection on HCT-116. At high mitochondrial membrane potential, JC-1 aggregates in the matrix of mitochondria and forms a polymer (J-aggregates), which produces red fluorescence. At low mitochondrial membrane potential, JC-1 could not aggregate in the mitochondrial matrix, and as a monomer, JC-1 could produce green fluorescence; scale bar, 100 μm. **(F)** The expression levels of apoptosis-related proteins were detected by western blot. **(G, H)** Bar graph of the quantitative result. Each value is the mean (± SD) from triplicate samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control. One-way analysis of variance followed by Tukey's *post hoc* multiple-comparisons test.

peripheral nuclear marginalization and chromatin condensation. Pictures I and III showed control group with clear cell spacing and intercellular connections, but not tight connections. In contrast, cells treated with ZDQ-0620 showed increased heterochromatin in the nucleus, and the chromatin condensed into apoptotic bodies in pictures II and IV.

In order to further clarify the potential mechanism of ZDQ-0620-induced apoptosis, mitochondrial membrane potential was detected by JC-1 staining assay after 48 h of treatment (**Figure 3E**). As the number of apoptotic cells increased, the proportion of red fluorescence and green fluorescence decreased gradually in a dose-dependent manner. To further verify these results, western blot was used to detect the expression of apoptosis-related proteins. **Figures 3F–H** indicated that ZDQ-0620 up-regulated the expression of Bax, cytochrome C, down-regulated the expression of anti-apoptotic protein Bcl-2. The expression levels of cleaved caspase-3/9 and cleaved PARP were also significantly increased ($p < 0.01$). These results suggest that ZDQ-0620 could activate the caspase-dependent apoptosis cascade through mitochondrial pathway, thereby inducing apoptosis of HCT116 cells.

ZDQ-0620 Effectively Blocks the PI3K/AKT/mTOR Signaling Pathway by Direct Targets PI3K

PI3K/mTOR and its downstream targets, 4EBP1 and P70S6K, are important factors for tumor cell survival and proliferation. To elucidate the anticancer mechanism of ZDQ-0620, western blot analysis was used evaluated the effects of ZDQ-0620 on the PI3K/AKT/mTOR pathway in HCT116. The results indicate that ZDQ-0620 could significantly suppress expression AKT and mTOR phosphorylation, which subsequently repressed phosphorylation of 4EBP1 and P70S6K (**Figure 4A**), and these effects were concentration-dependent (**Figures 4B, C**).

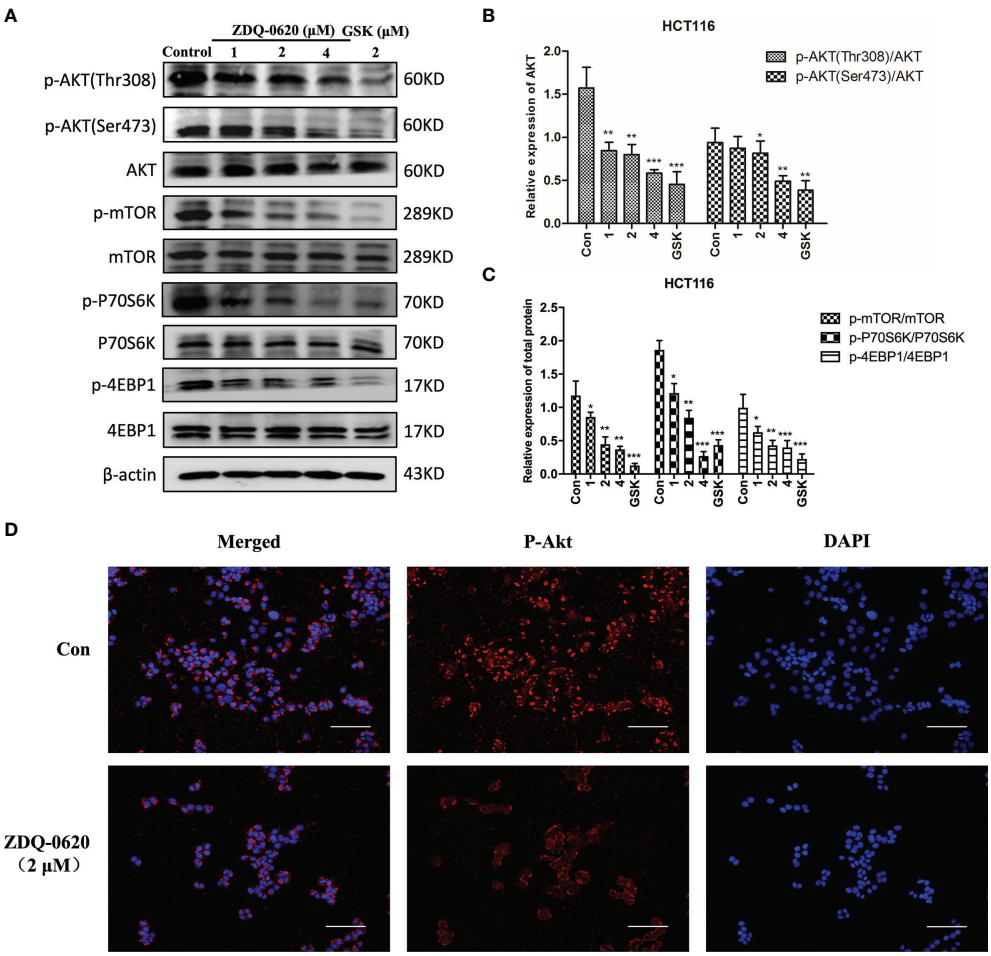

FIGURE 4 | ZDQ-0620 blocks the PI3K/AKT/mTOR pathway. **(A)** HCT116 cells were treated with ZDQ-0620 or GSK2126458 for 48 h following which western blotting was performed on the cell lysates. **(B, C)** The histogram shows average protein expression levels. **(D)** Immunofluorescence imaging of p-AKT in HCT116 cells treated with ZDQ-0620. Anti-rabbit p-AKT (Ser473) antibody was used for labeling and the nuclei were stained with DAPI; scale bar, 50 μm. Each value is the mean (± SD) from triplicate samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control. One-way analysis of variance followed by Tukey's *post hoc* multiple-comparisons test.

Immunofluorescence analysis also confirmed the same results, the phosphorylation levels of AKT were significantly inhibited by ZDQ-0620 in HCT116 cells (**Figure 4D**).

Then the target of the compound action was further identified. In order to explore the combination mode between ZDQ-0620 and PI3K, Maestro was used for molecular docking, and Discovery Studio 4.0 Visualizer was used to analyze the interaction mode. As shown in **Figure 5A**, Lys833 in the active pocket of PI3K forms a strong salt bridge with nitrogen on the ZDQ-0620 sulfonamide group, and forms a hydrogen bond with oxygen on the pyridine ring. In addition, the oxygen of the parent group of sulfonyl and benzooxazinone forms hydrogen bonds with Ser806 and Val882, respectively. The hydrophobic interaction between the morpholine ring and Lys890 further stabilized the binding of PI3K to ZDQ-0620. In conclusion, ZDQ-0620 can form a stable binding mode with PI3K, and the introduction of ethylmorpholine can increase the water-solubility and activity of ZDQ-0620. Then, the inhibitory activity of ZDQ-0620 against PI3 kinase was investigated. As shown in **Table 1**, ZDQ-0620 showed strong inhibitory activity against four PI3 kinase subtypes, and the inhibitory activity was below 160 nM. The inhibitory activity against PI3Kα was the best with IC50 of 0.5 nM. To further evaluate the binding character between ZDQ-0620 and PI3K, the CETSA assay was used with the treatment of HCT116 with or without ZDQ-0620. From CETSA experiments, the apparent aggregation temperatures (Tagg) were obtained with either ZDQ-0620 or DMSO, which could be compared, and substantial shifts demonstrated the binding of ZDQ-0620 and target proteins. As shown in **Figures 5B, C**, after ZDQ-0620 bound with PI3K, the thermal stabilization of PI3K were increased compared with the control groups (DMSO), and this thermal stabilization between ZDQ-0620 and PI3K was dose-dependent from $ITDRF_{CETSA}$ (**Figures 5D, E**). In conclusion, ZDQ-0620 can effectively blocks the PI3K/AKT/mTOR signaling pathway by direct targets PI3K.

TABLE 1 | IC_{50} of ZDQ-0620 against PI3K kinase *in vitro*.

Compound	PI3Kα(nM)	PI3Kβ (nM)	PI3Kγ (nM)	PI3Kδ (nM)
ZDQ-0620	0.5	93	55	152

ZDQ-0620 Inhibits the Migration, Invasion and Interaction of HCT116 and HUVEC

The PI3K/AKT signaling pathway plays an important role in cancer cell migration and invasion. To determine the effects of ZDQ-0620 on colonic carcinoma cell migration and invasion ability, we first performed wound healing and invasion assays in

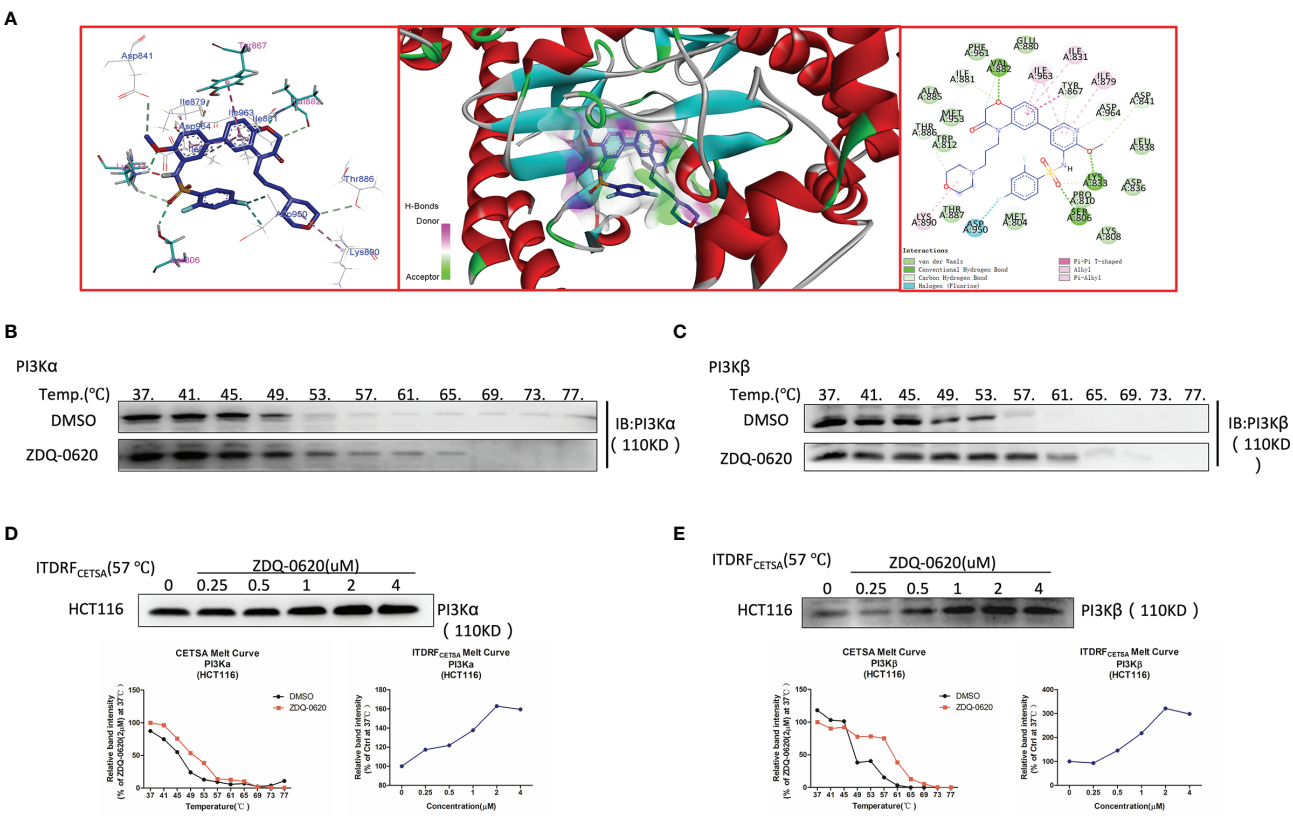

FIGURE 5 | ZDQ-0620 inhibits PI3K target activity. **(A)** Predicted binding modes for ZDQ-0620 with PI3Kα (PDB code: 4JPS). **(B–E)** A cellular thermal shift assay (CETSA) and an isothermal dose-response fingerprinting (ITDRF)cetsa were used to evaluate the thermal stability of ZDQ-0620 bound to PI3Kα/β.

HCT116 cell line. For wound healing and transwell migration assay, as expected, ZDQ-0620 significantly inhibited HCT116 cell metastasis in a concentration-dependent manner (**Figures 6A, B**). For transwell invasion assay, the results showed that the inhibition of invasiveness ability concentration-dependently increased (**Figure 6B**).

Angiogenesis is closely related to tumor metastasis and invasion. The migration and invasion ability of vascular endothelial cells is an important factor affecting neovascularization. Next, we investigated the effects of ZDQ-0620 treatment on HUVEC cell migration and invasion. Compared with control, the migration ability of HUVEC cells decreased gradually with the increase of ZDQ-0620 concentration (**Figures 6C, D**). Similarly, as shown in **Figure 6D**, ZDQ-0620 also inhibited the invasion of HUVEC cells in a concentration-dependent manner. These results indicate that ZDQ-0620 can significantly inhibit the migration and invasion ability of HUVEC cells *in vitro*, thus inhibit angiogenesis.

The interaction between tumor cells and vascular endothelial cells can play a synergistic role and promote the occurrence and progression of tumor. To investigate the effect of ZDQ-0620 on the interaction between HCT116 and HUVEC, the transwell chamber was used to verify the cell co-culture interaction. HUVEC was inoculated into the upper layer of transwell, and HCT116 was inoculated into the lower layer. As shown in **Figure 6E**, the presence of HCT116 cells in the lower layer significantly improved the HUVEC metastasis and invasion ability in the upper compartment. The migration and invasion levels were significantly inhibited with the addition of ZDQ-0620. Similarly, HCT116 was inoculated into the upper layer of transwell and HUVEC into the lower layer. Compared with the

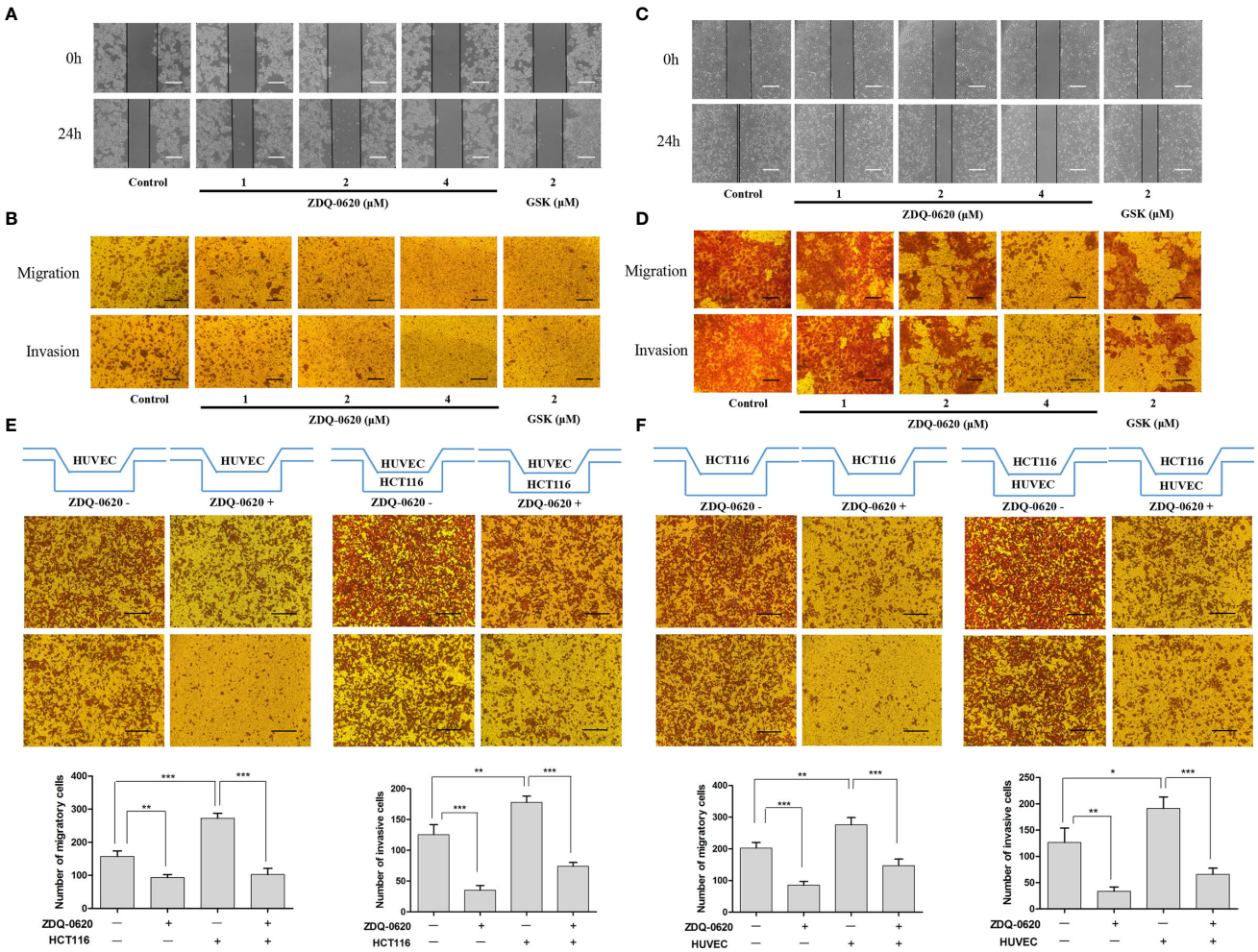

FIGURE 6 | ZDQv-0620 inhibits the migration, invasion and interaction. **(A, C)** Cell migratory ability was tested using a wound healing assay; scale bar, 50 μm. **(B, D)** Cell invasiveness was assessed using a Transwell assay with Matrigel; scale bar, 50 μm. **(E)** HUVECs were inoculated alone in the upper chamber or co-cultured with HCT116 cells in the lower chamber with or without ZDQ-0620 (2uM) for comparison, Scale bar =100 μm. **(F)** HCT116 was inoculated alone in the upper chamber or co-cultured with HUVECs cells in the lower chamber with or without ZDQ-0620 (2uM) for comparison. A histogram of the invaded and migrated rates is shown at the bottom. Each value is the mean (± SD) from triplicate samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control. One-way analysis of variance followed by Tukey's *post hoc* multiple-comparisons test.

uninoculated cells in the lower layer, the migration and invasion of HCT116 in the upper layer were significantly increased after inoculation with HUVEC. The migration and invasion ability were also significantly inhibited with the addition of ZDQ-0620 (**Figure 6F**).

ZDQ-0620 Inhibits Endothelial Cell Tube Formation and Vasculogenic Mimicry (VM) of HCT116

Endothelial differentiation (tube formation) is necessary for angiogenesis. ZDQ-0620 had little effect on the survival of HUVEC cells (**Figure 1C**), suggesting that ZDQ-0620 had low cytotoxicity to HUVEC. After treatment with ZDQ-0620, the tube formation of HUVEC cells was investigated, and it was observed that ZDQ-0620 significantly inhibited the catheterization of HUVEC cells in a concentration- (**Figures 7A–C**) and time-dependent manner (**Figures 7D–F**), ZDQ-0620 reduces the number of tubules and nodes.

The formation of VM leads to poor prognosis and promotes tumor hematogenous metastasis. Next, the ability of HCT116 to simulate microtubule formation in endothelial cells was verified, and the effect of ZDQ-0620 on the angiogenic mimicring-inhibiting activity of HCT116 was investigated. As shown in **Figure 7G**, HCT116 formed obvious microtubule connections.

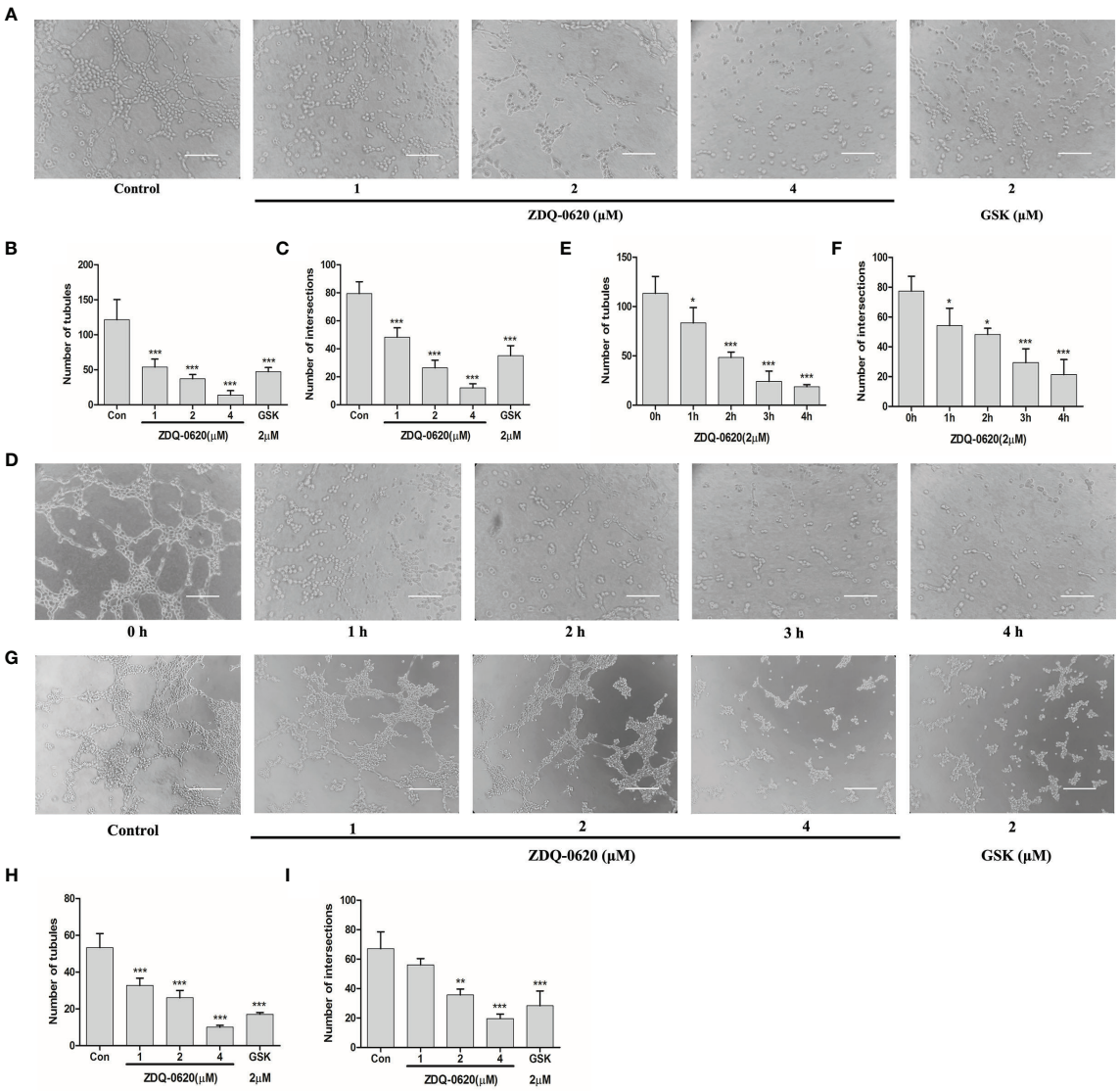

FIGURE 7 | ZDQ-0620 inhibits endothelial cell tube formation and vasculogenic mimicry (VM) of HCT116. **(A, D)** ZDQ-0620 inhibited tube formation in HUVECs. Scale bar, 100 μm. **(B, C, E, F)** Histogram showing the average number of cells in the five random areas on each filter under each condition. **(G)** Decrease of VM formation in HCT116 cells after ZDQ-0620 treatment, Scale bar =100 μm. **(H, I)** The number of microtubules and the number of microtubule nodes decreased after treatment with different concentrations of ZDQ-0620. Each value is the mean (± SD) from triplicate samples; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs*. the control. One-way analysis of variance followed by Tukey's *post hoc* multiple-comparisons test.

ZDQ-0620 could significantly inhibit the formation of angiogenic mimicry in a concentration-dependent manner (**Figures 7H, I**). Among them, the number of tubules and nodes in angiogenic mimicry concentration-dependently decreased after ZDQ-0620 treatment. In conclusion, ZDQ-0620 can inhibit the formation of angiogenic mimicry in HCT116 and play an indirect anti-angiogenic role.

ZDQ-0620 Suppresses VEGF-Induced Neovascularization of Rat Aortic Ring and CAM

In order to further verify the anti-angiogenic activity of ZDQ-0620, we first utilized rat aortic ring sprouting in *ex-vivo*. According to the results in **Figure 8A**, VEGF group (control) significantly stimulated the germination of rat aortic ring vessels and formed tubules covering the aortic ring. However, ZDQ-0620 could significantly inhibit VEGF-induced vascular germination in a concentration-dependent manner.

In addition, chicken embryo chorioallantoic membrane (CAM) was used to simulate angiogenesis *in vivo*. As shown in **Figure 8B**, compared with the blank group, VEGF could accelerate the new generation and growth of allantoic membrane blood vessels. VEGF group had more branches of vascular network, thicker vascular diameter and formed a dense vascular network after 48h treatment. However, ZDQ-0620 could significantly inhibit VEGF induction with sparse distribution of vascular network and low bifurcation degree, and its inhibitory activity was significantly stronger than that of positive drug GSK2126458. It is suggested that ZDQ-0620 can inhibit the angiogenesis *in vivo*. These data validate that the effects of ZDQ-0620 on the initiation stage of angiogenesis can be translated into the final suppression of microvessel formation.

DISCUSSION

The PI3K/AKT/mTOR pathway plays a critical role in the occurrence and progression of colorectal cancer (19, 20). Excessive activation of various receptor tyrosine kinases (such as insulin-like growth factor (IGF) and epithelial growth factor receptor (EGFR)) can also promote abnormal activation of this pathway (21). In addition, genes in the PI3K/AKT/mTOR pathway change most frequently in human cancers (22). Due to these somatic mutations, abnormal activation of this pathway is associated with cell transformation, tumorigenesis, angiogenesis and cancer progression (10, 23). Therefore, the PI3K/AKT/mTOR pathway has been a popular target for antitumor drug research in the past decades (24). However, the optimal treatment strategy for this pathway has not been established in CRC.

In this study, we investigated the anti-cancer effects of 4-aminoquinazoline derivative ZDQ-0620, and further elucidated its mechanism as a novel PI3K inhibitor. For the first time, we reported that ZDQ-0620 has excellent activity on proliferation, migration, invasion and angiogenesis of CRC cells by blocking the PI3K/AKT/mTOR pathway. And the activity of ZDQ-0620 was significantly better than that of the positive drug GSK2126458, a PI3K inhibitor.

Since the regulation of PI3K/AKT/mTOR pathway can promote cell survival and proliferation, our study shows that

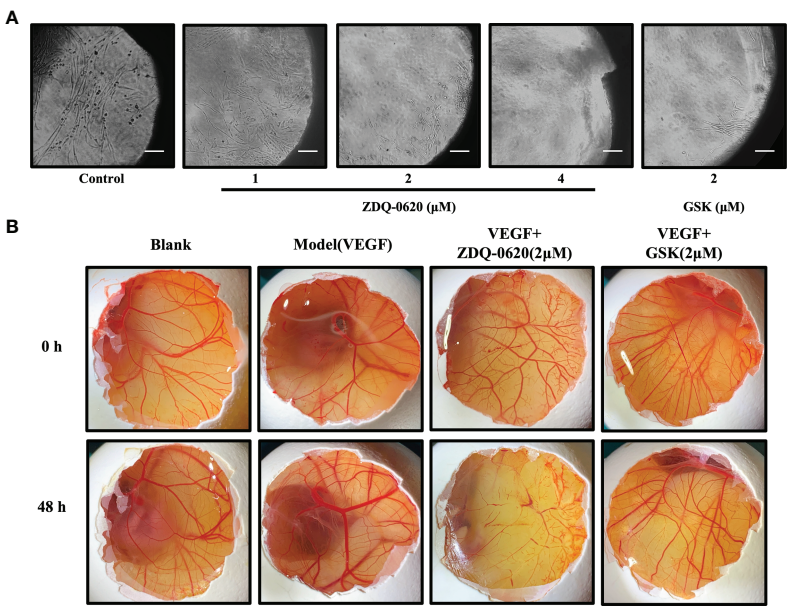

FIGURE 8 | ZDQ-0620 inhibits angiogenesis *ex vivo* and *in vivo*. **(A)** ZDQ-0620 inhibited microvascular sprouting in rat aortic rings; scale bar, 100 μm. **(B)** Chicken eggs were treated with vehicle, VEGF (50 ng/mL), or VEGF+ZDQ-0620 or GSK2126458. Representative images from separate experiments are shown. All experiments were repeated three times.

the reduction of HCT116 cell proliferation by ZDQ-0620 seems to be related to the PI3K/AKT/mTOR pathway. So we first used western blot assay to identified that ZDQ-0620 remarkably suppressed the activation of AKT, mTOR, p70S6K, and 4EBP1 in CRC cancer cell HCT116, thus inhibited PI3K/AKT/mTOR pathway. Moreover, vitro molecular docking results indicated that ZDQ-0620 inhibited 50% PI3K subtype activity (IC50) below 152 nM dose. The IC50 inhibition rate of PI3Kα reached 0.5nM, which was significantly stronger than other subtypes, with a difference of about 1000 times. It is suggested that ZDQ-0620 may be a PI3Kα target inhibitor, rather than a generic PI3K inhibitor. So, CETSA was further used to evaluate the binding ability of PI3K and ZDQ-0620. By CETSA assay, both ZDQ-0620 and DMSO obtained apparent aggregation temperatures (TAGG) that could be compared and showed significant changes indicating that ZDQ-0620 binds to the target protein (25). Western blot results suggested that ZDQ-0620 could significantly improve the binding ability with PI3K target protein, and the binding strength was dose-dependently. CETSA results confirmed that the compound ZDQ-0620 may target PI3K. Meanwhile, the CETSA Melt Curve showed that the binding ability of ZDQ-0620 to PI3Kα was significantly stronger than that of ZDQ-0620 to PI3Kβ, confirmed that the compound ZDQ-0620 may target PI3Kα.

Apoptosis plays an important role in the process of tumor proliferation and is a key mechanism to inhibit the growth of cancer cells (26). At the same time, the molecular mechanisms of apoptosis has also been confirmed to play an important role in the anti-tumor effect of PI3K/AKT/mTOR pathway (26–28), by activating various apoptotic signals or inhibiting survival signals (29, 30). In this study, we demonstrated that ZDQ-0620 can induce the apoptosis of HCT-116 cells through G0/G1 cell-cycle arrest. Bcl-2 is a key pro-apoptotic regulatory factor and its overexpression is associated with CRC (31). In addition, the Bcl-2 associated death promoters Bax is important target substrates for AKT (32–34). Bax is a pro-apoptotic protein that can be transported to mitochondria following the induction of cell death (31). Meanwhile, an increase in the Bax/Bcl-2 ratio upregulates the levels of cleaved caspase-3, leading to PARP cleavage and, consequently, irreversible apoptosis. In the present study, we found that ZDQ-0620 induces HCT-116 cell apoptosis by suppressing the expression of cleaved Bcl-2 and promoting that of Bax, cleaved caspase-3/9, and PARP. This suggested that the mitochondrial pathway may be the main mechanism underlying ZDQ-0620-induced apoptosis in CRC. In the PI3K/AKT/mTOR signaling pathway, AKT activates mTOR, thereby accelerating the transcription of genes required for cell cycle progression through the regulation of the downstream targets, 4EBP1 and p70S6K (35–37). Herein, we confirmed that the antiproliferative activity of ZDQ-0620 was related to G0/G1 cell cycle arrest, an effect that is likely achieved through a reduction in the expression levels of PI3K/AKT/mTOR pathway-related proteins.

When activated, the PI3K/AKT/mTOR signaling pathway not only modulates the translation of proteins involved in cell transformation and proliferation, but is also involved in tumor metastasis, invasion (38), and angiogenesis (39). AKT plays an important role in tumor invasion and metastasis by positively regulating the expression of MMP-2 (40). MMP-2 subsequently enhances the migration of cancer cells by degrading the extracellular matrix while also promoting metastasis through the induction of angiogenesis (41). In angiogenesis, the PI3K/AKT pathway is stimulated by multiple signals, including endothelial VEGF (42), and regulates multiple key steps through the phosphorylation of downstream target substrates, such as mTOR (43). In addition, the PI3K/AKT/mTOR pathway is associated with VEGF-induced endothelial signaling (43, 44). In Zang's study (45), they identified that VEGF is a key factor in regulating angiogenesis and can be secreted from tumor cells. It was also confirmed that anti-tumor drugs inhibited the interaction between cancer cells and HUVECs by inhibiting VEGF. In addition, VEGF expression is regulated by NF-κB and PI3K/AKT signaling pathways; there is increasing evidence that PI3K pathway is involved in VEGFA dysregulation (46–49). The activation of endothelial PI3K/AKT/mTOR pathway signals can promote the survival of cultured microtubules *in vitro* (44) and tumor blood vessels *in vivo* (50, 51). Our results confirmed that ZDQ-0620 inhibits the migration, invasion, and angiogenesis of CRC cells, which may be related to a decrease in AKT phosphorylation levels or inhibit the secretion of chemokines, such as VEGF. These observations indicate that inhibition of the PI3K/AKT pathway may be the key mechanism underlying the anti-cancer effects of ZDQ-0620.

In conclusion, ZDQ-0620 was identified as being a PI3K target inhibitor, and displayed excellent anti-CRC activity, including the inhibition of cell proliferation, migration, invasion, protein synthesis, and angiogenesis, *via* the blocking of the PI3K/AKT/mTOR signaling pathway both *in vitro* and *in vivo*. Additionally, ZDQ-0620 was found to induce G0/G1 cell cycle arrest and mediate cell apoptosis through the mitochondrial pathway. Combined, these findings indicated that ZDQ-0620 has potential as a novel anti-cancer drug targeting the PI3K/AKT/mTOR pathway in CRC to inhibit tumor initiation and progression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee for Animal Experiments of Shenyang Pharmaceutical University.

AUTHOR CONTRIBUTIONS

QZ and HD for supervision and funding acquisition. XQ for conceptualization, methodology, validation, formal analysis,

writing - original draft, writing - review & editing and project administration. ML for investigation and resources. Others for editing and proofread. All authors read and approved the final manuscript.

FUNDING

This research was supported by Liaoning Natural Fund Guidance Plan (Number: 2019-ZD-0446).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Oncology

ORIGINAL RESEARCH
published: 06 April 2022
doi: 10.3389/fonc.2022.862116

Variations in Circulating Levels of Angiopoietin-2 Over Time Are Predictive of Ramucirumab–Paclitaxel Therapy Outcome in Advanced Gastric Cancer: Results of Prospective Study

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The combination of paclitaxel and ramucirumab is the second-line therapy of choice in the treatment of advanced gastric cancer. To date, no biomarkers are available in gastric cancer to predict the outcome of antiangiogenic therapy. The present prospective study included 35 patients undergoing second-line therapy with ramucirumab and paclitaxel. Serum samples were systematically collected from the beginning of therapy and at each cycle until disease progression. Multiplex analysis of a panel of angiogenic factors identified markers for which the changes at defined time intervals were significantly different in patients with progression-free survival ≤3 (Rapid Progression Group) compared to those with progression-free survival >3 (Control Disease Group). Comparative analysis revealed significantly different results in the two groups of patients for VEGFC and Angiopoietin-2, both involved in angiogenesis and lymphangiogenesis. VEGFC increased in the progressive-disease group, while it decreased in the control-disease group. This decrease persisted beyond the third cycle, and it was statistically significant compared to the basal level in patients with longer progression-free survival. Angiopoietin-2 decreased significantly after 2 months of therapy. At progression time, there was a significant increase in VEGFC and Angiopoietin-2, suggesting the activation pathways counteracting the blockade of VEGFR2 by ramucirumab. Overall results showed that a greater change in VEGFC and Angiopoietin-2 levels measured at the

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Edited by:
Antonio Giovanni Solimando, University of Bari Aldo Moro, Italy

Reviewed by:
Antonella Argentiero, National Cancer Institute Foundation (IRCCS), Italy
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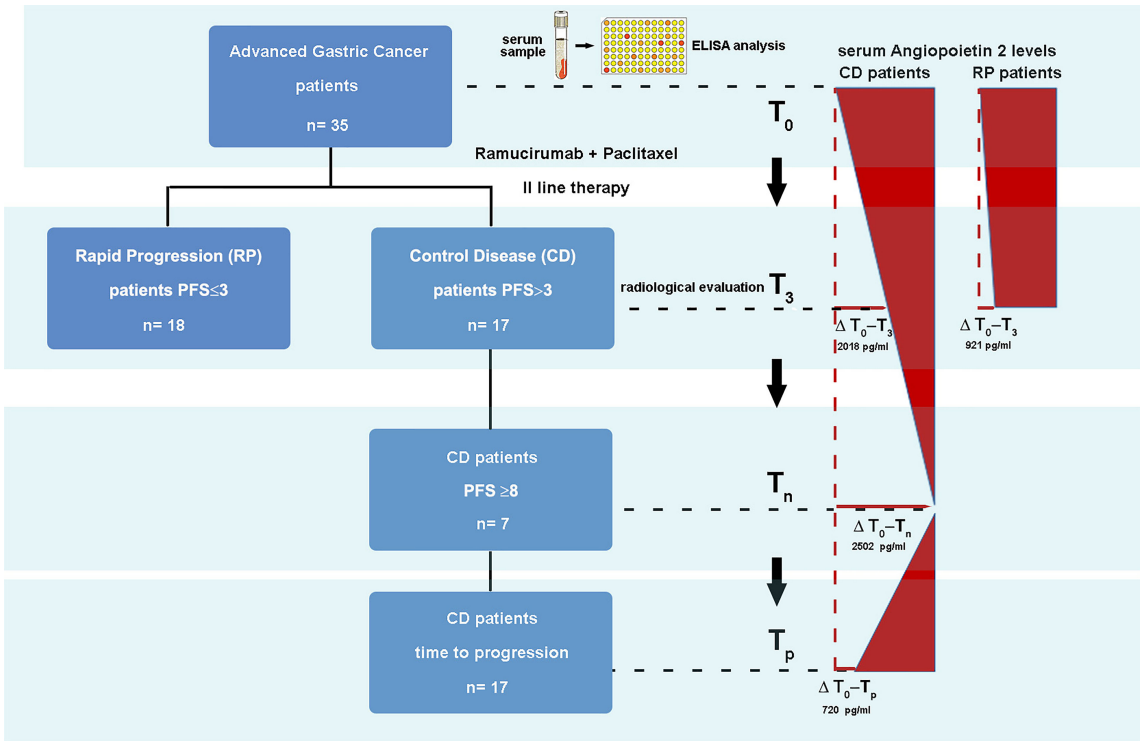
Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *25 January 2022*
Accepted: *25 February 2022*
Published: *06 April 2022*

Citation:
D'Alessandro R, Refolo MG, Schirizzi A, De Leonardis G, Donghia R, Guerra V, Giannelli G, Lolli IR, Laterza MM, De Vita F, Messa C and Lotesoriere C (2022) Variations in Circulating Levels of Angiopoietin-2 Over Time Are Predictive of Ramucirumab–Paclitaxel Therapy Outcome in Advanced Gastric Cancer: Results of Prospective Study. Front. Oncol. 12:862116. doi: 10.3389/fonc.2022.862116

beginning of the third cycle of therapy corresponded to a lower risk of progression and thus to longer progression-free survival.

Keywords: angiogenesis, gastric cancer, target therapy, biomarkers, cancer progression



GRAPHICAL ABSTRACT |

INTRODUCTION

Gastric cancer (GC) currently remains a global health burden, as it ranks as the fifth most common cancer and the fourth leading cause of cancer-related death worldwide. In 2020, more than 1 million new cases were diagnosed globally, and nearly 760,000 deaths occurred (1). The incidence of GC is the highest in Eastern Europe, Eastern Asia, and South America. In Western countries, 80% of patients are diagnosed with unresectable advanced-stage disease or develop a recurrence within 5 years of curative-intent surgery. Thus, the prognosis of advanced GC remains poor with a 5-year survival rate of <30% for all stages and <4% for metastatic disease (2, 3).

Evidence highlights that GC is characterized by a close interdependence between molecular subtype and the angiogenic and immune profile of the tumor microenvironment. As in other solid tumors, several cytokines and growth factors play a dual detrimental role in the tumor microenvironment, as they promote both tumor angiogenesis and immunosuppression. Vascular endothelial growth factors (VEGFs) are the most prevalent and potent promoters of angiogenesis. VEGF family members are involved at different levels in the regulation of the cancer-immunity cycle, producing substantial changes that ultimately contribute to creating a microenvironment that allows the tumor to evade immune surveillance (4, 5).

The actions of VEGFs (VEGFA, VEGFC, and VEGFD) on endothelial cells (ECs) are mediated primarily through the binding and activation of VEGFR-2 [6]. VEGFD and VEGFC mainly bind VEGFR3 involved in lymphangiogenesis (6, 7). Although VEGFC and VEGFD have a high degree of homology, they have different functionalities. VEGFD has a stronger angiogenic potential than VEGFC, which predominantly binds VEGFR3 and acts mainly in the lymphatic system (8, 9). The recent finding of the production of different VEGF ligands and receptors (VEGFRs) in epithelial cancer cells suggests a direct role for these ligands and their receptors in the autocrine control of some biological processes in cancer cells (10–12). The expression levels of VEGFs and VEGFRs in GC correlate with disease prognosis (13). These data represent the scientific rationale for targeting the VEGF pathway in patients with GC.

Ramucirumab, a fully human immunoglobulin IgG1 monoclonal antibody targeting VEGFR-2, is the first

antiangiogenic agent to demonstrate activity for advanced GC in a second-line setting. In two pivotal phase III double-blind, placebo-controlled trials, ramucirumab was showed to significantly improve survival when used for therapy either alone (5.2 vs. 3.8 months, hazard ratio (HR) = 0.776, p = 0.047) or combined with paclitaxel (PTX) (9.6 vs. 7.4 months, HR = 0.807, p = 0.017) (14, 15). In the phase III RAINBOW trial, ramucirumab plus PTX improved progression-free survival (PFS) by 1.5 months (median 4.4 vs. 2.9; HR = 0.635, p > 0.0001), with increased response rate (28% vs. 16%, p = 0.0001) and disease control rate (80% vs. 64%, p > 0.0001) as well (15). Real-world data have been shown to support the efficacy and safety of ramucirumab also in daily clinical practice (16).

Although retrospective studies considered VEGF and its receptors as possible biomarkers in gastric carcinoma, the importance of prospective studies evaluating changes in these factors during therapy was underlined (17–21). Furthermore, in a recent study, Van Cutsem and colleagues have investigated a panel of angiogenic markers in patients from the RAINBOW cohort and highlighted some pharmacodynamic and prognostic relationships (22). However, to date, no reliable biomarkers have been identified to select those patients who more likely will benefit from ramucirumab treatment.

The aim of this prospective study is to investigate circulating angiogenic biomarkers in patients with GC undergoing second-line treatment with ramucirumab and PTX.

MATERIALS AND METHODS

Study Design, Serum Sample Collection, and Analysis

The current study provided the enrollment of patients with advanced GC undergoing a second treatment line with ramucirumab and PTX. In this prospective analysis, sera from 35 patients collected before starting therapy and at the first infusion of each cycle were considered. The study was approved by the ethics committee (prot. n°139/c.e. 28-06-2017). Patients provided written informed consent for the collection of blood samples for biomarker analysis. ELISA analysis was performed on serum samples corresponding to basal level (T_0), the second cycle of therapy (T_2), the third cycle of therapy (T_3), and time of radiological and clinical disease progression (T_p). Regarding VEGFC, Angiopoietin-2 and VEGFR3 were also considered the sixth (T_6) and ninth cycles of therapy (T_9). The comparative analysis presented in this study considered times T_0, T_3, T_6, and T_p. In the analysis, two different groups of patients were distinguished, based on the clinical evaluation after 3 months of treatment: patients who presented disease progression and patients who presented disease control (partial response or stable disease). A total of 13 angiogenetic molecules were analyzed in the serum using two different panels, according to the manufacturer's instructions with a multiplex bead suspension array kit using Bio-PlexMagPIXSuspension Array System. In panel 1 EGF, Angiopoietin-2 (Ang2), PLGF, VEGFC, VEGFD, FGF2, and VEGFA were analyzed, and in panel 2, there were PDGF, sTIE-2, sEGFR, sVEGFR1, sVEGFR3, and sVEGFR2. These analytes were split into two panels to prevent cross-interferences between beads during the analysis. Each serum sample was analyzed in duplicate, and mean factor concentrations were reported in pg/ml. The serum levels of the aforementioned analytes were dosed beforehand and in different phases of the treatment, in order to associate the variations in their expression with clinical outcomes. Furthermore, linear regression analysis was performed to identify any significant association among VEGF, its receptor, and the other investigated molecules. These results were correlated with the clinical data of each patient.

Biomarker Detection

In view of interesting results obtained from multiplex beads suspension array analysis, for three of the 13 angiogenetic molecules, a uniplex ELISA was performed to better investigate previous data. VEGFC and Ang2 were quantified using ELISA Kits—QuantikineQuicKit ELISA (R&D Systems, Minneapolis, MN, USA), and sVEGFR3 was dosed using VEGF Receptor 3/FLT4 Human ELISA Kit Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Statistical Analysis

The primary objective of our analysis was to evaluate the relationship between disease progression and single factors involved in the advanced GC.

Patients' characteristics were reported as mean ± SD (M ± SD) for continuous variables and as frequencies and percentages (%) for categorical variables.

The normal distribution of quantitative variables was tested using the Kolmogorov–Smirnov test.

The variations of single factor measured on individual patients were calculated as differences among baseline time and subsequent evaluation time, and for testing the variations between basal values and those in subsequent times, the sign test was used.

For comparisons of single parameters between the groups as Control and Rapid Progression, the Wilcoxon rank-sum (Mann–Whitney) test was used for continuous variables, so long as the variables were not distributed normally.

Linear regression was used to evaluate the association of individual markers on VEGA, sVEGFR2, Ang2, and VEGFC, where the R-squared is expressed as the goodness-of-fit measure for linear regression models because this statistic indicates the percentage of the variance in the dependent variable that the independent variables explain collectively.

The variation of VEGFC, VEGFR3, and Ang2 between basal time and the third cycle of therapy was divided into tertiles. For studying the time between entry to a study and a subsequent event, such as the progression of the disease, the Cox model was used. The Cox model is a statistical technique for exploring the relationship between the disease progression of a patient and several explanatory variables, and it allows us to estimate the hazard (or risk) of progression for an individual, given its prognostic variables measured as categorical. The Cox proportional hazard model was fitted to the data, and the proportional hazard assumption was evaluated by means of the

Schoenfeld residuals test (SRT). Model fitting was evaluated by means of the Akaike information criterion (AIC) and Bayesian information criterion (BIC). Risk estimators were expressed as HR and 95% CI. When testing the null hypothesis of no association, the probability level of error at two tails was 0.05. All the statistical computations were made using STATA (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC).

RESULTS

In the present study, 35 patients with advanced GC undergoing second-line therapy based on ramucirumab and PTX were enrolled. The population was divided into two groups based on the type of response estimated from the first radiological evaluation. In the Control Disease (CD) group, 17 patients who presented response disease or stable disease were included, whereas in the Progression Disease (PD) group, 18 patients were included. In **Table 1**, the population characteristics were reported. The median PFS was of 2.8 and 8.8 months for the PD and CD groups, respectively.

The detection of circulating angiogenic biomarkers levels was performed in multiplex or uniplex array at predefined timing during treatment, as described in the *Materials and Methods*. Comparing the two groups of patients, basal levels of VEGFA, VEGFC, PLGF, and Ang2 in the CD group were higher than those in the PD group (**Table 2**).

On the other hand, the basal levels of VEGFR2, VEGFR3, and sTie2 receptors were lower in the CD group in comparison to the levels of the same receptors detected in the group of patients with progressive disease. The differences, as shown in **Table 2**, were not statistically significant, although they were approaching significance for VEGFR2 ($p = 1.12$) and VEGFR3 ($p = 1.19$) receptors.

The mean values of serum levels of each marker were compared between the two groups at time T_3 versus time T_0 (**Tables 3A**, **B**). At time T_3, a significant increase in the levels of the VEGFA, VEGFD, and PLGF ligands was observed with respect to basal levels in both groups. The results for the VEGFC ligand differed between the two groups of patients. In particular, there was a decrease in VEGFC levels in patients within the CD group and an increase in those with the progressive disease when T_3 and T_0 were compared (**Tables 3A**, **B**). Considering VEGF receptor levels, a notable decrease was detected in both groups of patients, and the reduction was significant in the case of VEGFR3. In addition, a significant decrease in the Ang2 factor and its receptor sTie2 was detected in both groups when T_3 and T_0 were compared (**Table 3**). The analysis of serum levels of other angiogenic factors investigated, including EGF, FGF2, PDGF, and sEGFR, revealed no significant differences in expression levels during treatments in all patients examined (data not shown).

Interestingly, the comparative analysis of the T_0–T_3 deltas (ΔT_0–T_3) for each analyte in the two groups of patients revealed that the ΔT_0–T_3 for VEGFC was positive in the CD group (where the factor decreased by 9.2%) and negative in the PD group (where the factor increased by 31%). Furthermore, the ΔT_0–T_3

TABLE 1 | Clinicopathological features of enrolled patients (n = 35).

Patient features		Category of patients	
		PD[1]	CD[1]
Enrolled patients		18	17
Age, mean (years)		62	67
Gender	*Male*	12	12
	Female	6	5
Tumor features			
Location	*Gastroesophageal Junction*	6	3
	Fundus of stomach	1	3
	Gastric body	8	6
	Antrum of stomach	4	7
	Whole stomach	1	/
Pathological type	*Intestinal*	4	4
	Diffuse	12	13
	Mixed	2	/
Pathological differentiation	*High differentiation*	1	/
	Medium differentiation	3	3
	Poor differentiation or undifferentiation	14	14
HER2 status	*Positive*	2	1
	Negative	16	16
Primary tumor present	*Yes*	7	3
	No	11	14
Peritoneal metastasis	*Yes*	9	9
	No	9	8
Number of metastatic organs	*0–2*	15	15
	≥3	3	2
Second line of treatment PFS (months)		2.8	8.8

PFS, progression-free survival.

[1]*CD, control disease; PD, progression disease (refer to the first radiological evaluation).*

TABLE 2 | Differences between serum basal levels of biomarkers in control disease group and progression disease group.

*Biomarkers	Basal levels (T_0)		p^
	CD patients (n = 17)	PD patients (n = 18)	
VEGFC#	6,391.62 ± 3,855.81	5,379.77 ± 2,247.34	0.50
Ang2#	3,519.88 ± 1,491.77	3,183.22 ± 1,530.03	0.55
PLGF	4.43 ± 8.10	2.16 ± 1.63	0.29
VEGFD	260.00 ± 122.43	278.80 ± 264.54	0.36
VEGFA	179.71 ± 119.77	162.87 ± 104.50	0.73
sVEGFR1	32.71 ± 76.77	9.83 ± 3.84	0.21
sVEGFR2	2,119.51 ± 772.96	2,660.58 ± 961.85	0.12
sVEGFR3#	23,256.74 ± 10,117.86	30,979.99 ± 14,826.57	0.19
sTie2	2,591.33 ± 1,158.65	2,902.55 ± 1,313.44	0.39

CD, control disease; PD, progression disease (refer to the first radiological evaluation).
**Concentration pg/ml, mean ± SD.*
^Wilcoxon rank-sum (Mann–Whitney) test.
#Detected with uniplex ELISA.

for Ang2 was significantly greater (p = 0.05) in CD patients than in the PD patients (**Table S1**). Patients within the CD group were followed up until the time of progression (T_p).

Comparing the serum levels of the investigated ligands at time T_3 with those at T_p, a further increase in the VEGFA and PLGF levels was detected; moreover, this increase was statistically significant for VEGFD (p = 0.0002) (**Table 4**). Similar results were obtained for VEGFC and Ang2. In the case of Ang2, the increase at the time of progression in comparison with T_3 was significant (p = 0.0005) (**Table 4**). In addition, the comparative analysis of receptor levels at time T_3 versus time T_p revealed an increase in both VEGFRs and Tie2 receptor levels (**Table 4**).

In patients with control of disease after the first radiological evaluation (CD group), the levels of VEGFC, VEGFR3, and Ang2 were evaluated at T_0, T_3, T_6, and the time of progression of disease (T_p). In the analysis, the CD group was divided into two subgroups: one included patients with PFS > 6 months (n = 11) and the other patients with a PFS > 8 months (n = 6) (**Figure 1**).

TABLE 3A | Trend of serological biomarkers at the first radiological evaluation on patients with control (**A**) and progression disease (**B**).

*Biomarkers	CD patients (n = 17)		p^
	Basal levels (T_0)	3° Cycle (T_3)	
VEGFC#	6,391.62 ± 3,855.81	5,801.32 ± 2,855.72	1.00
Ang2#	3,519.88 ± 1,491.77	1,501.46 ± 679.81	<0.0001
PLGF	4.43 ± 8.10	34.30 ± 30.22	0.0003
VEGFD	260.00 ± 122.43	524.51 ± 248.90	<0.0001
VEGFA	179.71 ± 119.77	420.09 ± 238.92	0.0003
sVEGFR1	32.71 ± 76.77	15.98 ± 26.64	0.63
sVEGFR2	2,119.51 ± 772.96	2,009.90 ± 863.59	0.33
VEGFR3#	23,256.74 ± 10,117.86	3,118.64 ± 2,714.44	<0.0001
sTie2	2,591.33 ± 1,158.65	2,005.41 ± 831.52	0.01

TABLE 3B |

*Biomarkers	PD patients (n = 18)		p^
	Basal levels (T_0)	3° Cycle (T_3)	
VEGFC#	5,379.77 ± 2,247.34	7,066.78 ± 4,122.82	0.24
Ang2#	3,183.22 ± 1,530.03	2,262.26 ± 1,629.11	0.007
PLGF	2.16 ± 1.63	35.92 ± 24.96	<0.0001
VEGFD	278.80 ± 264.54	460.38 ± 295.60	0.0003
VEGFA	162.87 ± 104.50	453.80 ± 203.49	<0.0001
sVEGFR1	9.83 ± 3.84	12.27 ± 9.33	0.33
sVEGFR2	2,660.58 ± 961.85	2,324.60 ± 1,371.55	0.14
VEGFR3#	30,979.99 ± 14,826.57	6,081.25 ± 4,102.31	<0.0001
sTie2	2,902.55 ± 1,313.44	2,361.33 ± 1,094.16	0.05

CD, control disease; PD, progression disease (refer to the first radiological evaluation).
**Concentration pg/ml, mean ± SD.*
^Sign test.
#Detected with uniplex ELISA.

TABLE 4 | Trend of serological biomarkers from the first radiological evaluation to time of progression.

*Biomarkers	CD patients (n = 16)[§]		p^
	3° Cycle (T_3)	time of progression (>T_p)	
VEGFC[#]	5,865.57 ± 2,936.65	6,741.09 ± 4,434.30	0.80
Ang2[#]	1,519.55 ± 697.87	2,792.62 ± 1,067.43	0.0005
PLGF	35.18 ± 30.99	54.71 ± 51.41	0.58
VEGFD	495.33 ± 225.04	704.95 ± 302.45	0.0002
VEGFA	423.70 ± 246.28	566.17 ± 339.50	0.58
sVEGFR1	16.17 ± 27.50	38.60 ± 101.97	0.27
sVEGFR2	2,063.73 ± 861.95	3,492.69 ± 5,275.96	0.58
VEGFR3[#]	3,193.42 ± 2,785.32	4,232.80 ± 3,834.45	0.45
sTie2	2,075.69 ± 804.96	2,569.63 ± 2,227.84	0.58

CD, control disease.
**Concentration pg/ml, mean ± SD.*
^Sign test.
[§]One patient undergoing therapy.
[#]Detected with a uniplex ELISA.

In both subgroups, there was a reduction of all three analyzed markers from time T_0 to T_3. The decrease was highly pronounced in the case of VEGFR3 and Ang2, while in the case of VEGFC a relevant but non-significant reduction was observed. A further decrease was detected from time T_3 to T_6, to a higher extent in VEGFC and Ang2, with respect to VEGFR3. At the time of progression, all three analyzed angiogenetic factors showed an increase in serum level over T_6 that was clearly evident for Ang2 and minor or insignificant for VEGFR3. Interestingly, while the decrease in serum levels detected for all three factors in intervals from T_0 to T_3 and from T_3 to T_6 was greater in the group of patients with PFS > 8 months compared to that with PFS > 6 months, the detected increase in the interval from T_6 to T_p was PFS independent (**Figure 1** and **Table S2**).

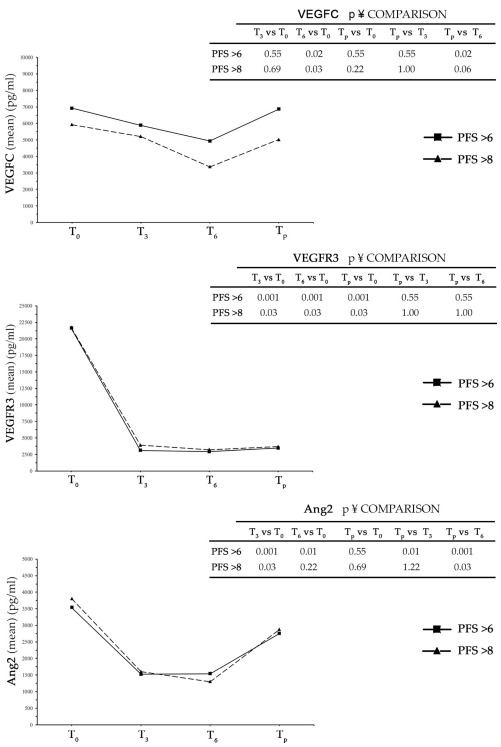


FIGURE 1 | In the three graphs is shown trends over time of VEGFC, VEGFR3, and Ang2 in patients with PFS > 6 months and PFS > 8 months. PFS, progression-free survival.

To assess whether marker changes at time T_3 were predictive for the therapeutic outcome, the univariate analysis was performed using the Cox model to calculate an HR of progression. The ΔT_0–T_3 values of each marker were divided into tertiles such that each comprised 33% of patients. The first tertile was considered as a reference (HR = 1). As shown in **Table 5** for VEGFC, all patients with ΔT_0–T_3 higher than 400 pg/ml had a probability for progression decreased by 25% compared to patients of the reference category. Considering the VEGFR3 receptor, in patients with ΔT_0–T_3 between 19,288.89 and 24,622.22 pg/ml, the probability of the disease progression was halved (51%) compared to that of the reference group. In contrast, when the VEGFR3 ΔT_0–T_3 was greater than 24,622.22 pg/ml, the probability for progression increased by 84%. In the case of Ang2, for ΔT_0–T_3 greater than 2,350 pg/ml, the risk of progression decreased significantly by 58% compared to the reference group (**Table 5**).

It can be observed, in the graphs shown in **Figure S1**, how the cumulative hazard varied over time for each of the three tertiles. In order to find possible correlations between the biomarkers examined, a linear regression analysis of the basal levels of VEGFA, VEGFR2, VEGFC, and Ang2 was performed with respect to each of the markers examined. We found a slight but significant association of VEGFC with its receptor VEGFR3 ($\beta = 0.19$, se(β) = 0.08, p = 0.03, CI = 0.01–0.037, $R^2 = 0.26$) and a slight but significant association of Ang2 with VEGFR3 ($\beta = 0.09$, se(β) = 0.03, p = 0.005, CI = 0.03–0.16, $R^2 = 0.42$) (**Table S3**).

DISCUSSION

Tumor growth and progression rely on the tumor vascular network for the necessary supply of oxygen and nutrients. Tumor angiogenesis is the result of a program finely tuned by

TABLE 5 | Correlation between marker changes at time T_3 and therapy outcome.

Biomarkers	HR	se (HR)	p	95% CI
VEGFC (pg/ml)				
<–1,000 [Ref. Category]	1			
–1,000 to 400	1.03	0.46	0.94	0.43–2.50
≥ 400	0.75	0.33	0.51	0.31–1.78
VEGFR3 (pg/ml)				
<19,288.89 [Ref. Category]	1			
19,288.89–24,622.22	0.49	0.23	0.13	0.20–1.24
≥24,622.22	1.84	0.80	0.16	0.79–4.30
Ang2 (pg/ml)				
<892.86 [Ref. Category]	1			
892.86–2,350.00	0.78	0.33	0.56	0.33–1.81
≥2,350.00	0.42	0.19	0.05	0.17–1.00

HR, hazard ratio; se(HR), standard error HR. CL, Confidential Interval.

a plethora of growth factors, EC proliferation, extracellular matrix (ECM) remodeling, and stromal cell interactions. Several pro-angiogenic factors have been identified; the most important of them is represented by the VEGF family. Blockade of VEGFRs or ligands by neutralizing antibodies are among the most studied therapeutic approaches in preclinical and clinical research for inhibition of angiogenesis. Despite the promising results, the benefits of these therapeutic weapons are reduced due to the phenomena of induced or acquired resistance that is implemented through the activation of alternative angiogenic mechanisms that bypass the block exerted by specific inhibitors (23). The selection of patients who may benefit from a given therapeutic approach has been made possible in many cancers by the knowledge of biomarkers with predictive value. However, the availability of markers is very limited in the case of antiangiogenic therapy (21). The identification of biomarkers for antiangiogenic therapy in GC is even more complex due to the extreme molecular heterogeneity of this type of cancer (24).

The members of the VEGF family, including ligands and receptors, are studied as principal candidates for predictive/prognostic biomarkers, and their high serum levels have been associated with a poor prognosis of GC (25–28). To date, retrospective studies focused on the analyses of baseline levels of circulating markers have failed to identify biomarkers predictive for response to antiangiogenic therapy in advanced GC (17, 29). Therefore, more recent prospective studies have focused on changes in some circulating markers over time compared to baseline levels measured before initiation of therapy. In this line, the recent prospective study conducted by Van Cutsem and colleagues on patients from the RAINBOW cohort did not identify specific predictive biomarkers for response to treatment with ramucirumab and PTX. However, the authors found a trend of response in plasma levels of VEGFD, PLGF, and Ang2 during therapy. The plasma levels of VEGFD and PLGF increased from baseline during treatment and decreased after treatment suspension. Instead, Ang2 showed a decrease during treatment and increase upon treatment suspension (22).

In this framework, the present prospective study was aimed to investigate whether VEGFs and VEGFRs change during the pharmacological treatment and to identify possible correlations with clinical outcomes. Furthermore, the study was extended at some of the principal angiogenic factors known to be involved in ramucirumab resistance like PDGF, PIGF, and EGF including also angiogenesis modulators such as Ang2/sTIE-2 (17, 30, 31).

Thirty-five patients with advanced GC undergoing second-line therapy with ramucirumab and PTX were included in the study, and an analysis of selected angiogenic biomarkers levels by serum sampling over multiple time points was performed. The population, according to the response at the first radiological evaluation, was divided into "Control Disease Group" and "Progression Disease Group," with median PFS of 8.8 and 2.8 months, respectively. In a first comparative analysis, the basal levels of biomarkers in the two groups were compared. This analysis revealed differences between the groups, although not statistically significant for the markers examined. The basal levels of VEGFA, VEGFC, PLGF, and Ang2 were higher in the CD group than in the PD group, whereas the basal levels of VEGFR2, VEGFR3, and sTie2 receptors were higher in the PD group.

To assess any differences between CD and PD patients, the levels of the different markers measured at baseline were compared with those measured at the beginning of the third cycle of therapy and with those found at the time of radiological and clinical disease progression.

The results showed that the levels of VEGFA, VEGFD, and PLGF ligands tend to increase significantly already during the first months of therapy. This result is explained by the displacement of the main VEGFR2 ligands, such as VEGFA, VEGFD, and PLGF, due to the ramucirumab binding. The trend of VEGFC was different in the two groups, with a decrease in its levels in the CD group and an increase in the PD group. As a result, the ΔT_0–T_3 of VEGFC was positive in the CD group (where the factor decreased by 9.2%) and negative in the PD group (where the factor increased by 31%). This finding is of particular interest since it could be related to an inhibition of the VEGFR3/VEGFC axis and thus of lymphangiogenesis. In contrast to VEGF ligands, VEGFR levels decreased during therapy. There was also a decrease in serum Ang2 levels during therapy, but the degree of this reduction was significantly greater in the CD group than in the PD group. Accordingly, the ΔT_0–T_3 value of Ang2 was significantly higher in the CD group than in the PD group.

It is widely accepted that Ang2 overexpression regulates vascular remodeling independently of VEGF, thus constituting a possible mechanism of acquired resistance during anti-VEGF therapies (32).

A compromised vasculature leads to hypoxic conditions resulting in the production of signal-activating molecules that create a microenvironment with an immunosuppressive phenotype devoid of effector T cells. Therefore, VEGF and Ang2 can be considered not only as the main players in the angiogenic switch but also as powerful immune modulators (4, 5). Several studies demonstrated that upregulation of VEGF, through interaction with VEGFR2, is responsible for the Ang2 overexpression by ECs in the stroma surrounding the tumor. Moreover, it is well known that high levels of circulating Ang2 correlate with poor prognosis in several tumors. Results from the AVAGAST study showed that this factor could be considered as a prognostic marker in advanced GC treated with bevacizumab (30). The blockage of VEGFR2 due to ramucirumab binding could explain the observed Ang2 decrease (30, 32, 33).

In all patients examined, the analysis of serum levels of the other biomarkers did not show a strong pattern of their expression during treatment, and accordingly, they were not considered in subsequent comparative analyses. Patients in the CD group were followed up until progression disease. In this group of patients during treatment, a continuing increase in the levels of VEGFA, VEGFD, and PLGF was detected until progression disease. Conversely, the levels of VEGFC presented a rapid increment at the time of progression after the initial decrease. In addition, there was an increase in the levels of VEGFRs and Tie2 receptors and a further significant increase in the levels of Ang2. The extent of the changes in VEGFC and Ang2 over time was greater and more significant both when the analysis included time after the third cycle (ΔT_0–T_6) of therapy and when the analysis was restricted to a subgroup of patients with longer PFS (PFS > 8 months). The results described suggested a crucial role of the VEGFC/VEGFR3 and Ang2/Tie2 axes in determining response to therapy. Furthermore, the decrease in angiogenic markers such as Ang2 and VEGFC could be also related to the vessel "normalization window" and a permissive immune phenotype (4).

To assess the predictive value of the ΔT_0–T_3 of these markers, the univariate analysis using the Cox model was performed. The results of this analysis showed that a greater change in VEGFC and Ang2 levels measured at the beginning of the third cycle of therapy corresponded to a lower risk of progression and thus a longer PFS. In the case of Ang2, for ΔT_0–T_3 greater than 2,350 pg/ml, the risk of progression decreased significantly by 58% [HR = 0.42, se(HR) = 0.19; p = 0.05 (95% CI 0.17–1.00)]. Therefore, the ΔT_0–T_3 of Ang2 may be considered as an outcome predictor of ramucirumab–PTX therapy. The limitations of the study were related to the small number of patients recruited, which makes it difficult to stratify the analyses performed on the basis of parameters such as age, sex, and clinicopathological characteristics of the tumor. In addition, further studies are needed to establish possible correlations between changes in circulating biomarkers over time and their expression *in situ* in the tumor and surrounding microenvironment, since there are indications that the levels of these markers are associated with a different outcome depending on their expression site [17]. Unfortunately, the analyses did not show significant correlations between basal VEGFC and Ang2 levels, although slight but significant relationships between VEGFC and VEGFR3 and between Ang2 and VEGFR3 were detected.

Nevertheless, the rapid increase in VEGFC and Ang2 at the time of progression could suggest the activation of alternative pathways, represented by VEGFC/VEGFR3 and Ang2/Tie2 able to counteract the VEGFR2 blockade by ramucirumab. These results support the idea, already present in the literature, that there is a close correlation between angiogenesis and lymphangiogenesis and that this is crucial for tumor progression and spread. It is known that the upregulation of Ang2 by lymphatic ECs is induced by the action of VEGFC and its binding to VEGFR2 (34).

CONCLUSIONS

This prospective study focused on the analysis of serum levels of angiogenic biomarkers in patients with advanced GC undergoing second-line therapy with ramucirumab and PTX. All VEGF family members as well as Ang2 and its receptor Tie2 were considered. Sera were sampled at each cycle of therapy until the time of progression. The aim of our study was to identify possible predictive markers and to evaluate whether variations in a given marker over time could be predictive for therapeutic outcomes. Overall results indicated that patients with longer PFS presented higher baseline levels of VEGFs and Ang2 compared to those with shorter ones. None of the baseline markers were found to be predictive for outcomes to therapy. However, the results clearly showed that a greater decrease in VEGFC and Ang2 levels measured at the beginning of the third cycle of therapy corresponded to a lower risk of progression and thus a longer PFS. Significantly, changes in Ang2 levels greater than or equal to 2,350 pg/ml decreased the risk of progression by 58%. In addition, there was a significant increase in VEGFC and Ang2 at the progression time, which could suggest the activation of alternative pathways such as VEGFC/VEGFR3 and Ang2/Tie2 that may counteract the blockade of VEGFR2 by ramucirumab. These findings support the rationale that dual inhibition of Ang2 and VEGFRs could increase the vessel normalization window. Furthermore, this combined blockade elicits antitumor immunity; therefore, cotargeting of angiogenesis and immune checkpoints could improve the efficacy of GC therapy (35).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of I.R.C.C.S "Giovanni Paolo II" Cancer Institute of Bari (Italy), n°139/c.e. 28-06-2017. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization: RD'A, MR, and CL. Methodology: RD'A, MR, AS, and GL. Software: RD and VG. Validation: RD, CM,

and CL. Formal analysis: RD'A, MR, and VG. Investigation: RD'A, MR, AS, and GL. Resources: GG, CM, IL, and CL. Data curation: RD'A, RD, VG, and CL. Writing—original draft preparation: RD and CL. Writing—review and editing: ML, FV, GG, CM, and CL; visualization, RD'A, MR, AS, and CM; supervision, GG, CM, FV, and CL. Project administration: GG, CM, and CL. Funding acquisition: CM. All authors have read and agreed to the published version of the manuscript.

FUNDING

This research was funded by the Italian Ministry of Public Health (n.7/2017).

ACKNOWLEDGMENTS

The authors thank all the participating patients, all the medical oncologists, and the nursing staff of the oncology units. The authors thank Andrijana Klajn, PhD, for the careful English revision.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.862116/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Oncology

REVIEW
published: 19 May 2022
doi: 10.3389/fonc.2022.897927

Resistance Mechanisms of the Metastatic Tumor Microenvironment to Anti-Angiogenic Therapy

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Angiogenesis describes the formation of blood vessels from an existing vascular network. Anti-angiogenic drugs that target tumor blood vessels have become standard of care in many cancer entities. Though very promising results in preclinical evaluation, anti-angiogenic treatments fell short of expectations in clinical trials. Patients develop resistance over time or are primarily refractory to anti-angiogenic therapies similar to conventional chemotherapy. To further improve efficacy and outcome to these therapies, a deeper understanding of mechanisms that mediate resistance to anti-angiogenic therapies is needed. The field has done tremendous efforts to gain knowledge about how tumors engage tumor cell and microenvironmental mechanisms to do so. This review highlights the current state of knowledge with special focus on the metastatic tumor site and potential therapeutic relevance of this understanding from a translational and clinical perspective.

Keywords: anti-angiogenic therapy, resistance, metastatic microenvironment, angiogenesis, tumor microenvironment

OPEN ACCESS

Edited by:
Alessandro Passardi, Scientific Institute of Romagna for the Study and Treatment of Tumors (IRCCS), Italy

Reviewed by:
Kevin Dzobo, University of Cape Town, South Africa
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

Received: *16 March 2022*
Accepted: *21 April 2022*
Published: *19 May 2022*

Citation:
Schiffmann LM, Bruns CJ and Schmidt T (2022) Resistance Mechanisms of the Metastatic Tumor Microenvironment to Anti-Angiogenic Therapy. Front. Oncol. 12:897927. doi: 10.3389/fonc.2022.897927

INTRODUCTION

Angiogenesis is a biological process that describes the formation of new blood vessels from an existing vascular network. It is essential in many physiological processes including embryonical development or the female reproductive system (1). It is furthermore highly relevant in many diseases.

When a developing malignant lesion reaches a critical size, diffusion does not sufficiently cover the increased demand for nutrients and oxygen. The core of this lesion becomes hypoxic leading to the stabilization of HIF-1 alpha. This induces the upregulation of many target genes that foster tumor progression. Among them are several so-called pro-angiogenic genes that orchestrate the 'angiogenic switch' by which the tumor recruits blood vessels from the surrounding healthy tissues enabling exponential tumor growth (2, 3). Besides this 'classical' mode of sprouting angiogenesis tumors engage other mechanisms of vascularization such as intussusceptive angiogenesis or vasculogenic mimicry (4, 5).

Long before tumor angiogenesis was viewed 'officially' as one central 'hallmark of cancer' that is crucial for tumor progression at the primary tumor site and metastatic dissemination (6–8), Judah Folkman in the 1970s coined the hypothesis that a malignant tumor could be forced to regression by attacking its vasculature (9, 10). Propelled by this postulation many growth factors and signaling

pathways that mediate (tumor-) angiogenesis have been discovered and plethora of substances were developed to inhibit or modulate angiogenic cascades in tumors in the following. Studies from Hurwitz and Kabbinavar 2003 and 2004 first demonstrated that Bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF) improved response rates and prolonged survival in patients with metastatic colorectal cancer (11, 12). Accordingly, anti-angiogenic therapies, mainly bevacizumab and recently ramucirumab, an anti-VEGFR2 antibody, have become an important part of many tumor therapies including in colorectal cancer, gastric cancer, renal cell cancer, ovarian cancer and non-small-cell lung cancer (13). Until now most of the clinically approved anti-angiogenic drugs target the VEGF signaling pathway.

Compared to initial prospects which based on very promising experimental basic research and preclinical data (14–16) as well as pivotal clinical trials (11, 12), anti-angiogenic therapies fell short of expectations regarding efficacy, both as a single agent and in combination with chemotherapy. Correspondingly, patients develop resistance towards anti-angiogenic therapies that clinically present in the same way as refractoriness against conventional chemotherapy which occurs during disease progression (17). In this light, one of the major challenges of (tumor)-angiogenesis research is to identify modes of resistance and develop strategies to overcome them.

In parallel to the Hurwitz Trial researchers sought to find prove and insights in how VEGF blockade with bevacizumab exactly works to inhibit tumor growth and progression. It became clear that the main mode of action of pharmacological VEGF withdrawal is the correction of functional and structural tumor blood vessel abnormalities. This has been summarized by Jain under the term 'vascular normalization' (18).

It became clear that not only different tumor cell derived pro-angiogenic growth factors contribute to resistance against VEGF blockade, but also tumor stromal cells crucially mediate the efficacy and response to VEGF targeted therapies.

Preclinical evaluation studies in mice exploring the efficacy of anti-angiogenic therapies have mainly been performed in disease models that only partially mimic clinical cancer situations. Many experimental findings are based on subcutaneous tumor models that involve a large primary tumor at best with metastasis at a single organ site and often without metastasis. Clinical evaluation and application of anti-angiogenic therapy, beside very few indications, take place in stage IV situations, often as second- or third-line therapy. This altogether makes preclinical and clinical findings often difficult to compare. Still cancer patients for the most part die from disseminated metastatic disease and anti-angiogenic therapy is mostly used in this disease stage. It is therefore very likely that metastatic lesions and their tumor microenvironment significantly contribute to resistance to anti-angiogenic therapies. This review will give a focused overview over the current state of knowledge of mechanisms of resistance that is mediated by the tumor microenvironment with specific respect to the metastatic tumor site and its potential clinical implications.

ALTERNATIVE PATHWAYS

The simplest concept of resistance to VEGF inhibition is the compensatory upregulation of alternative pathways. Accordingly, several dual or multi-targeting approaches that involve mainly the angiopoietin-2 (ANG-2)/TIE2 axis, platelet derived growth factor receptor beta (PDGFR-beta) signaling and fibroblast growth factor receptor (FGFR) signaling (19–25) have been developed and tested preclinically and are e.g. with drugs such as regorafenib or nintedanib clinically approved concepts. Combined VEGF/PDGF signaling blockade has also been tested in a phase I/II trial with promising efficacy and acceptable toxicities, but further clinical studies are lacking until now (26).

Targeting the Angiopoietin/TIE2 Axis

Targeting or manipulating ANG-2/TIE2 signaling has been demonstrated to show beneficial effects on tumor vascularization, vascular normalization and prolonged survival in murine models of multimodal treatment strategies (27, 28) (29). Clinical studies testing ANG-2/angiopoietin-1/TIE2 inhibition with various substances failed to mirror the promising preclinical results which is presumably due to the complex context-dependent impact of the angiopoietin/TIE2 axis on the endothelium and other tumor stromal cells such as myeloid derived cells (30).

Targeting both VEGF and ANG-2 had additive effects on tumor growth, vascularity and vascular normalization in preclinical models by various mechanisms (13, 15, 17, 23–25). The eagerly awaited McCAVE trial failed to demonstrate a relevant advantage of combined ANG-2 and VEGF blockade with vanucizumab, a dual humanized monoclonal antibody binding both, VEGF and ANG-2, compared to bevacizumab when both drugs were combined with mFOLFOX-6 in previously untreated metastatic colorectal cancer (31). These results were unexpected based on previous trials and have to be further substantiated (32).

One of the perennial questions also here remains how findings from preclinical models that focus on primary tumor growth can be translated into stage IV clinical diseases. One phenomenon highly relevant for systemic cancer disease that seems to be tightly connected to resistance to anti-VEGF therapy that can potentially overcome by ANG-2 inhibition or ANG-2/TIE2 manipulation is the recruitment of myeloid cells to primary tumors and metastatic lesions.

TUMOR-INFILTRATING IMMUNE CELLS

Tumor-infiltrating myeloid cells constitute the majority of the cellular tumor stroma. They can hinder or foster tumor progression depending on the disease entity, stage and treatment modality, specifically in the context of anti-angiogenic treatment (17, 24, 28–30). Accordingly, with respect to angiogenesis tumor-infiltrating macrophages and neutrophils contribute to resistance to anti-angiogenic therapy in multiple ways (33, 34).

CD11b$^+$ GR1$^+$ Cells

A broad spectrum of neutrophils, macrophages and myeloid-derived suppressor cells (MDSCs) characterized by positivity for CD11b and GR1 (Ly6G/C) have been found to be associated with refractoriness to anti-VEGF therapy in multiple murine tumor models (35). This was at least partially mediated by a cross-talk between granulocyte colony-stimulating factor (G-CSF) and bone-marrow derived Bombina variegate peptide 8 (Bv-8) (33). Targeting Bv-8 with a specific antibody in conjunction with metronomic gemcitabine improved outcomes in a murine model of pancreatic cancer by counteracting pro-angiogenic and pro-metastatic effects of tumor-infiltrating MDSCs (36), compare **Figure 1**.

Whether targeting bone-marrow derived sources of resistance to anti-angiogenic therapy can be translated into the clinic warrants further careful investigation specifically in the context of metastasis.

Tumor-Infiltrating Macrophages/ Neutrophils Primary Tumor Versus Metastasis

The role of tumor-infiltrating neutrophils apparently seems to be divergent depending on the tumor stage. While their occurrence is beneficial at early stages of CRC tumorigenesis (37), increased infiltration of local lymph node or distant organ metastasis with CD177$^+$ neutrophils predicted poor outcome to bevacizumab containing chemotherapy in patients with stage IV colorectal cancer (25). Resistance to anti-angiogenic therapy in an anti-VEGF therapy refractory murine model could be overcome by combined inhibition of anti-VEGF and ANG-2 inhibitory treatment (25). There are several potential explanations how ANG-2 blockade can render anti-VEGF treatment induced neutrophil recruitment. First, a specific subset of tumor-infiltrating immune cells express TIE2 (TIE2 expressing monocytes, TEMs) which would directly be targeted by ANG-2 blockade (38). Second, ANG-2 renders the endothelium more sensitive to immune cell binding and infiltration towards the parenchyma/tumor (39, 40).Third, combined anti-VEGF and ANG-2 inhibition enhanced anti-tumor activity of CD8$^+$ cytotoxic T-cells and showed complementary effects with immunotherapy (41). Furthermore, blockade of VEGF enhances endothelial adhesion molecules which most likely acts synergistically with the above named mechanisms (42, 43). All mentioned mechanism can be seen as relevant for an unresected primary tumor and for metastatic lesions.

There is ample evidence that VEGF inhibition triggers the recruitment and priming of neutrophils fueling metastasis and progression. An increased neutrophil/lymphocyte ratio predicts outcome of patients with colorectal cancer independent of anti-VEGF treatment (44). VEGF blockade in an experimental model of neutrophil-driven metastasis promoted disease progression (45). Furthermore, increased systemically circulating neutrophils were associated with poor prognosis in patients receiving bevacizumab containing chemotherapy (46). A particular role of metastasis-infiltrating macrophages was recently defined in colorectal cancer metastasis. Proangiogenic VEGFR1$^+$ macrophages in colorectal liver metastases predicted survival in patients, which was also true for circulating VEGFR1$^+$ monocytes in these individuals (47).

The exact mechanisms how circulating and metastasis infiltrating neutrophils/macrophages promote cancer progression remain to be elucidated, but certainly more studies that discriminate between primary tumor and metastatic site (25, 47) are urgently needed.

T-Cells/Immunotherapy

Immunotherapy against cancer mostly with immune checkpoint inhibitors (IT) has been integrated into treatment regimens of many

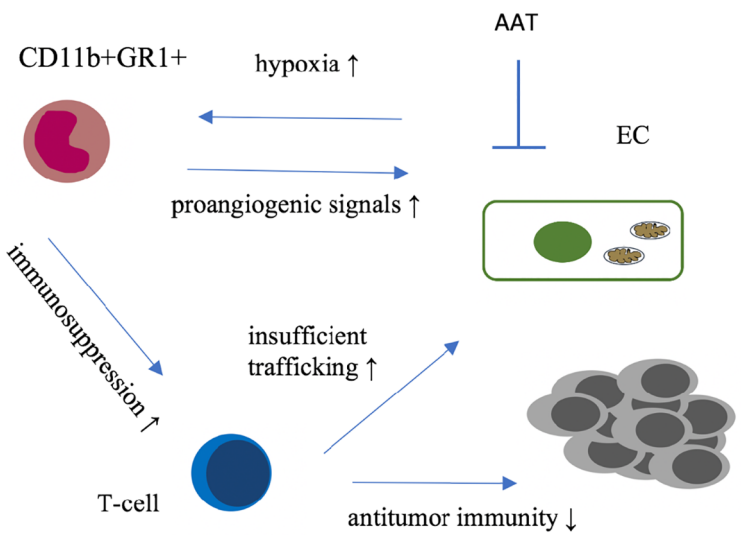

FIGURE 1 | Graphical summary of immune microenvironmental interactions involved in resistance to antiangiogenic therapies. GR1+ cells (i) infiltrate tumors as response to AAT-induced hypoxia and secrete proangiogenic factors; (ii) Furthermore these immune cells are suspected to suppress T-cell activity mitigating anti-tumor immunity. Together with insufficient trafficking of immune cells along structurally and functionally insufficient blood vessels theses mechanisms provide a rational for complementary anti-tumor activity of vascular normalizing AAT and immunotherapy. AAT, antiangiogenic therapy; EC, endothelial cell.

cancer entities (48). There is strong evidence that efficacy of the anti-tumor immune response is significantly hampered by specific characteristics of the tumor vasculature and the pro-angiogenic microenvironment. For example, $CD8^+$ T-cell infiltration into tumors is disturbed in part due the structural defective and dysfunctional vascular system, T-cell effector functions are manipulated and pro-angiogenic molecules can promote $CD8^+$ T-cell exhaustion (49–51) whereas M2-like macrophages and certain subtypes of T-cells secrete proangiogenic factors thereby directly foster tumor angiogenesis (52), see also **Figure 1**.

Accordingly, based on many preclinical studies, combining IT and anti-angiogenic therapy has been suggested as a promising synergistic concept (53). Which patients and to which cost regarding side effects will benefit from combining anti-angiogenics and IT will be deciphered in clinical trials that are currently running for several indications, in general these combinations have already been approved by the FDA (54) (see also **Table 1**). One potential factor that might influence tumor entity specific response to this combination therapy is the sheer abundance of immune cells (e.g. macrophages, T-cells) which differs rigorously between different types of cancer (55).

VESSEL CO-OPTION

Tumors do not exclusively engage neoangiogenesis to recruit and hold a vascular system available. Tumor cells can also grow along existing vasculature of the diseased organ without inducing neoangiogenesis, a term called vessel co-option [38,39].

Accordingly, the main target for current clinically approved anti-angiogenic therapy is far less relevant as the vasculature is not dependent on VEGF.

Vessels histologically proliferate less and exert an increased pericyte coverage as indicators for a mature, non-activated vascular systems. It is very important to notice that the simplest measure of tumor vascularity, the microvessel density, does not indicate which type of vascularization, angiogenesis or vessel co-option is present in a tumor (56)

Vessel Co-Option as Challenge to Target Metastatic Vessels

Especially in metastatic lesions vessel co-option is a frequently observed characteristic of tumor progression and a long-suspected cause of resistance to anti-angiogenic therapy (57). The occurrence of vessel co-option was demonstrated for lung metastasis (58), liver metastasis (59) and brain metastasis (60) among others.

Frentzas and colleagues were able to connect histopathological growth patterns of these metastases that involve vessel co-option to poor response to bevacizumab (61). They could demonstrate that nearly half of the examined CRC liver metastases were vascularized by vessel co-option not 'classical' angiogenesis and that patients that suffer from metastatic disease which is driven by vessel co-option have a poor histopathological response and particularly detrimental outcome to bevacizumab containing oncologic treatment.

This work furthermore demonstrated that tumor cells require actin-related protein 2/3 complex (Arp2/3) to successfully perform vessel- co-option. Accordingly, knockdown of ARPC3 a subunit of Arp2/3 blocked cancer cell motility thereby inhibiting vessel co-option and re-sensitizing tumors to anti-angiogenic therapy containing cytostatic treatment (61).

Summarizing, vessel co-option might be a major cause why anti-angiogenic treatment is ineffective for example in a large proportion of patients with CRC liver metastases.

Future efforts should focus on two things: (i) to design clinical trials to prospectively prove that response to and outcome after bevacizumab containing chemotherapy depends on histopathological growth patterns involving vessel co-option, (ii) develop treatment strategies that inhibit both vessel co-option and neoangiogenesis, especially in the context of metastatic disease. Furthermore, it is highly relevant to further clarify the role of anti VEGF therapy with bevacizumab or other drugs in multi-modal treatment strategies. The notion that upfront surgery followed by chemotherapy plus bevacizumab improves patients overall survival compared to upfront surgery plus chemotherapy without bevacizumab in patients with metastatic colorectal cancer underscores how relevant this might be (62).

Another challenge is to develop and clinically evaluate techniques that can pre-therapeutically define the histopathological growth pattern which could guide clinical treatment decisions, e.g. in individual multimodal treatment concepts involving chemotherapy +/- targeted therapy prior surgery (e.g. resection of colorectal liver metastasis) or vice versa (63).

METABOLIC REPROGRAMMING OF THE TUMOR MICROENVIRONMENT

Endothelial Cell Metabolism

From a metabolic perspective (neo)-angiogenesis is a highly demanding cellular process. Endothelial cells (ECs) that under

TABLE 1 | overview of some currently recruiting clinical trials investigating anti-angiogenic therapy in conjunction with cancer immunotherapy.

Entity	Interventional arm	NCT number	year of registration
Hepatocellular carcinoma	Ablative therapy* + Bevacizumab + Atezolizumab	NCT04727307	2021
Breast cancer	Paclitaxel + Bevacizumab + Atezolizumab	NCT04732598	2021
Melanoma	Nivolumab+ Axitinb	NCT04493203	2020
Breast Cancer	Paclitaxel + Bevacizumab + Atezolizumab	NCT04408118	2020
Rectal cancer	atezolizumab + bevacizumab	NCT04017455	2019
NSCLC	sintilimab + bevacizumab	NCT04213170	2019

*NSCLC, non-small cell lung cancer. * radiofrequency ablation.*

quiescent, steady state conditions line the inner surface of each blood vessel, maintain their cellular homeostasis under opulent conditions. They consume low amounts of energy while being exposed to the most comprehensive environment, the blood stream. When a growing malignant lesion secretes proangiogenic signaling molecules that activate endothelial cells this relation between supply and demand is completely shifted. The growing vessel, initially mainly constituted by the endothelial sprout, elongates towards a nutrient poor and hypoxic, acidic environment. To execute this challenging task, endothelial cells undergo a 'metabolic' switch that involves upregulation of key metabolic pathways. The knowledge of endothelial specific metabolic features is just beginning to be expanded, especially the specific role of tumor endothelial cells. From a clinical perspective endothelial cell metabolism offers many opportunities to explore novel therapeutic targets that might contribute to overcome resistance to growth factor targeted strategies.

Endothelial Cell Predilection for Glycolysis

Glycolysis is until now the best characterized metabolic pathway in endothelial cells. Specifically, tumor endothelial cells upregulate their glucose metabolism by several mechanisms. This is noteworthy as tumor cells are also considered to use mainly 'aerobic' glycolysis as energy resource and to fuel side pathways. Among other things the following: i) tumor ECs upregulate the glucose transporter GLUT-1 (64), ii) tumor ECs directly or indirectly upregulate the expression of rate limiting glycolytic enzymes, e.g. Phosphofructokinase-2/fructose-2,6-bisphosphatase (PFKFB), specifically its isoenzyme PFKFB3 (65) and iii) ECs express high amounts of lactate transporters (e.g. MCT1) (66).

Knockdown of endothelial cell PFKFB3 inhibited vessel sprouting *in vitro* and vivo. The fact that manipulation of the endothelial cell glycolytic metabolism was able to alter endothelial cell sprout differentiation showed the immense role of endothelial cell metabolism that might overrule even growth factor receptor signals (67). This suggested that endothelial cell metabolism as a growth factor independent engine of vessel sprouting and angiogenesis might contribute to resistance to anti-angiogenic therapy (68).

PFKFB3 as Novel Anti-Angiogenic Target

Indeed, PFKFB3 has then been proven to represent a promising target to reduce pathological angiogenesis in tumors and other diseases (58, 62, 63). Partial genetic or pharmacological inhibition of PFKFB3 was shown to normalize the tumor vasculature and reduce invasiveness in several tumor mouse models. This was accompanied not by reduced tumor growth at the primary tumor site, the conventional read out for efficacy of anti-angiogenic drugs, but by better control of tumor metastasis indication improved vascular normalization. Especially the small molecule compound 3-(3-pyri- dinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), an inhibitor of PFKFB3 was shown to control metastasis at an intermediate well tolerable dose in preclinical studies (65).

Inhibition of PFKFB3 with 3PO was shown to exert complementary effects with VEGF blockade by bevacizumab in an orthotopic PDX mouse model of glioblastoma (69). This was mediated by a prolonged vascular normalization window and improved delivery of chemotherapy indicating that inhibition of EC glycolysis might contribute to resistance towards antiangiogenic therapy in glioblastoma.

Role of Endothelial Oxidative Phosphorylation in Tumor Angiogenesis

Based on early pioneer work endothelial cells have long been viewed as similar to cancer cells to exert a 'warburg-like' metabolic phenotype (58, 59). This included the presumption that ECs have very few and dysfunctional mitochondria (70).

This has recently been amended as mitochondrial metabolism and oxidative phosphorylation (oxphos) indeed play an important role in activated endothelial cells and are indeed functionate (71, 72).

Manipulating endothelial cell mitochondrial metabolism has broad effects on endothelial cell integrity and function (71, 73–75). Pharmacological targeting of the mitochondrial respiratory chain and genetic ablation of mitochondrial oxidative phosphorylation reduced tumor growth and vascularity in mice. Surprisingly, metastatic dissemination was increased in mice were endothelial cells lacked functional oxphos (71). The genetic approach included a maximum achievable Cre recombination mediated gene deletion. These results are probably comparable to maximum blockade of EC glycolysis from others (76). Dose escalation to higher doses of 3PO showed a higher efficacy regarding tumor growth reduction (in comparison to lower doses) of primary tumors in mice but failed to control metastasis (76). It remains to be elucidated whether this effect is specific for the manipulation of tumor vessel metabolism or a general phenomenon (compare section 'dosing of anti-angiogenic therapies). It is also possible that manipulation of endothelial cell metabolism whether it is cytosolic glucose metabolism or mitochondrial metabolism induces cellular signaling processes that directly facilitate metastatic dissemination.

Lactate as Alternative Substrate and Signaling Molecule

Potential metabolism related pathways that could contribute to resistance to anti-angiogenic therapy are lactate induced signaling pathways. Endothelial cells were shown to be highly activated by tumor cell derived lactate which induces a NF-κb/Interleukin-8 driven proangiogenic stimulus (77). Beside this lactate induced signaling cues it is possible that ECs take up lactate to metabolize it to pyruvate which is then catabolized *via* the respiratory chain to generate ATP by oxidative phosphorylation (71) a form of metabolic symbiosis similar to processes in the brain (78, 79). Beside the fact that lactate might serve as an alternative substrate in conditions where glucose is scarce, e.g. in the tumor microenvironment, elimination of lactate by endothelial cells might alleviate lactate induced acidity and might limit proangiogenic lactate induced signaling, compare **Figure 2**.

Endothelial NF-κB and Metastasis

Activated NF-κB signaling in ECs was shown to be associated with poor pericyte coverage. Targeting EC glycolysis reduced NF-κB signaling, tightened EC intercellular junctions and increased pericyte coverage which might in part explain favourable results on metastasis (65).

Interestingly, endothelial specific transgenic mice, that express a 'superinhibitory' mutant of iκBα, leading to impaired NF-κB downstream signaling in endothelial cells, showed an impaired endothelial barrier. This resulted in increased metastasis indicating that dysfunctional endothelial NF-κB signaling increases the risk or dynamic of tumor cell dissemination (80). This is not implicitly in contrast to each other, but rather highlights a highly prominent role of endothelial cell NF-κB signaling in cancer progression and metastasis that warrants further investigation. Accordingly, therapeutic approaches that might interfere with EC NF-κB signaling should be carefully designed to modulate overactivation of this pathway without totally inhibit NF-κB related control of endothelial homeostasis. Whether and how direct or indirect targeting of NF-κB signaling in ECs that has been designed to treat inflammatory diseases (81) can be exploited as anti-angiogenic therapies has to be further evaluated.

Modulation of TAM Metabolism as Therapeutic Opportunity

Another aspect that could contribute to novel pharmacological opportunities to inhibit tumor and stromal metabolism to overcome resistance of the metastatic tumor microenvironment is to gain a deeper understanding of how stromal cells interact with each other on a metabolic level and how tumor cells and stroma cells co-operate to foster tumor progression (compare section above). E.g. tumor associated macrophages (TAMs) can be manipulated towards a hyper-glycolytic metabolic phenotype thereby 'steeling' glucose from endothelial cells which results in vascular normalization, lowers hypoxia and decreases metastasis (82). Tumor derived lactate acted as a signaling molecule that polarizes TAMs toward an M2-like differentiation thereby contributing to tumor progression (82, 83). Studies that characterize the metabolic phenotype of TAMs are urgently needed to find out whether and how TAM metabolism contributes to tumor progression, metastasis and resistance to anti-angiogenic therapy.

Tumor Cell Metabolism and Anti-Angiogenic Treatment

Another aspect is how targeting tumor and stromal metabolism can influence efficacy of anti-angiogenic compounds. Navarro et al. could demonstrate that vascular normalization by anti-angiogenic therapy modulates tumor cell metabolism away from glycolysis towards OxPhos. This sensitized tumor cells to the mitochondrial inhibitor ME344. ME344 acted synergistically with several anti-angiogenic compounds among them regorafenib which showed resistance as a single-agent (84). A phase 0/I trial demonstrated an increased efficacy of ME344 plus bevacizumab compared to bevacizumab as monotherapy in treatment naïve breast cancer (85). Besides the fact that this concept is innovative it is one of the very few trials that demonstrates efficacy of anti-angiogenic/targeted therapies without conventional chemotherapy and beyond in the neoadjuvant setting. Whether and how this concept is effective in metastatic diseases has to be further pursued.

DOSING OF ANTI-ANGIOGENIC THERAPIES

Accumulating evidence suggests that dosing of anti-angiogenic therapy is more complex than previously thought, especially compared to intense multi-substance chemotherapy regimens.

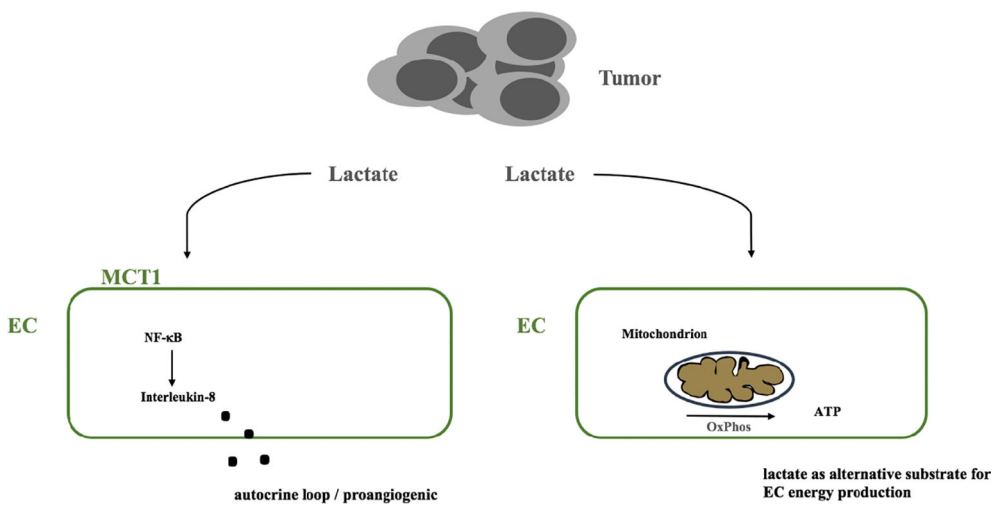

FIGURE 2 | Model of the potential dual role of tumor-derived lactate. (i) as a signaling molecule that triggers a NF-κB dependent autocrine proangiogenic program *via* IL-8 and (ii) as an alternative substrate that ECs metabolize *via* the respiratory chain to produce ATP. MCT1, monocarboxylate transporter 1; EC, endothelial cell; OxPhos, oxidative phosphorylation; ATP, adenosintriphosphate; NF-κB, nuclear factor kappa B.

Dose escalation of bevacizumab from 5 mg/kg to 10 mg/kg in combination with fluorouracil and leucovorin failed to further improve survival and response to treatment compared to fluorouracil and leucovorin alone in patients with metastatic colorectal cancer. Furthermore, only the lower dose of bevacizumab showed a significant improvement in response rates not the high dose (11).

Reasons for this clinical finding can be multifaceted. Besides biases from the study design and patient recruitment of this study a potential mechanism behind this is related to the window of normalization, in which structural and functional abnormalities of insufficient tumor vessels become corrected improving delivery of chemotherapy. This is dose and time-dependent and varies from tumor entity to entity and potentially from patient to patient making clinical application and patient selection even more complicated (86, 87). Preclinical data strongly support the context that a maximum reduction of both tumor and stromal cell derived VEGF can cause detrimental effects rather than improving cancer outcome (88, 89). Additionally, dose reductions of VEGF inhibition alone or in combination with the inhibition of other pro-angiogenic pathways demonstrated to be superior to higher doses, e.g. in terms of hypoxia (22, 23).

Prior to the introduction of novel anti-angiogenic treatments to clinical application, lessons learned regarding the importance of dosing of anti-angiogenic therapies should be considered.

ECM COMPONENTS OF THE TUMOR STROMA

Empty Basement Membrane Sleeves

Another important question with high clinical relevance is how the (metastatic) tumor reacts on a therapy pause due to drug intolerance, scheduled drug holiday or prior surgery. In several murine tumor models, both murine orthotopic and subcutaneous models, intense VEGF withdrawal eliminates the endothelial compartment of a tumor blood vessel but spares vascular support structures, e.g. the basement membrane and pericytes (14, 82, 83). Following interruption of VEGF blockade endothelial cells rapidly regrow into these scaffolds (90).

Besides of tumor cells and stromal cells solid tumors are composed of extracellular matrix (ECM).

It was observed that VEGF blockade induces the deposition of extracellular matrix (ECM) consisting of collagen I and IV, hyaluronic acid and glycosaminoglycans. This is the case in both murine primary tumors, murine and human metastasis (22, 91). Constant deposition of these ECM components over time contributes to an increased stiffness within tumors. This contributes to therapy resistance by several proposed mechanisms. The increased intratumoral mechanical force compresses tumor blood vessels which hinders delivery of cytostatic therapy (92–94).

ECM Deposition in Response to VEGF Inhibition in Mice and Humans

Desmoplastic stromal compositions are known to be associated with poor patient outcome e.g. in pancreatic cancer independent of anti-angiogenic therapy (95), it is therefore particular detrimental that VEGF inhibition might even exacerbate this situation and potentially contributes to primary resistance of anti-angiogenic drugs in several cancer entities. A potential strategy to neutralize deposition of extracellular matrix as a response to VEGF inhibition in colorectal cancer liver metastases has been proposed in murine tumor models. Additional therapy with polyethylene glycol conjugated (PEG) hyaluronidase in combination with VEGF inhibition led to a significant reduction of hyaluronic acid in murine colorectal liver metastases compared VEGF blockade as monotherapy (91). Combination treatment of B20.4-1.1, a monoclonal VEGF neutralizing antibody, and PEG- hyaluronidase significantly improved tumor tissue perfusion with Hoechst 33342, a surrogate marker for delivery of cytostatic therapy compared to B20 alone. Furthermore, the combination therapy in conjunction with 5-FU significantly prolonged mice survival compared to B20 alone.

To summarize, deposition of excessive amounts of extracellular matrix components as response to anti-VEGF therapy might represent a targetable mechanism of acquired resistance of the metastatic microenvironment which warrants further investigation.

STIFFNESS/METASTASIS-ASSOCIATED FIBROBLASTS

Primary tumors and metastasis are composed of tumor cells and stromal cells and a considerable amount of extracellular matrix (ECM). Structurally and functionally this tumor ECM composes basement membranes of mainly tumor blood vessels and the ECM of the interstitium (96). The latter besides mechanical and secretory functions that are comparable to healthy organs, significantly contributes to cancer disease progression (97) by several mechanisms. Besides storing growth factors (97) and serving as migration scaffold for several cell types, the ECM contributes to a mechanical phenomenon called tumor stiffness. Stiffness is defined as the capacity of a tissue to resist mechanical force and is composed in tumors mainly by the ECM. Increased tumor stiffness has been identified as a prognostic factor correlated with poor prognosis in several cancer entities (98). A significant determinant of stiffness in tumors is the activation state of cancer associated fibroblasts (CAFs). Activated CAFs induce a constant production of extracellular matrix components such as collagen I and fibronectin, growth factors and employ contractile forces that transforms tissue composition to increase stiffness (92, 93). Increased tissue stiffness has long been considered as resistance factor for anti-angiogenic therapy. Specifically, a role as resistance factor for efficacy of anti-angiogenic therapy in metastasis has recently confined by Shen et al. They could demonstrate that colorectal cancer liver metastases (CRCLM) show a significantly higher rate of stiffness than primary colorectal tumors. Increased stiffness was mainly driven by activation of metastasis associated fibroblasts (MAFs). These MAFs together with the non-cellular tumor

stroma composed a proangiogenic microenvironment. MAF activation and stiffness could be targeted by inhibitors of the renin-angiotensin-system (RAS). In CRCLM pharmacological targeting of metastasis stiffness with RAAS inhibitors produced favorable outcomes in conjunction with bevacizumab and chemotherapy compared to chemotherapy and bevacizumab alone (99), compare **Figure 3**. These findings elaborate a mode of resistance against anti-angiogenic therapy specifically for the metastatic environment and suggest a potent and already clinically approved strategy to overcome this mode (100).

YAP/TAZ as Multi-Faceted Approach to Overcome Resistance

Another very interesting aspect is the role of endothelial YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) which are important regulators of vascular development (101, 102) and are controlled by VEGF and also by mechanical signals (103). Accordingly, YAP/TAZ is involved in both, signaling of the therapeutic target and a potent resistance mechanism of anti-angiogenic therapy in metastatic disease (99). It was recently shown that genetic and pharmacological targeting of endothelial YAP/TAZ inhibits primary colorectal cancer tumor growth in mice. YAP/TAZ nuclear localization was induced by VEGF and TNF and could be inhibited by Verteporfin, a YAP/TAZ inhibitor, in a STAT3 dependent manner (104). Whether pharmacological YAP/TAZ manipulation (105) with verteporfin can be exploited to render (also metastatic) resistance to anti-angiogenic therapy has to be further explored.

DISCUSSION

Anti-angiogenic therapies have become part of many mostly palliative treatment regimens. After very successful preclinical work and promising first clinical trials 20 years ago, anti-angiogenic therapies failed to revolutionize anti-cancer therapies. Resistance appears after time similar to conventional cytostatic drugs. Tremendous efforts have been performed to uncover potential mechanisms of resistance to anti-angiogenic therapies. Though still nearly two decades after clinical approval of bevacizumab, targeting VEGF is the only broadly clinically applied antiangiogenic concept, not only in colorectal cancer.

One major burden in the development of first-generation anti-angiogenic therapy was to disregard several initially already evident facts: (i) subcutaneous murine tumor models are very different to polytopic metastasized human cancers (ii) vessel co-option is insufficiently targetable with VEGF inhibition (iii) though VEGF is a very potent proangiogenic factor many other cytokines can drive angiogenesis instead (iv) the complex microenvironment(s) of polytopic metastasized cancer diseases exploits a plethora of mechanisms to foster tumor progression independent of VEGF.

Accordingly, future studies should engage models that involve metastasis and test their hypothesis in (ideally) large human cohorts. The field has to balance a difficult bargain between two challenges: first, to bring novel strategies that apparently are more effective than 'just' inhibiting VEGF quickly to clinical application, among them combined VEGF and ANG-2 blockade or novel metabolism targeted strategies such as PFKFB3 inhibition; and second, to exclude as best as possible that these interventions produce detrimental unwanted modulations of the tumor and its microenvironment that exhaust the beneficial effects that were pronounced in preclinical studies. This has the potential to further improve patients' outcome in colorectal cancer, brain cancer, ovarian cancer, esophagogastric cancer and many other entities (106).

Additionally, serum biomarkers and radiologic tools, e.g. image guided determination of the vascular normalization

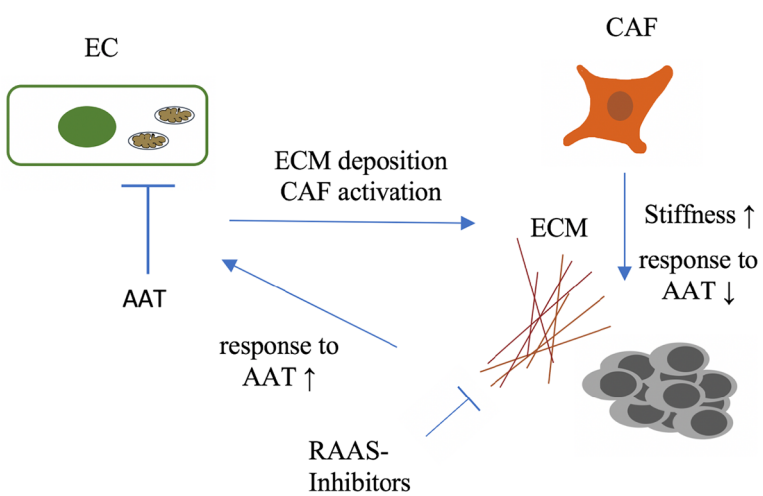

FIGURE 3 | Complex interactions between endothelium and cellular and non-cellular components of the extracellular tumor matrix. Increased tumor stiffness which is in part provoked by AAT itself leads to poor response to AAT and chemotherapy. Tumor stiffness can be targeted by inhibitors of the RAAS, sensitizing patients to therapy. EC, Endothelium; ECM, extracellular matrix; CAF, cancer-associated fibroblast; AAT, anti-angiogenic therapy; RAAS, renin-angiotensin-aldosteron-system.

windows (107) are urgently needed to be able to pre-select patients. This would spare unnecessary or even harmful treatments for individuals and uncountable costs for health care systems.

AUTHOR CONTRIBUTIONS

All three authors conceptualized and wrote the paper. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
published: 30 June 2022
doi: 10.3389/fphar.2022.797805

Atractylenolide III Attenuates Angiogenesis in Gastric Precancerous Lesions Through the Downregulation of Delta-Like Ligand 4

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Edited by:
Cornelis F. M. Sier,
Leiden University, Netherlands

Reviewed by:
Zhi-Hang Zhou,
Chongqing Medical University, China
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Specialty section:
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

Received: 19 October 2021
Accepted: 16 May 2022
Published: 30 June 2022

Citation:
Gao Y, Wang J, Zhao M, Xia T, Liu Q, Chen N, Liao W, Zeng Z, You F and Zeng J (2022) Atractylenolide III Attenuates Angiogenesis in Gastric Precancerous Lesions Through the Downregulation of Delta-Like Ligand 4. Front. Pharmacol. 13:797805. doi: 10.3389/fphar.2022.797805

Background: Blocking and even reversing gastric precancerous lesions (GPL) is a key measure to lower the incidence of gastric cancer. Atractylenolide III (AT-III) is a mainly active component of the Atractylodes rhizome and has been widely used in tumor treatment. However, the effects of AT-III on GPL and its mechanisms have not been reported.

Methods: H & E staining and AB-PAS staining were employed to evaluate the histopathology in the gastric mucosa. In parallel, CD34 immunostaining was performed for angiogenesis assessment, and transmission electron microscope for microvessel ultrastructural observation. Investigation for the possible mechanism *in vivo* and *in vitro* was conducted using immunohistochemistry, RT-qPCR and western blotting.

Results: In most GPL specimens, AT-III treatment reduced microvascular abnormalities and attenuated early angiogenesis, with the regression of most intestinal metaplasia and partial dysplasia. Meanwhile, the expression of VEGF-A and HIF-1α was enhanced in GPL samples of model rats, and their expressions were decreased in AT-III-treated GPL rats. Moreover, DLL4 mRNA and protein expression were higher in GPL rats than in control rats. DLL4 protein expression was significantly enhanced in human GPL tissues. In addition, AT-III treatment could diminish DLL4 mRNA level and protein expression in the MNNG-induced GPL rats. *In vitro* study showed that in AGS and HGC-27 cells, DLL4 mRNA level and protein expression were significantly decreased after AT-III treatment. However, AT-III had no significant regulatory effect on Notch1 and Notch4.

Conclusion: AT-III treatment is beneficial in lessening gastric precancerous lesions and attenuating angiogenesis in rats, and that may be contributed by the decrease of angiogenesis-associated HIF-1α and VEGF-A, and downregulation of DLL4.

Keywords: gastric precancerous lesions, angiogenesis, DLL4, atractylenolide III, microvessel

INTRODUCTION

Gastric precancerous lesions (GPL) refer to the pathological changes of intestinal metaplasia (IM) and dysplasia accompanied by chronic atrophic gastritis, which are positively associated with the incidence of gastric cancer (GC) (Huan et al., 2015). Therefore, it is an effective measure to pay attention to the early diagnosis and treatment of GPL for the secondary prevention of GC. The pathogenesis of GPL is still unclear, and endoscopic mucosal dissection is currently recommended as the main treatment for severe dysplasia and early gastric cancer (Pimentel-Nunes et al., 2019). However, in clinical practice, there is no specific treatment for most GPL patients. Thus, it is essential for us to find new and effective treatments for GPL.

At present, many researchers have focused more attention on natural bioactive components due to their high activity and low cytotoxicity (Ambriz-Pérez et al., 2016; Leyva-López et al., 2016; Villarreal-García et al., 2016). Atractylodes macrocephala is one of the traditional Chinese medicinal herbs, which has obvious curative effects on anorexia, dyspepsia and diarrhea (Wei et al., 2011; Zheng et al., 2012). Atractylenolide III (AT-III) is the main bioactive component of Atractylodes macrocephala and it has been proved to possess pharmacological properties include anti-inflammatory, antioxidative, anti-tumor and anti-angiogenesis effects (Hoang et al., 2012; Wang et al., 2015; Huai and Ding, 2020; Bailly, 2021; Sheng et al., 2021). A previous study demonstrated that AT-III might play a gastroprotective role in ethanol-induced acute gastric ulcer by reducing extracellular matrix damage (Wang et al., 2010). Recently, AT-III has been found to inhibit the proliferation of gastric cancer cells and induce apoptosis of gastric cancer cells, thus playing an anti-tumor role (Ji et al., 2019). In addition, AT-III, as an anti-tumor agent, restricts the recruitment of new blood vessels required for tumor formation and growth by inhibiting angiogenesis (Wang et al., 2015). However, the anti-angiogenesis mechanisms of AT-III in GPL treatment are still unclear.

Angiogenesis, defined as the basic process of forming new blood vessels from pre-existing ones, is closely associated with tumor growth, invasion and metastasis (Judah, 2002; Nienhüser and Schmidt, 2017). Therefore, targeting angiogenesis is the focus of tumor therapy. Hypoxia inducible factor-1α (HIF-1α) is the main drive factor of angiogenesis, and abnormal activation of HIF-1α leads to VEGF overexpression to a large extent, which is critical for angiogenesis (Arany et al., 2008; Tirpe et al., 2019). Vascular endothelial growth factor-A (VEGF-A), also called VEGF, is considered to be a major regulatory factor of tumor angiogenesis, which can stimulate tumor angiogenesis and increase tumor vascular permeability. (Hoeben et al., 2004; Korpanty et al., 2011). More importantly, recent reports have found that DLL4/Notch signaling is the most significant of all the signaling pathways involved in tumor angiogenesis (Yen et al., 2015; McKeage et al., 2018). In humans, four Notch receptors (Notch 1–4) and five ligands (delta-like ligands 1, 3, 4 and Jagged 1 and 2) have already been identified. Among these, Notch1, Notch4, and DLL4 were confirmed to play a pivotal role in angiogenesis (Sainson and Harris, 2008). As the specific ligand for Notch1 and Notch4, DLL4 expression is closely related to tumor angiogenesis and metastasis (Benedito et al., 2009; Li et al., 2011; Miao et al., 2017). Studies have shown that DLL4 overexpression is associated with TNM stage and distant metastasis in GC patients, indicating an association with poor prognosis in GC patients (Ishigami et al., 2013; Du et al., 2014). Although the role of the molecules in promoting angiogenesis in gastric cancer has been reported in recent years, what role they might play in GPL remains unclear.

In the research, the effects of AT-III on GPL angiogenesis and expression of angiogenesis related factors were observed. We hoped to test the hypothesis that the anti-angiogenesis properties of AT-III are related to the regulation of angiogenesis-associated markers HIF-1α and VEGF-A, as well as the DLL4/Notch signaling pathway. Our results may provide experimental evidence for AT-III to inhibit GPL angiogenesis.

MATERIALS AND METHODS

Animals and Ethics Statement

Half male and half female SD rats, weight 180–200 g, were provided by Chengdu Dashuo Experimental Animal Co., Ltd. The rats were fed standard rat chow at room temperature of 22–24°C, relative humidity of 40–60% and light-dark cycle of 12 h. The rats were given adaptive feeding for 1 week before the experiment. All animal procedures are approved and permitted by the Institutional Animal Care and Use Committee (Animals use license: SCXK-2020-030, ethical approval number: 2019-17/24).

Clinical Tissue Samples

56 cases of GPL gastric mucosa and 46 cases of normal gastric mucosa were collected from the Teaching Hospital of Chengdu University of Traditional Chinese Medicine, and retrospectively analyzed. Formalin fixed and paraffin-embedded tissue samples were stored at room temperature. All specimens were confirmed by pathological examination. This present study was permitted by the Institutional Review Board of the Teaching Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu, China) (approval no. 2018KL-023). Each participant included in the study signed the written informed consent.

Establishment of Gastric Precancerous Lesions Model in Rats and Drug Administration

The experiment flow chart was shown in **Figure 1**. Briefly, SD rats were randomly divided into four experimental groups ($n = 10$ per group): control group (treated with distilled water and physiological saline), model group (treated with MNNG and physiological saline), high-dose Atractylenolide III group (treated with MNNG and AT-III, 2.4 mg/kg/d) and low-dose Atractylenolide III group (treated with MNNG and AT-III, 1.2 mg/kg/d) (cat. no. BZP0374, Hefei Bomei Biotechnology Co., Ltd., China). The GPL rats model was set up based on the literatures (Saito et al., 1970; Tatematsu et al., 1988). To

FIGURE 1 | Flowchart of experimental design. 1) Normal group (treated with distilled water and physiological saline, control group); 2) Model group (treated with MNNG and physiological saline); 3) Atractylenolide III (2.4 mg/kg) group (treated with MNNG and high dose AT-III); 4) Atractylenolide III (1.2 mg/kg) group (treated with MNNG and low dose AT-III). MMNG, N-methyl-N′-nitro-N-nitrosoguanidin.

establish the GPL rat model, the rats were given MNNG at 5 ml/kg by gavage once a week and allowed to drink MNNG solution (200 μg/ml) (cat. no. M0527, Tokyo Chemical Industry Co., Ltd., Japan) *ad libitum*, and underwent starvation and satiety conversion every other day. At the end of 9th week, 2 model rats were randomly selected and sacrificed, and then detected for GPL. At the 10th week, rats in the high-dose and low-dose Atractylenolide III groups were given AT-III at 2.4 mg/kg and 1.2 mg/kg by gavage, respectively, while rats in the control group and model group were intragastric with physiological saline (10 ml/kg) for 10 weeks, once a day. At the end of 20th week, all rats were sacrificed with sodium pentobarbital (140 mg/kg i. p.) after 12 h fasting. Following sacrifice by cervical dislocation, stomachs were harvested immediately.

Cell Culture

Human gastric cancer cell lines (AGS and HGC-27) were obtained from the Centre Laboratory of Affiliated Hospital of Chengdu University of TCM. Cells were divided into 3 groups: control group, high-dose AT-III group (120 μM), low-dose AT-III group (80 μM). Cell lines were cultured in RPMI-1640 medium (Gibco, United States) containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2 saturated humidity. CCK-8 assay was used to determine relative cell viability after AT-III treatment for 24 h. The IC_{30} and IC_{50} of AT-III at 24 h in HGC-27 and AGS cells were found to be 80 and 120 μM, respectively.

Pathological Analysis

Gastric tissues were removed and fixed overnight in 10% neutralized formalin, followed by dehydration with alcohol and xylene. Then, the 3-μm paraffin-embedded sections were prepared and dipped in hematoxylin and eosin (H & E) using standard protocols. According to the manufacturer' sintroductions, the types of intestinal metaplasia were examined by Alcian Blue-Periodic Acid Schiff (AB-PAS staining). The paraffin sections were deparaffinized to water. The sections were stained with Alcian Blue staining solution for 5–10 min. The slices were oxidized with 1.0% periodic acid solution for 10 min, rinsed in running water for several minutes, and washed twice in distilled water. The sections were stained with Schiff solution for 15–30 min without light, rinsed with running water for 5–10 min. After the slices were dried, the nucleus was lightly stained with Mayer hematoxylin for about 1 min, rinsed with running water for several minutes, dehydrated with gradient alcohol, transparented with xylene and sealed with neutral gum. Neutral mucins in normal mucosa were stained magenta, while acidic mucins in IM lesion were stained blue. The morphological changes of the sections were observed by light microscope (IX71; Olympus Corporation) and the incidence of GPL in the rats were analyzed (Tytgat, 1991; Riddell, 1995). The magnifications used were ×100 and ×200.

Evaluation of Microvessel Density

The expression of CD34 was detected by immunohistochemical staining (IHC) to assess microvessel density (MVD) in gastric mucosa. In order to measure MVD, we used the method described by Weidner to perform the quantitative vessel counts (Vermeulen et al., 1996). To be specific, the tissue sections were scanned at low-power magnification (×40 and ×100) to identify areas with the highest angiogenesis (also known as hot-spot). Then, counting the stained microvessels in 3 random views of the "hot-spot" area at high-power (×200). The microvascular density value was figured as the mean value of the 3 field counts (×200).

Transmission Electron Microscopy

The ultrastructure of the gastric mucosa was observed using TEM. The gastric mucosa tissue specimens (1 mm^3) were fixed in 2.5% glutaraldehyde prepared in phosphate buffer for 2.5 h, and re-fixed in 1% osmium tetroxide in phosphate buffer for 2 h. The tissues were washed with buffer, dehydrated in gradual ethanol, then dipped twice in a mixture of acetone and epoxy resin, and embedded in capsules filled with epoxy resin, heated overnight at 70°C, and 70-nm ultrathin sections were sliced with LKB microtome. Images were observed and imaged by TEM (H-7650; Hitachi Ltd.) and used to describe the ultrastructure of microvessel. The magnification was ×10,000.

Immunohistochemical Staining

Gastric sections were embedded in paraffin and cut into 3 μm slices for IHC assay. The sections were heated at 97°C for 20 min, soaked in 3% hydrogen peroxide solution for 15 min and blocked

with 5% bovine serum albumin for 30 min. The sections were incubated overnight at 4°C with primary antibodies against CD34 (lot ZDP0112111, R & D Systems, United States), VEGF-A (lot GR116031-1, Abcam, United Kingdom), HIF-1α (lot L1212, Santa Cruz Biotechnology, United States), Notch1 (ab52301, Abcam, United Kingdom), Notch4 (ab184742, Abcam, United Kingdom), and DLL4 (ab7280, Abcam, United Kingdom). Then, the sections were stained with diaminobenzidine and counterstained with hematoxylin to detect the results. Three fields were randomly selected under the light microscope for photographing. Quantification of expression levels was determined by mean of integrated optical density and analyzed by Image Pro Plus 6.0 software (Media Cybernetics, Inc.). The magnification used was ×200.

Western Blot

Total protein was extracted with Radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors, and the protein concentration was measured by the BCA assay. Equal quantities of the total protein were loaded into wells and separated using 10% SDS-PAGE, and then transferred electrophoretically to polyvinylidene fluoride membranes. After blocking in 5% nonfat dry milk for 2 h, the membranes were incubated overnight with primary antibodies at 4°C. The dilution ratios of primary antibodies in the experiment were as follows: DLL4 (1: 1000), Notch1 (1: 1000), Notch4 (1: 1000), and GAPDH (1: 1000) (lot 00093663, Proteintech Co., Ltd., United States). Next, the membranes were washed with PBST, then the secondary antibodies were added and incubated at 37°C for 1 h. The gray values of the bands were quantified and normalized to GAPDH by the Image-Pro Plus software version 6.0 (Media Cybernetics, Inc.).

Real-Time Quantitative RT-PCR

TRIzol kit (G3013; Servicebio) was used to extract total RNA from tissues and cells. The RT-qPCR was performed with SYBR Green qPCR Mix kit and 7500 Fast Real-Time PCR System (Applied Biosystems Inc.) to detect the mRNA levels of Notch1, Notch4, and DLL4. The differences of amplification were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in rats were as follows: *DLL4* forward 5′-TGCCACTTCGGT TACAC-3′ and reverse 5′-TGACACATTCGTTCCTCTC-3′; *Notch1* forward 5′-AGCCAGTAAGCCAAGT-3′ and reverse 5′-ACAGTCCATCCTCAGTT-3′; *Notch4* forward 5′-CAG CCCGAGCAGATGTAGGA-3′ and reverse 5′-CGGCGTCTG CTCCCTACTGT-3′; *18S* 5′-ACGGCTACCACATCC-3′ and reverse 5′-CAGACTTGCCCTCCA-3′. The human DLL4 primer was Cat#HQP013577 (Hs-QRP-20948 DLL4; GeneCopoeia, Inc) and GAPDH primer was Cat#HQP006940 (Hs-QRP-20169 GAPDH; GeneCopoeia, Inc).

Statistical Analysis

The data analyses were performed by SPSS 23.0 software (IBM Inc.). All quantitative data were presented as mean ± SEM. One-way ANOVA was used to evaluate the differences between groups, Tukey method was used for homogeneous data and Dunnett's T3 method was used for non-homogeneous data. Unpaired Student's t-test was used to analyze the differences in DLL4, notch1 and notch4 expressions between GPL group and normal group. Pearson's χ^2 test and Fisher's exact test were performed to assess DLL4, notch1, and notch4 expression and clinicopathological characteristics. $p < 0.05$ was considered statistically significant.

RESULTS

Atractylenolide III Improves the General Condition and Alleviates Histopathological Changes of the Gastric Mucosa in Gastric Precancerous Lesions Rats

The control rats appeared relaxed, moved quickly, ate well, and had hard, grainy feces. In contrast, the model rats seemed less energetic, moved slowly, ate less and had diarrhea. The body weight of the model rats decreased remarkably compared with the control group ($p < 0.01$). AT-III administration (concentration of 1.2, 2.4 mg/kg) could partly preserve the body weight of rats ($p < 0.05$; $p < 0.01$; **Figure 2A**). These results suggest that AT-III prevents GPL-associated body weight loss.

We used H & E staining to assess the histopathological changes of gastric mucosa. Our data indicated that there was no intestinal metaplasia or dysplasia in the control group, and the difference was statistically significant compared with the model group ($p < 0.01$). The incidence of GPL in the model group, AT-III administration groups (1.2 mg/kg, 2.4 mg/kg) was 90% (9/10), 50% (5/10), and 30% (3/10), respectively. There were significant differences between the AT-III group (2.4 mg/kg) and the model group ($p < 0.05$). As shown in **Table 1**. Morphologically, rats in the control group exhibited normal macroscopic appearance of gastric mucosa. The glands and structure of gastric epithelial cells were normal, and there was little or no inflammatory infiltration in gastric epithelium under light microscope. In contrast, the gastric mucosa of the model rats appeared as dark red, with poor lustrousness, little mucus and rough surface. Light microscope revealed the arrangement of gastric mucosa glands was irregular and crowded and back-to-back tubular structure. In addition, the gastric epithelial cells showed enlarged and hyperchromatic nuclei, increased nuclear-cytoplasmic ratio, loss of nuclear polarity, and gastric mucosa stimulated cavity fusion, suggesting dysplasia lesion of the gastric mucosa. As expected, atypical hyperplasia and inflammatory infiltration were inhibited to varying degrees in most rats treated with AT-III (**Figures 2B,C**). These observations suggested that AT-III can inhibit or even reverse the process of dysplasia and protect gastric mucosa.

The degree of intestinal metaplasia was evaluated using AB-PAS staining. In the control rats, the neutral mucins in gastric mucosa were stained red, indicating no intestinal metaplasia. In the model group, gastric mucosa with lesion of intestinal metaplasia were stained blue or purple. After AT-III intervention, we found that intestinal metaplasia was visibly regressed as compared with that in the model rats

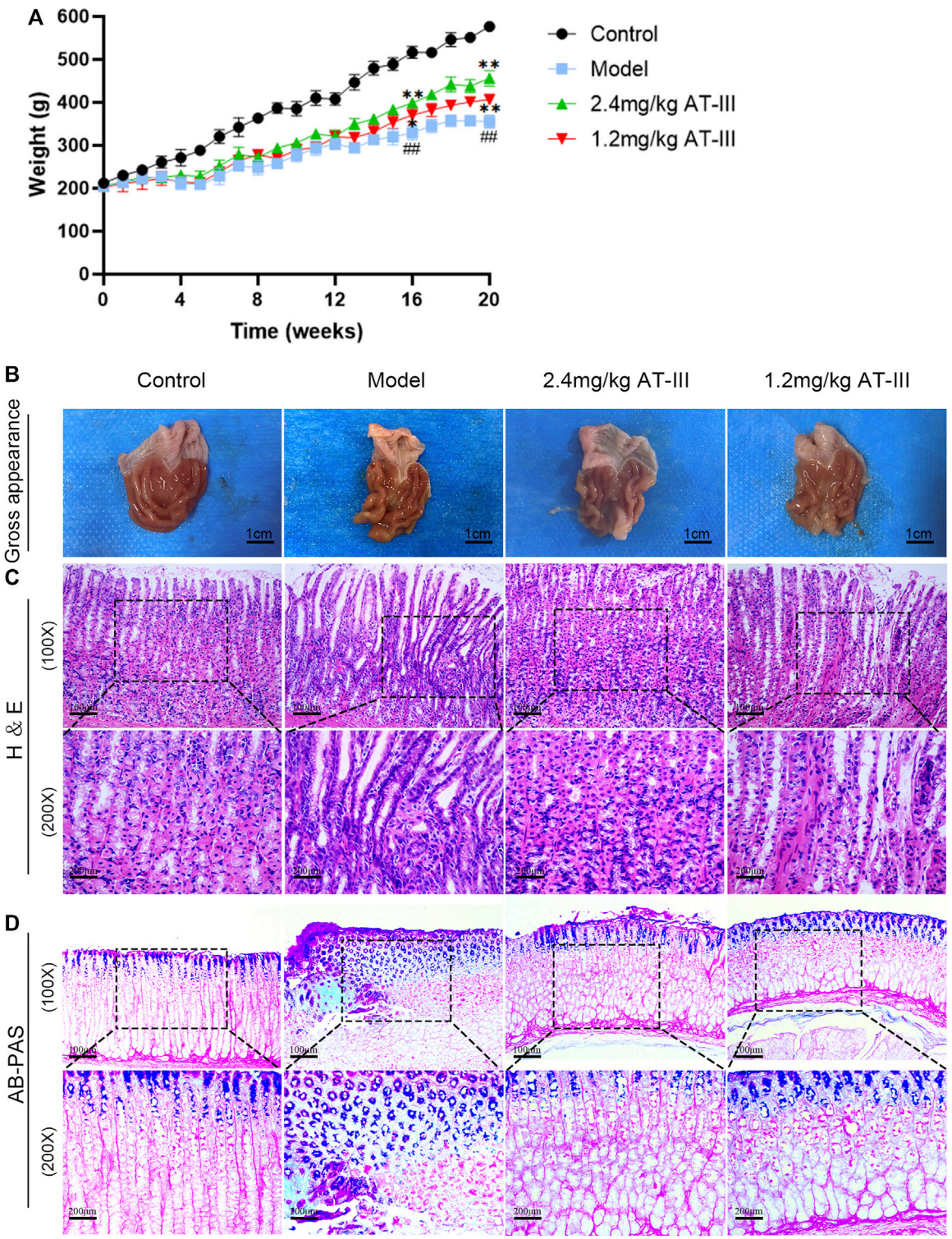

FIGURE 2 | Effects of AT-III on pathomorphology of dysplasia, gastric intestinal metaplasia and body weight in GPL rats. **(A)** Effect of AT-III treatment on body weight of GPL rats. **(B)** Gross evaluation of the gastric mucosa. **(C)** Representative images of H & E staining of the gastric epithelium (×100, ×200). **(D)** Evaluation for intestinal metaplasia using AB-PAS staining (×100, ×200). $^{\#\#}p < 0.01$ versus the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus the model group. Abbreviations: AT-III, Atractylenolide III; GPL, gastric precancerous lesions; H & E, hematoxylin and eosin; AB-PAS, alcian blue-periodic acid schiff.

TABLE 1 | The incidence rate of GPL in each group.

Group	Number	Intestinal metaplasia			Dysplasia			GPL incidence (%)
		Mild	Moderate	Severe	Mild	Moderate	Severe	
Control group	10	0	0	0	0	0	0	0
Model group	10	1	2	1	1	1	3	90
1.2 mg/kg AT-III group	10	2	1	0	0	1	1	50
2.4 mg/kg AT-III group	10	1	1	0	1	0	0	30

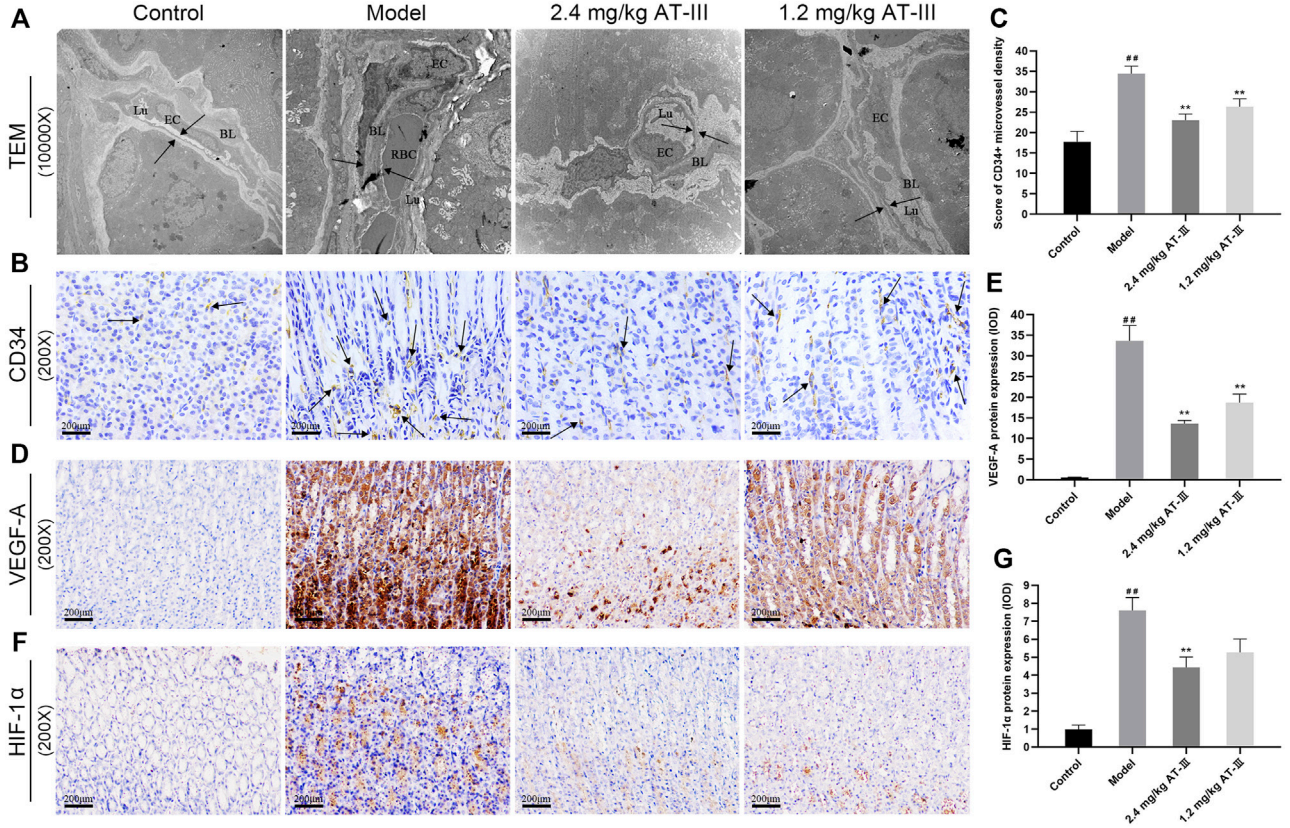

FIGURE 3 | Evaluation of microvessel density in gastric mucosa. **(A)** Representative images of ultrastructures of epithelial cells using TEM (×10000). **(B)** CD34-labelled microvessels in gastric mucosa of each group (IHC, ×200). Expression of VEGF-A **(D)** and HIF-1α **(F)** protein in different gastric mucosa (IHC, ×200). Effect of AT-III on CD34 levels **(C)**, VEGF-A **(E)** and HIF-1α **(G)** protein expressions in gastric mucosa from various groups ($n = 10$). Data are presented as mean ± SEM. RBC, red blood cell; EC, endothelial cell; BL, basal lamina; Lu, lumen. $^{\#\#}p < 0.01$ versus the control group; $^{**}p < 0.01$ versus the model group. RBC, red blood cell; EC, endothelial cell; BL, basal lamina; Lu, lumen; TEM, transmission electron microscopy; AT-III, Atractylenolide III; IHC, immunohistochemistry; SEM, standard error of mean.

(**Figure 2D**). Our observation showed that AT-III could effectively reverse gastric intestinal metaplasia in GPL rats.

Atractylenolide III Ameliorates Microvascular Abnormalities

The morphological changes of microvessels in gastric mucosa were observed under TEM. The vascular inner diameter of microvessels in the control group was normal, the basal lamina was smooth and the structure was clear, complete and continuous with uniform thickness and homogeneous electron density. In contrast, we found that the microvascular lumen was dilated, the vascular inner diameter was significantly reduced, the basal lamina was thickened and rough, and the basement membrane was irregular and discontinuous in model rats. Partial or complete occlusion of some vascular lumens by erythrocytes was also observed. In the low-dose AT-III group (1.2 mg/kg), vascular lumen inner diameter of rats was mild to moderately reduced, the basal lamina surface was still rough, and part of the basal lamina was broken and discontinuous.

Furthermore, in the high-dose AT-III group (2.4 mg/kg), the capillary wall of rats was relatively smooth, the basal lamina is slightly fractured and discontinuous without obvious thickening, the inner diameter of vascular lumen was slightly decreased. Most of the nuclear membrane is clear and intact, and the distribution of nuclear chromatin is normal (**Figure 3A**). Therefore, AT-III intervention showed a potent protective effect on microvascular abnormalities in GPL rats.

Atractylenolide III Reduces the CD34-Labled Microvessel Density and Decreases VEGF-A and HIF-1α Protein Expressions

The expression of the angiogenesis marker CD34 in GPL tissues was detected by immunohistochemistry to analyze the effect of AT-III on angiogenesis. The number of $CD34^+$ microvessels was increased in most GPL tissues, suggesting active angiogenesis, whereas these microvessels were sparse in control tissues. Furthermore, we noticed that more GPL rats with dysplasia had a larger number of microvessels than IMs, and severe dysplasia had more microvessels than mild or moderate dysplasia. In contrast, we noted a significant reduction in $CD34^+$ microvascular counts in many AT-III-treated rats. These results demonstrated that $CD34^+$ microvessel density levels were significantly increased in model rats compared to the control group. But it decreased markedly after AT-III intervention, indicating that AT-III effectively reduced angiogenesis in GPL rats (**Figures 3B,C**).

IHC was used to evaluate whether VEGF-A and HIF-1α inhibition was associated with anti-angiogenic ability of AT-III. The data confirmed that HIF-1α and VEGF-A were sparsely expressed in normal gastric mucosa, while HIF-1α and VEGF-A protein expression were increased in GPL rats. As expected, the expressions of HIF-1α and VEGF-A protein in gastric mucosa were significantly decreased after AT-III intervention, and the difference was statistically significant ($p < 0.01$), but AT-III had no significant inhibitory effect on HIF-1α protein at a concentration of 1.2 mg/kg ($p > 0.05$). Interestingly, we observed that the reduction of HIF-1α and VEGF-A in GPL tissues was often accompanied by the attenuation of $CD34^+$ expression, suggesting that inhibition of HIF-1α and VEGF-A might be beneficial in AT-III-alleviated angiogenesis (**Figures 3D–G**).

DLL4 is Over-Expressed in Human Samples of Gastric Precancerous Lesions

In order to verify the high expression of Notch1, Notch4, and DLL4 in GPL, we investigate the expression of Notch1, Notch4, and DLL4 in 56 human GPL specimens and 46 normal specimens. We observed high DLL4 expression in 62.5% (35/56) of the GPL specimens and 41.3% (19/46) of the normal specimens by IHC. At the same sites, we found strong Notch1 expression in 7.1% (4/56) of the GPL specimens and 4.3% (2/46) of the normal specimens. In addition, we observed high Notch4 levels in 14.3% (8/56) of the GPL specimens and 6.5% (3/46) of the normal specimens. We noticed that the DLL4 immunoreactivity was notably stronger in the human GPL specimens than in the healthy controls (**Figures 4A,D**), while Notch1 and Notch4 were not overexpressed in human samples of GPL (**Figures 4B,C,E,F**). This evidence supports the association of DLL4 expression with increased angiogenesis in GPL. DLL4 expression was significantly correlated with advanced GPL pathology but not age, gender, location of lesion and Hp infection (**Table 2**).

Atractylenolide III Diminishes DLL4 Protein Expression and mRNA Level in Gastric Precancerous Lesions Rats

We further examined the expression levels of Notch1, Notch4 and DLL4 in GPL rats to determine the possible mechanism of GPL angiogenesis. The expression of DLL4 protein in gastric mucosa was observed by immunohistochemistry and analyzed by Western blotting. As shown in **Figures 5A–C**, normal gastric mucosa did not or barely express DLL4, while diffuse and intense cytoplasmic labeling, found in most cases of GPL rats, could be markedly diminished by AT-III. Statistically, GPL rats showed an increased DLL4 protein expression compared with the control group ($p < 0.01$), while AT-III intervention reduced the over-expression. Furthermore, we found that AT-III had a stronger inhibitory effect at a concentration of 2.4 mg/kg on DLL4 over-expression ($p < 0.01$). However, AT-III may have little effect on Notch1 and Notch4 expression (without statistical significance) (**Figures 5D–F**).

Quantitative analysis of protein expression proved that DLL4 protein expression was elevated in GPL rats compared with control group ($p < 0.05$), whereas AT-III treatment reduced the over-expression ($p < 0.01$) and did not significantly inhibit Notch1 and Notch4 ($p > 0.05$). (**Figures 5G–J**). Furthermore, RT-qPCR analysis confirmed that DLL4 mRNA level in GPL rats was significantly higher than that in controls ($p < 0.01$). After AT-III intervention, DLL4 mRNA level of rats was dramatically reduced ($p < 0.05$). The data suggested that AT-III could efficiently inhibit DLL4 mRNA level in model rats, but Notch1 and Notch4 mRNA levels were not significantly decreased (**Figures 5K–M**).

Atractylenolide III Down-Regulates DLL4 Protein Expression and mRNA Level in Human Gastric Cancer Cell Lines

DLL4 protein expression was measured by western blotting in human gastric cancer cell lines. The results revealed that AT-III treatment (concentration of 80, 120 μM) down-regulated DLL4 protein expression in AGS and HGC-27 cell lines ($p < 0.05$; $p < 0.01$). Furthermore, AT-III showed a better inhibitory effect at a concentration of 120 μM ($p < 0.01$). And the difference in the gray value was statistically significant ($p < 0.01$; **Figures 6A–D**).

RT-qPCR detection for DLL4 gene expression in AGS and HGC-27 cells. Our results indicated that the mRNA expression of DLL4 was obviously decreased in the AT-III group (80 μM) compared with control group ($p < 0.05$). The expression of

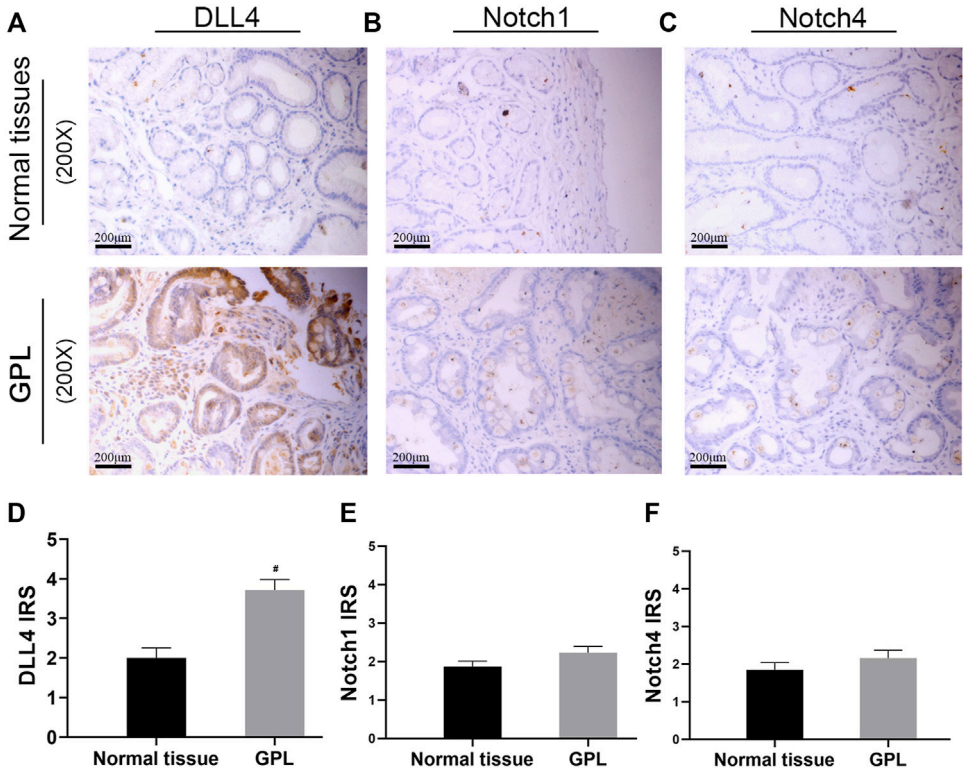

FIGURE 4 | DLL4 mRNA expression is upregulated in gastric cancer and DLL4 protein is over-expressed in human GPL specimens. Representative IHC images demonstrating the expression of DLL4 **(A)**, Notch1 **(B)** and Notch4 **(C)** in GPL and normal tissues (×200). Semi-quantitative analysis of DLL4 **(D)**, Notch1 **(E)** and Notch4 **(F)** protein expression in human specimens (n = 102). $^{\#}p < 0.05$ versus the control group. Data are presented as mean ± SEM. Abbreviations: AT-III, Atractylenolide III; GPL, gastric precancerous lesions; GC, gastric cancer; IHC, immunohistochemistry; SEM, standard error of mean.

DLL4 gene was further decreased in the AT-III group (120 μM) ($p < 0.01$; **Figures 6E,F**). All these results clearly indicated that DLL4 protein expression and mRNA level was inhibited after AT-III treatment in AGS and HGC-27 cell lines. However, AT-III treatment did not significantly inhibit the protein expression of Notch1 and Notch4 ($p > 0.05$; **Figures 6G–J**).

DISCUSSION

It is well known that GPL is the key stage in the progression of GC. Therefore, early intervention for GPL is important for reducing the morbidity of GC (Huang et al., 2015; Malik et al., 2017). AT-III is a Chinese traditional herb with a long medicinal history isolated from the dried rhizome of Atractylodes macrocephala. In recent years, many basic and clinical studies have shown that AT-III has anti-cancer effects (Ji et al., 2019; Bailly, 2021; Sheng et al., 2021). However, the effects of AT-III on GPL has not been reported. In the research, we observed that AT-III administration could alleviate IM and partial dysplasia in GPL rats, suggesting that AT-III is beneficial in protecting against gastric precancerous lesions.

Folkman's tumor angiogenesis theory is critical to the treatment of malignant tumors and precancerous lesions (Folkman, 1971). Therefore, inhibiting angiogenesis may be an attractive strategy to prevent and treat malignant transformation of gastric mucosa, which can reduce the morbidity of GC patients. In this research, we found that $CD34^{+}$ MVD in GPL rats was remarkably higher than that in the normal gastric mucosa, which supported the hypothesis of angiogenesis in GPL rats. More importantly, the gastric mucosa often showed a higher $CD34^{+}$ microvessel count in more severe lesions. There was more microvessels in dysplasia than in intestinal metaplasia, and more microvessels in severe dysplasia than in mild or moderate dysplasia. After administration of AT-III, the angiogenesis marker CD34 was significantly decreased in GPL tissues.

HIF-1α and VEGF are considered to be classic factors controlling multiple proangiogenic processes in hypoxic tumors (Rey et al., 2017). A study focusing on the correlation between VEGF and the degree of progression of GPL found that VEGF was overexpressed in GPL, and its expression increased with the severity of gastric mucosal gland atrophy and intestinal metaplasia (Zhao et al., 2019). Experimental evidence suggests that HIF-1α was activated in the early stages of GC. The over-expression of HIF-1α was positively correlated with tumor infiltration depth, MVD and VEGF expression in gastric cancer, and patients with HIF-1α (+)/VEGF (+) had a relatively poor prognosis (Fu et al., 2019). It is worth mentioning that early angiogenesis

TABLE 2 | Correlation between DLL4 positivity and clinicopathological characteristics.

	Notch1		Notch4		DLL4	
	Low	High	Low	High	Low	High
Age						
<60	33	3	31	5	12	24
≥60	19	1	17	3	9	11
p value						
	0.643		0.909		0.388	
Gender						
Male	33	3	31	5	7	18
Female	19	1	17	3	14	17
p value						
	0.643		0.909		0.187	
Location of lesion						
Body	10	0	9	1	6	4
Angle	10	1	10	1	4	7
Antrum	26	1	21	6	9	18
Multiple	6	2	8	0	2	6
p value						
	0.160		0.374		0.407	
Hp infection						
Negative	22	0	16	6	12	10
Positive	30	4	32	2	9	25
p value						
	0.095		0.025		0.034	
Histopathological category						
Normal gastric epithelium	44	2	43	3	27	19
Gastric precancerous lesions	52	4	48	8	21	35
p value						
	0.551		0.208		0.033	
Intestinal-type metaplasia	24	2	24	2	8	18
p value						
	0.882		0.189		0.333	
High-grade intraepithelial neoplasia	12	1	9	4	5	8
Low-grade intraepithelial neoplasia	16	1	15	2	8	9
p value						
	0.844		0.197		0.638	

found in GPL tissues is usually accompanied by activation of HIF-1α and VEGF-A. And we noted that AT-III could inhibit VEGF-A expression in GPL tissues at a concentration of 1.2 mg/kg and 2.4 mg/kg, and reduce HIF-1α expression at a concentration of 2.4 mg/kg.

Recent reports have shown that DLL4/Notch signaling pathway occupies an important role in angiogenesis, including Notch1, Notch4, and DLL4 as key targets (Kangsamaksin et al., 2014). Studies have confirmed that DLL4 expression is up-regulated in the tumor vasculature compared with normal vessels (Schadler et al., 2010; Liu et al., 2015). For example, the expression of DLL4 in clear cell renal tumor vessels was higher than that in normal renal tissues and related to vascular maturation (Huang et al., 2013). Furthermore, studies have indicated that the activation of Notch1, Notch4, and DLL4 is crucial in the initiation and progression of gastric cancer (Du et al., 2014; Qian et al., 2015; Huang et al., 2019). However, the possible role of the molecules in GPL remains unclear.

A previous study demonstrated that AT-III could inhibit breast tumor angiogenesis *in vitro* and *in vivo* (Wang et al., 2015). The possible mechanism is that AT-III suppressed Runx2 activation in endothelial cells, which contributed to the inhibition of MMPs and VEGF-VEGFR2 signaling as well as the anti-angiogenic properties of AT-III (Wang et al., 2015). In our research, DLL4 protein expression were up-regulated in human GPL specimens compared with normal specimens. We found similar results in the animal study, and different concentrations of AT-III treatment could significantly reduce the gene and protein expressions of DLL4. Furthermore, our cell experiments showed that DLL4 protein and gene expressions were markedly down-regulated after AT-III treatment in AGS and HGC-27 cells. The results suggested the role of DLL4 in angiogenesis and provide new ideas for anti-angiogenesis therapy of GPL.

More importantly, our animal and cell experiments showed that AT-III had a stronger inhibitory effect on DLL4 over-expression at a concentration of 2.4 mg/kg and 120 μM, which may be more ideal for GPL intervention, but we need to expand the sample size in the future to further verify our speculation. However, AT-III intervention showed no inhibitory effects on Notch1 and Notch4 expression. The possible reason is that Notch1 and Notch4 may not be the targets for AT-III treatment of GPL. Furthermore, the superiority of AT-III in GPL treatment and its detailed mechanisms merit further investigations.

In conclusion, it is encouraging that our data suggested that AT-III treatment could prevent the occurrence and progression

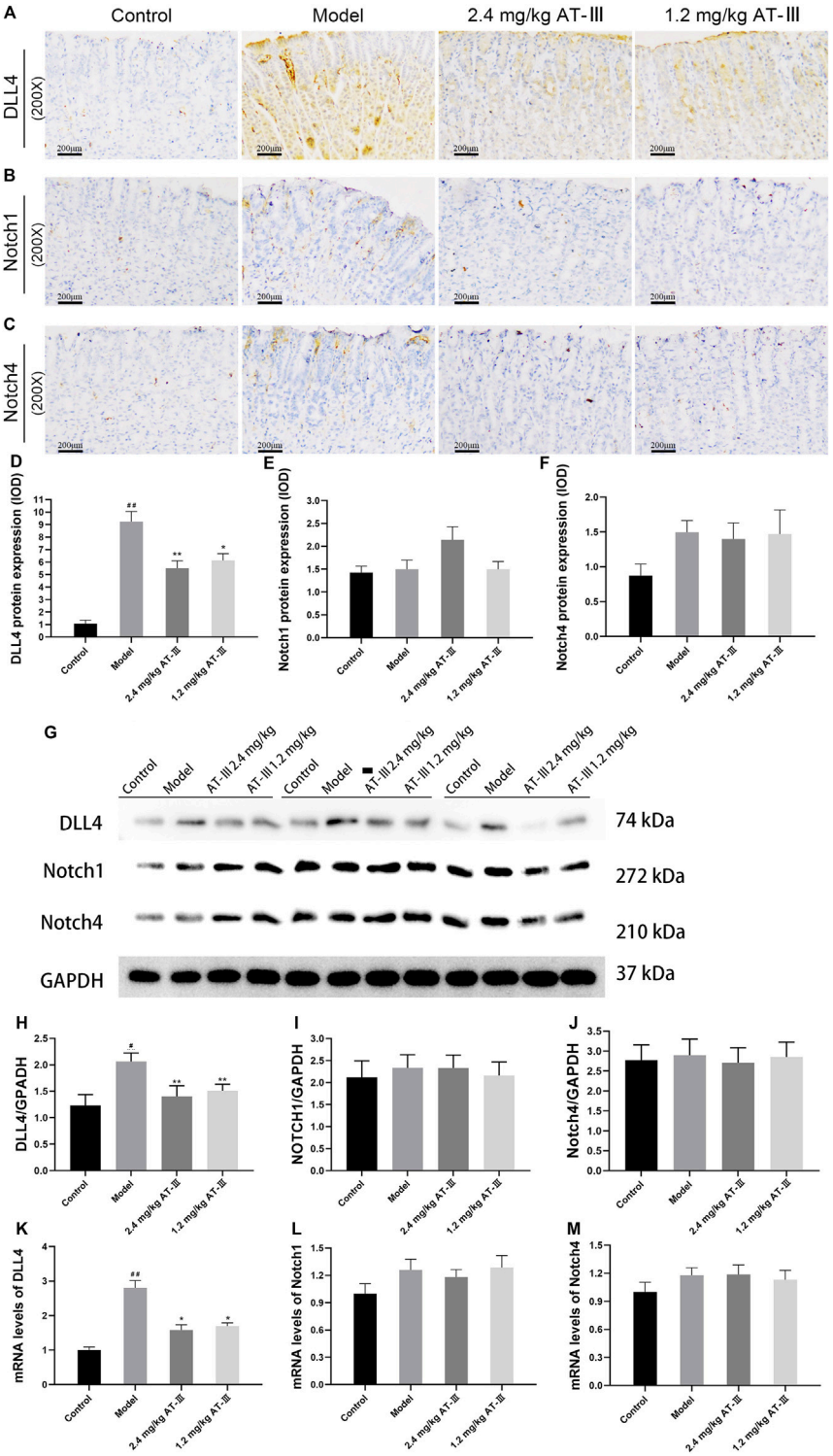

FIGURE 5 | The rat samples were detected by IHC. Expression of DLL4 **(A)**, Notch1 **(B)** and Notch4 **(C)** in gastric mucosa from various groups. Semi-quantitative analysis of DLL4 **(D)**, Notch1 **(E)** and Notch4 **(F)** protein expression levels in each group ($n = 10$). **(G)** Representative images of western blot proteins bands. Quantitative analysis of DLL4 **(H)**, Notch1 **(I)** and Notch4 **(J)** in western blotting bands ($n = 9$). Quantization for mRNA levels of DLL4 **(K)**, Notch1 **(L)** and Notch4 **(M)** in gastric mucosa from various groups ($n = 6$). **(J)** Representative western blotting bands of DLL4, Notch1 and Notch4. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus the model group. Data are presented as mean ± SEM. AT-III, Atractylenolide III; IHC, immunohistochemistry; SEM, standard error of mean.

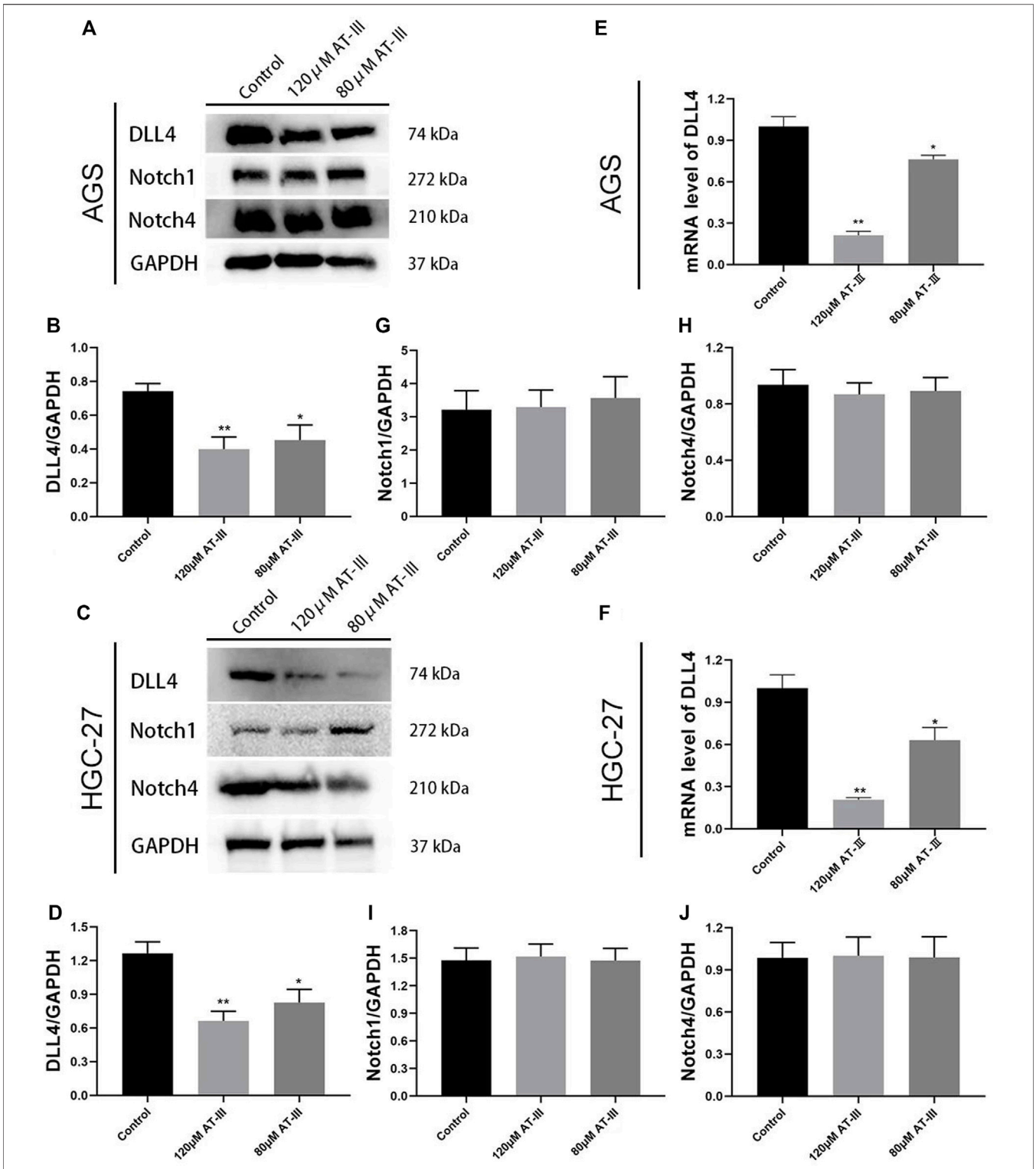

FIGURE 6 | Representative western blotting bands of DLL4, Notch1 and Notch4 in transfected AGS **(A)** and HGC-27 **(C)** cells. Quantitative analysis of DLL4 **(B,D)**, Notch1 **(G,I)**, and Notch4 **(H,J)** in western blotting bands ($n = 9$). RT-qPCR detection for DLL4 mRNA levels in transfected AGS **(E)** and HGC-27 **(F)** cells ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the control group. Data are presented as mean ± SEM. AT-III, Atractylenolide III; SEM, standard error of mean.

of GPL. The therapeutic effects may be associated with the inhibition of angiogenesis contributed by decreasing expression of angiogenesis-associated markers HIF-1α and VEGF-A, and by down-regulating DLL4. This study provided reliable experimental basis for the clinical treatment of GPL.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee, Hospital of Chengdu University of Traditional Chinese Medicine. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Medical Ethics Committee, Hospital of Chengdu University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

JZ and FY conceived the study. YG and JW wrote the manuscript. YG, MZ, and NC carried out the experiments. WL and ZZ collected the data. QL and TX performed the statistical analysis. All authors reviewed and approved the manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (grant nos. 82174346), Project of Sichuan Administration of traditional Chinese Medicine (grant no. 2021MS104), and the Science and Technology Project of Sichuan Province (grant no. 2022YFS0399).

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frontiers | Frontiers in Pharmacology

TYPE Case Report
PUBLISHED 10 August 2022
DOI 10.3389/fphar.2022.888106

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology

RECEIVED 02 March 2022
ACCEPTED 28 June 2022
PUBLISHED 10 August 2022

CITATION
Fu S, Li L, Li X, Wu Q, Wang X, Huang Y, Hu H and Cao D (2022), Case report: Long-term partial response of apatinib plus paclitaxel as second-line therapy in a patient with metastatic gastric cancer. *Front. Pharmacol.* 13:888106. doi: 10.3389/fphar.2022.888106

Case report: Long-term partial response of apatinib plus paclitaxel as second-line therapy in a patient with metastatic gastric cancer

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Gastric cancer is the second most prevalent cancer and the second leading cause of cancer-related death in China. The prognosis of metastatic gastric cancer is poor with a median overall survival of 8–10 months. Apatinib, an oral small-molecule, selective vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor, is approved as third-line or subsequent therapy for gastric cancer in China. Several recent small-scale studies and case reports showed that it may be great help in improvement of prognosis as second-line treatment in patients with advanced or metastatic gastric cancer. Here, we present a case of advanced gastric adenocarcinoma with multiple hepatic metastases who was treated with apatinib plus paclitaxel as second-line therapy, realized a long progression-free survival of 37 months. Until 29 January 2022, the disease remains an efficacy of partial response. We believe that the good outcome of this case is not an accident, because of the typically hyper-vascular of his liver metastases, the treatment toxicities of hypertension and proteinuria, all may be potential predictive biomarkers for anti-angiogenic treatments.

KEYWORDS

apatinib, second-line treatment, hyper-vascular, biomarker, metastatic gastric cancer

Introduction

According to an estimation of Chinese gastric cancer from 1990 to 2019, both the incidence and mortality rank second in China (He et al., 2021). The majority of newly diagnosed cases are locally advanced or metastatic diseases. Despite many treatment options available, including chemotherapy (e.g., platinums, taxanes, and fluoropyrimidines) and targeted therapy (e.g., ramucirumab and trastuzumab), the median overall survival (OS) of advanced or metastatic gastric cancer remains a dismal 8–10 months (Shah, 2015). Ramucirumab is a human IgG1 monoclonal antibody vascular endothelial growth factor receptor-2 (VEGFR-2) antagonist. Two international phase III randomized clinical trials, REGARD and RAINBOW, showed clear efficacy benefit of ramucirumab (Fuchs et al., 2014; Wilke et al., 2014). Based on these results, National Comprehensive Cancer Network (NCCN) recommend ramucirumab as a single agent or in combination with paclitaxel as treatment options for second-line or subsequent therapy in patients with advanced or metastatic gastric adenocarcinoma. However, ramucirumab is not available in China. Apatinib, a small-molecule VEGFR-2 tyrosine kinase inhibitor (TKI), has an anticancer effect though inhibition of angiogenesis, stimulation of apoptosis, suppression of cell proliferation, and inducing the effect of conventional chemotherapy drugs (Fathi Marouf et al., 2020). Apatinib was the first anti-angiogenic drug approved for treatment of advanced or metastatic gastric adenocarcinoma by China National Medical Products Administration (NMPA) and was recommended as a third-line or subsequent therapy. Despite several studies with small sample sizes revealed that apatinib was also effective for second-line treatment of advanced gastric adenocarcinoma (Zhang et al., 2018; Yang et al., 2020; Chen et al., 2021), the treatment of apatinib as a second-line agent remains unknown. Herein, we present a metastatic gastric adenocarcinoma patient with multiple hepatic metastases, treated with apatinib as the second-line therapy and realized a long progression-free survival (PFS) of 37 months up to now (29 January 2022).

Case description

In July 2017, a 66-year-old man attended to our hospital complaining of persistent pain in the upper abdomen for 3 years. His previous medical history was unremarkable. The thoracic and total abdomen computed tomography (CT) scan revealed gastric cardia tumor with multiple hepatic nodules. Subsequently, he underwent a gastric endoscopy and endoscopic biopsy, which revealed that primary lesion in the cardia of stomach and the mass pathology showed adenocarcinoma with negative human epidermal growth factor receptor 2 (Her-2). Based on these examinations, the patient was diagnosed with advanced gastric adenocarcinoma with multiple hepatic metastases (cT4NxM1, Stage IV). A total of eight cycles of chemotherapy with oxaliplatin and S-1 were administered. The gastric and hepatic lesions showed stable disease (SD). Five months after first-line treatment, the gastric and liver masses progressed on 17 December 2018 (Figure 1).

Before second-line treatment, we made comprehensive review and analysis of the dates of this case. The liver metastases of this case were typically hyper-vascular according to abdomen enhanced CT. We creatively used the average CT ratio (the density of the liver metastases/the density of abdominal aorta) during arterial phase on CT imaging to show the blood supply of liver metastasis. The ratio was 79/210 before second-line treatment. And then, considering that ramucirumab in combination with paclitaxel was recommend by NCCN as second-line treatment for patients with advanced or metastatic gastric adenocarcinoma, apatinib plus paclitaxel were selected for our case. The patient received apatinib (500 mg orally once daily) plus paclitaxel on 25 December 2018. After two cycles of therapy, the efficacy was assessed as partial response (PR). That average CT ratio was 38/281 with an obviously decreasing of the degree of enhancement (Figure 2). However, the patient developed hypertension with the highest blood pressure of 160 + mmHg, mild proteinuria (2+), and renal insufficiency with creatinine of 126 umol/L during second-line treatment. All above side effects were well controlled with appropriate treatment. The dose of apatinib was reduced to 250 mg/d on 27 April 2020. From 10 March 2021, the patient received apatinib (250 mg/d) alone for maintaining therapy (Figure 3). The recent CT examination was on 20 November 2021, which showed the disease remained an efficacy of PR. Up to now (29 January 2022), the patient demonstrated a PFS of 37 months and still received apatinib for maintaining therapy.

Discussion

In the second-line setting of advanced or metastatic gastric cancer, chemotherapy can improve survival in patients with good performance status who are fit for chemotherapy compared with best supportive care in randomized trials. The preferred second-line chemotherapy regimens include docetaxel, paclitaxel, and irinotecan. However, the benefit from second-line chemotherapy is limited. The outcome of second-line chemotherapy in gastric cancer is extremely poor with a median OS of less than 6 months in clinical trials (Thuss-Patience et al., 2011; Kang et al., 2012; Ford et al., 2014). Ramucirumab, a VEGFR-2 antibody, has shown favorable results in two phase III clinical trials for patients with previously treated advanced or metastatic gastroesophageal cancers (Fuchs et al., 2014; Wilke et al., 2014). Based on these results, NCCN guidelines recommend ramucirumab as a single or in combination with paclitaxel as treatment options for second-line therapy in patients with advanced or metastatic gastric adenocarcinoma. However, ramucirumab is not approved by the NMPA. Apatinib,

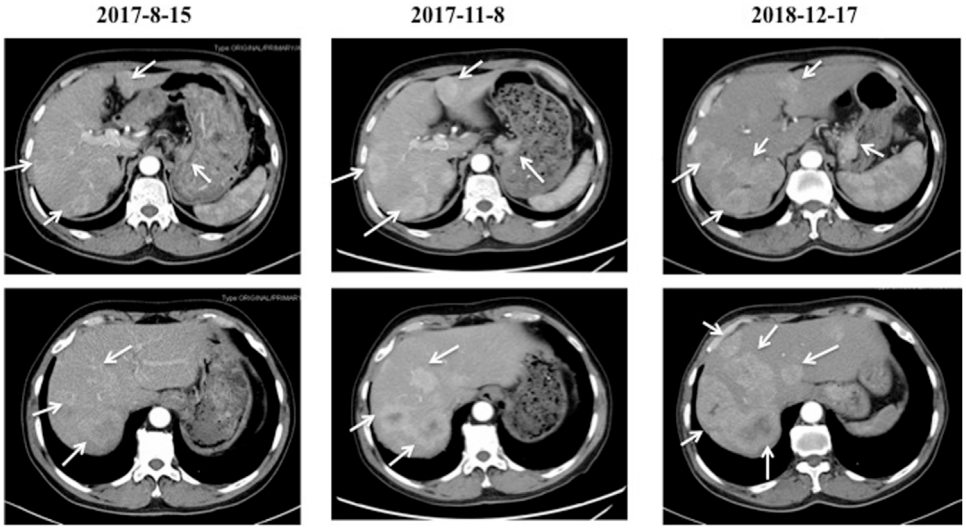

FIGURE 1
The CT images of the cardiac cancer and hepatic metastases before and after first-line chemotherapy. The cardiac tumor with multiple hepatic metastases were indicated on 15 August 2017. After two cycles of first-line chemotherapy with oxaliplatin and S-1, the cardiac and hepatic lesions showed stable disease (SD) on 8 November 2017. Five months after first-line treatment, the cardiac and liver masses were progressed on 17 December 2018.

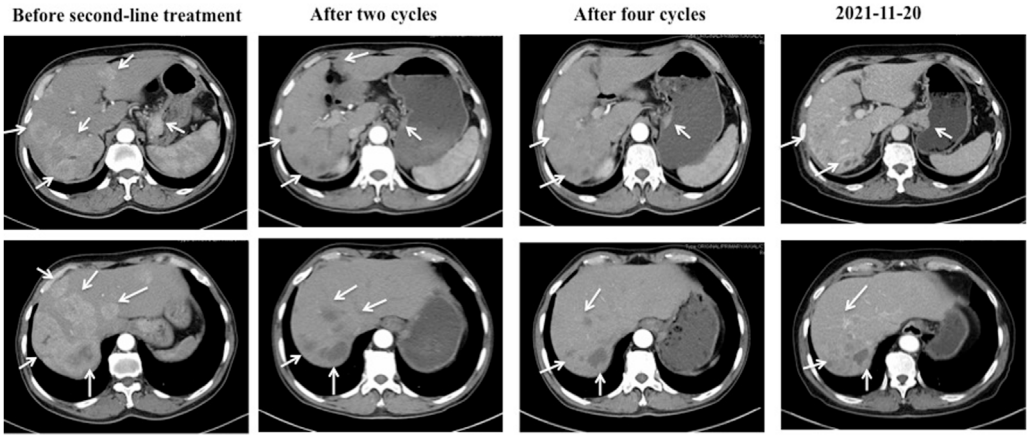

FIGURE 2
The CT images of the cardiac cancer and hepatic metastases during second-line treatment with apatinib and paclitaxel. Before second-line treatment, the CT showed markedly enlarged cardiac tumor and multiple hepatic metastases with abundant blood supply. The average CT ratio (the density of the liver metastases/the density of abdominal aorta) during arterial phase on CT imaging was 79/210. After two cycles of second-line therapy, the cardiac tumor and hepatic metastases showed a response of partial response (PR), and the ratio was 38/281 with an obviously decreasing of the degree of enhancement. Four cycles later, the efficacy remained PR. The recent CT examination was on 20 November 2021, which showed the disease remained an efficacy of PR.

a small-molecule VEGFR-2 tyrosine kinase inhibitor (TKI), is approved as a third-line or above therapy for advanced or metastatic gastric adenocarcinoma in China according to a phase III clinical study (Li et al., 2016). Several studies also showed that apatinib is an effective regimen for the second-line treatment. Zhang et al. firstly confirmed the clinical effectiveness of second-line apatinib for advanced gastric cancer with a median PFS of 4.43 months and median OS of 9.11months (Zhang et al., 2018). In Chen's study, compared with S-1, apatinib was superior in OS, showing a statistically significant difference (10.7 versus 8.1 months,

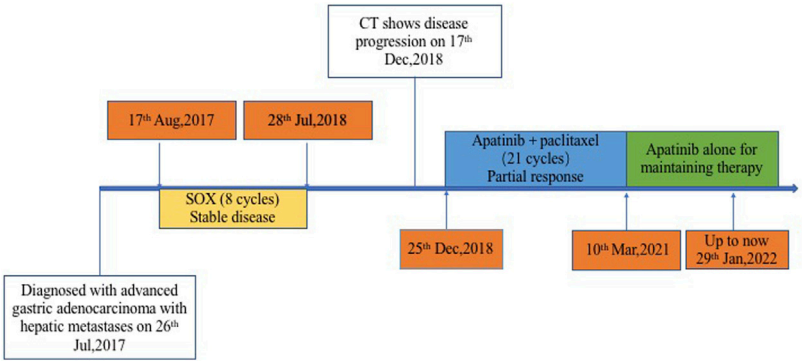

FIGURE 3
Timeline of management of this patient with metastatic gastric adenocarcinoma.

$p = 0.028$) (Chen et al., 2021). In this report, we present a metastatic gastric adenocarcinoma received apatinib in combination with paclitaxel as second-line therapy, who had an excellent PFS of 37 months up to now. To our knowledge, this case realized the longest PFS in patients received apatinib with or without chemotherapy as second-line or above therapy.

However, there are still many advanced or metastatic gastric cancer patients failed to benefit from anti-angiogenic drugs. The high cost of anti-angiogenic treatments makes it crucial to identify biomarkers which would help select responsible patients and improving the cost to benefit ratio. Although no biomarker is fully validated for this purpose, several candidates are currently under investigation.

Circulation molecules associated with angiogenesis are the most popular potential biomarkers. The results of studies in exploring the correlation between serum VEGF/VEGFR levels and response to VEGF inhibitor treatments were complex and inconclusive. According to AVAGAST trial, high baseline plasma VEGF-A associated with a trend towards improved OS and PFS in non-Asian patients with advanced gastric cancer treated with bevacizumab (Van Cutsem et al., 2012). While no significant association was observed in Asian patients. Another analysis also identified the potential predictive value of circulating VEGF-A and VEGFR-2 in patients with metastatic breast cancer received anti-angiogenic treatment (Miles et al., 2013). And VEGF-A is being evaluated prospectively in metastatic breast cancer in the MERiDiAN trial. Serum placental growth factor (PIGF), another VEGF family member which expression levels are significantly higher in gastric cancer, also increases in response to anti-VEGF treatment (Jain et al., 2009). Soluble VEGFRs (sVEGFRs) are presented in serum as the result of alternative splicing or membrane shedding, which has a high affinity for VEGFA, and has been demonstrated to act as a naturally produced VEGF antagonist (Inoue et al., 2000). Both circulating levels of PIGF and sVEGFR were being explored as predictive biomarkers of response to anti-angiogenic drugs.

Tissue-based VEGFs have so far not been shown to be promising biomarkers for anti-angiogenic treatments. There are limitations including invasiveness and the difficulty in standardizing immunohistochemical analysis. For instance, the results obtained from tumor samples provided as slides and blocks were different. Compared with blocks, loss of immunoreactivity more often happens to paraffin-embedded tumor tissue stored on slides. Moreover, the main pitfalls of using immunohistochemistry as a quantitative measure without the consensus of standardized tests include pre-analytic tissue processing and subjective scoring.

Imaging methods are emerging as potential pharmacodynamic biomarkers because of its noninvasiveness and reproducibility. Changes in dynamic MRI-based tissue vascular measures such as blood flow, blood volume, or permeability after anti-angiogenic treatments with bevacizumab or VEGFR TKIs have been shown to occur (Schmainda et al., 2014; Kichingereder et al., 2015). Previous studies classified hepatic tumors as hyper-vascular or hypo-vascular according to the degree of contrast enhancement during the arterial phase on CT images. Hepatic tumors, which with >50% of the lesions enhances more than the adjacent liver parenchyma, are classified as hyper-vascular tumors (Katyal et al., 2000). Theoretically, hyper-vascular tumors will highly benefit from anti-angiogenic treatments. In our case, the enhanced CT disclosed that >50% of the hepatic metastases were obviously enhanced with little central necrosis. The Hounsfield unit (HU) is a relative quantitative measurement of radio density used by radiologists in the interpretation of computed tomography (CT) images. We creatively used the average CT ratio (the density of the liver metastases/the density of abdominal aorta) during arterial phase on CT imaging to show the blood supply of liver metastasis. Before second-line treatment, the average CT ratio was 79/210. Encouragingly, after two cycles of second-line treatment with apatinib plus paclitaxel, the efficacy was evaluated as PR, and the

average CT ratio was 38/281 with an obviously decreasing of the degree of enhancement.

Some toxicities of anti-angiogenic drugs, including hypertension and proteinuria, can give indirect information about the outcome and might be prognostic factors. In previous studies, the rate of hypertension was consistently higher in patients treated with anti-angiogenic drugs. Anti-angiogenic drugs can reduce the production or biological activity of VEGF which is associated with decreased production of nitric oxide, causing vasoconstriction and indirectly leading to an immediate increase in blood pressure (Robinson et al., 2010). Early report suggested that the grade of hypertension might be related to the dose (Rugo et al., 2005). This point was confirmed in a meta-analysis which include seven randomized controlled trials (RCTs) (Zhu et al., 2007). Compared with low dose, high dose bevacizumab was associated with a significant increased risk of hypertension (relative risk, 7.5 versus 3.0). Whether the toxicity of hypertension correlates with better survival is controversial. Several small-scale clinical trials indicated that hypertension might be an effective biomarker and associated with a favorable OS (Khoja et al., 2014; Zhong et al., 2015). Treatment with anti-angiogenic drugs can also lead to proteinuria. A meta-analysis revealed that the summary rate of all-grade proteinuria was 13.3% among patients who were administered bevacizumab (Wu et al., 2010). Other VEGF-signaling inhibitors including apatinib and sunitinib have also been associated with proteinuria. Dose intensity was related to the incidence and the severity of proteinuria (Wu et al., 2010). Several studies attempted to explore the relationship between the incidence of proteinuria and clinical outcome but with inconsistent results. A retrospective analysis showed that proteinuria portends poorer survival in patients with metastatic colorectal cancer treated with anti-angiogenic drugs (Khoja et al., 2014). Another retrospective case series of 140 patients with recurrent glioblastoma reported converse outcome in which hypertension and proteinuria are associated with longer disease control (Carvalho et al., 2020). Our case developed hypertension, mild proteinuria, and renal insufficiency after treated with apatinib. All above side effects were well controlled with appropriate treatment. We reduced the dose of apatinib to 250 mg/d and regularly administrated blood pressure medication. After that, the patient's blood pressure was well controlled, the degree of proteinuria ranged from negative to 1+, and the renal function kept normal. Up to now (29 January 2022), this patient has maintained continuous PR.

After reviewing the previous published literatures about apatinib as second-line or above therapy in patients with advanced or metastatic gastric cancer, we are sure that the present case in our report realized the longest PFS. We believe that the encouraging outcome of our case is not accident. The typically hyper-vascular hepatic metastases, treatment toxicities of hyertension and proteimuria in this patient may be potential predictive biomarkers for anti-angiogenic tretment. In second-line therapy, apatinib combination with chemotherapy might be an alternative treatment for some selected advanced or metastatic gastric adenocarcinoma. Further well-designed prospective clinical studies are necessary to explore the efficacy of apatinib alone or combined with chemotherapy as a second-line treatment and the predictive biomarkers of apatinib in advanced or metastatic gastric cancer.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Sichuan University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SF, LL, and XL wrote the main manuscript. SF and LL revised the main manuscript according to the comments of reviews. DC made contribution to conception and design. QW, XW, YH, and HH performed the data collection. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors thank the patient for his agreement to the publication of the report.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Oncology

TYPE Review
PUBLISHED 17 August 2022
DOI 10.3389/fonc.2022.957068

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

RECEIVED 01 June 2022
ACCEPTED 28 July 2022
PUBLISHED 17 August 2022

CITATION
Lauricella E, Mandriani B, Cavallo F, Pezzicoli G, Chaoul N, Porta C and Cives M (2022) Angiogenesis in NENs, with a focus on gastroenteropancreatic NENs: from biology to current and future therapeutic implications. *Front. Oncol.* 12:957068. doi: 10.3389/fonc.2022.957068

Angiogenesis in NENs, with a focus on gastroenteropancreatic NENs: from biology to current and future therapeutic implications

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Neuroendocrine neoplasms (NENs) are highly vascularized malignancies arising from cells of the diffuse neuroendocrine system. An intricated cross-talk exists between NEN cells and the tumor microenvironment, and three main molecular circuits (VEGF/VEGFR pathway, FGF-dependent signaling and PDGF/PDGFR axis) have been shown to regulate angiogenesis in these neoplasms. Multiple randomized trials have investigated antiangiogenic agents over the past two decades, and sunitinib is currently approved for the treatment of advanced, progressive, G1/G2 pancreatic NENs. In recent years, two phase III clinical trials have demonstrated the efficacy and safety of surufatinib, a multi-tyrosine kinase angioimmune inhibitor, in patients with well-differentiated pancreatic and extrapancreatic NENs, and two studies of this agent are currently underway in Europe and US. The HIF-2α inhibitor belzutifan has recently received regulatory approval for the treatment of tumors arising in the context of Von-Hippel Lindau syndrome including pancreatic NENs, and a study of this drug in patients with sporadic tumors is presently ongoing. Combinations of antiangiogenic agents with chemotherapeutics and targeted drugs have been tested, with accumulating toxicities being a matter of concern. The potential of antiangiogenic agents in fine-tuning the immune microenvironment of NENs to enhance the activity of immune checkpoint inhibitors has been only partially elucidated, and further research should be carried out at this regard. Here, we review the current understanding of the biology of angiogenesis in NENs and provide a summary of the latest clinical investigations on antiangiogenic drugs in this malignancy.

KEYWORDS

cabozantinib, lenvatinib, pazopanib, carcinoid tumor, TKIs (tyrosine kinase inhibitors)

Introduction

Neuroendocrine neoplasms (NENs) are heterogeneous malignancies arising from cells of the diffuse neuroendocrine system. They are often characterized by an indolent behavior and the ability to secrete a variety of peptide hormones and biogenic amines (1). The incidence of NENs has steadily increased in the last four decades, and NENs currently constitute the second most prevalent cancer of the gastroenteropancreatic (GEP) tract (2). According to the 2019 WHO classification (3), GEP-NENs can be subdivided in well-differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs). Neuroendocrine tumors can be further subdivided in low-grade (G1), intermediate-grade (G2) and high-grade (G3) tumors according to their proliferative activity, and large series have proven the prognostic relevance of such a classification (4, 5).

Neuroendocrine tumors are highly vascularized malignancies, and their intratumor vessel density is estimated to be approximately 10-fold higher than in carcinomas (6, 7). This feature is not particularly surprising, as it recapitulates the microscopic architecture of normal endocrine glands which are characterized by a dense vascular network facilitating hormone secretion. In this context, evidence demonstrates that the aberrant activation of the hypoxia-inducible factor-1 (HIF-1) transcriptional program is a frequent event in NETs, driving the production of large amounts of proangiogenic molecules such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), semaphorins and angiopoietins (8).

Clinical strategies encompassing angiogenesis inhibition have a definite place in the therapeutic armamentarium against NETs. The oral tyrosine kinase inhibitor (TKI) sunitinib is currently approved for pancreatic NETs (panNETs) (9), and a variety of new antiangiogenic agents are presently under clinical scrutiny for both GEP and bronchopulmonary (BP) NETs (10). In this review, we provide an overview of the current understanding of the molecular events driving neoangiogenesis in NENs, also discussing present and future therapeutic applications of antiangiogenic agents in the clinical arena.

Angiogenesis in NETs

Tumor angiogenesis is a complex process through which a neoplasm creates its own vascularization, essential for obtaining the oxygen and nutrients necessary to grow beyond a certain, and well defined, volume. Moreover, this vascularization provides an access to the bloodstream that the tumor uses to metastasize. This is true for almost all malignancies, including NETs (7). Angiogenesis is tightly regulated by a complex balance between pro- and anti-angiogenic molecules, and a cross-talk exists between endothelial cells, pericytes and tumor cells. Indeed, while anti-apoptotic factors supporting the tumor growth are released by activated endothelial cells of the newly formed vessels, pro-angiogenic molecules are produced in turn by tumor cells, thereby sustaining the so called "angiogenic switch" and engaging neovascularization (11). In this context, pericytes can stimulate an autocrine VEGF-mediated prosurvival signaling in endothelial cells, further promoting neovascular sprouting and, indirectly, tumor growth (12). Influenced by the same family of molecular cues driving angiogenesis, tumor lymphangiogenesis has also a key role in metastasis formation, and possibly resistance to antiangiogenic therapy. In this context, VEGFs other than VEGF-A have been described to mediate the outgrowth of lymphatic vessels in NETs thereby leading to progression to stages of greater malignancy (13–15).

The vascular alterations observed in NETs are both quantitative and qualitative. Extensive neovascularization in the presence of low endothelial proliferation is indeed a hallmark of well-differentiated NETs, while a lower intratumor microvascular density is typically observed in poorly differentiated carcinomas (8, 16). Such a phenomenon, named as "neuroendocrine paradox", is possibly related to the capability of well-differentiated NET cells to retain their normal precursors' ability to stimulate the formation of a dense vascular network, with the angiogenesis of poorly differentiated neoplasms being instead primarily dependent on proliferation-induced hypoxia. The newly formed blood vessels are structurally and functionally aberrant in NETs (7). In particular, endothelial cells appear to contain multiple fenestrations (which are also typical of normal endocrine glands) and trans-endothelial channels while basement membranes are discontinuous and lack pericyte coverage, thus resulting in increased interstitial fluid pressure, vessel tortuosity and leakiness, as well as frequent hemorrhage.

As depicted in Figure 1, three main molecular circuits regulate angiogenesis in NETs: the VEGF/VEGFR pathway, the FGF-dependent signaling and the PDGF/PDGFR axis (17). Vascular endothelial growth factor is constitutively expressed by normal neuroendocrine cells. Its expression is retained in up to 80% of GEP-NETs, where it drives angiogenesis through interaction with VEGFR-1 and VEGFR-2. The expression of VEGF is higher in well-differentiated malignancies with respect to poorly differentiated NENs, and parallels the expression of its receptors on both tumor and endothelial cells (8, 18–20). Tumor and stromal cells are not the only sources of VEGF in NETs, as tumor-infiltrating neutrophils have been shown to mobilize latent VEGF from the extracellular matrix through the release of metalloproteinase 9 (MMP-9), at least in mice (21). Mechanistically, VEGF acts in an autocrine or paracrine fashion triggering both vascular endothelial mitogenesis and

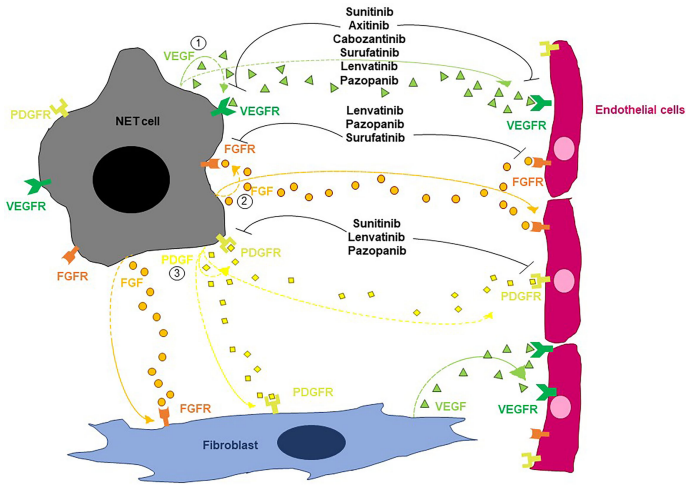

FIGURE 1
Schematic overview of the main pathways regulating angiogenesis in NETs. By acting on endothelial cells, VEGF stimulates both vascular endothelial mitogenesis and permeability. FGFs trigger endothelial cell migration, proliferation and differentiation as well as vessel formation. PDGF contributes to the angiogenic process by stimulating the recruitment of pericytes and the resulting vessel coverage. Multiple TKIs can interfere with the angiogenic process in NETs.

permeability *via* activation of the Notch signaling in endothelial cells (18, 20, 22, 23). Evidence from the RIP1-Tag2 transgenic mouse model demonstrates that VEGF exerts a critical role throughout the whole course of the multistage process of pancreatic endocrine tumorigenesis (19). In particular, the selective knockout of *VEGF* in β cells of RIP1-Tag2 mice dampens both angiogenic switch and neovasculature formation in dysplastic islets, thus preventing the growth of panNETs (24). As in other cancers, the production of VEGF by NET cells is primarily regulated by local oxygen availability through the sensing activity of HIF-1 (23, 25). In this context, evidence demonstrates that panNETs arising in patients with Von-Hippel Lindau disease, a condition characterized by uncoupled oxygen levels/HIF-1 activity, show a distinct proangiogenic molecular signature when compared with sporadic panNETs, thus suggesting that different evolutionary trajectories are followed by these two entities (26).

A second angiogenic pathway modulating the progression of NETs involves FGF and its cognate receptors. The family of FGF is known to comprise 23 members (although there are only 18 FGFR ligands) and exerts multiple functions through the activation of FGFRs (27). This pathway has both direct and indirect effects on angiogenesis. Indeed, while directly stimulating endothelial cell migration, proliferation and differentiation as well as vessel formation and maturation, FGF also acts as a key regulator of proangiogenic molecules including VEGF and angiopoietins (28, 29). Fibroblast growth factor-1 and fibroblast growth factor-2 are expressed in approximately 40% and 100% of GEP-NENs respectively, while fibroblast growth factor receptor (FGFR) 1-4 are expressed by the 68-88% of these malignancies (30, 31). Fibroblast growth factor has a key role in maintaining tumor angiogenesis after an initiation phase primarily guided by the VEGF signaling, and the inhibition of the FGF/FGFR axis suppresses neoangiogenesis and tumor growth in the RIP1-Tag2 transgenic mouse model (32). Evidence demonstrates that FGF is a critical driver of VEGF-independent revascularization of panNETs and can therefore mediate evasive resistance to antioangiogenic therapy (33, 34).

The PDGF/PDGFR axis is another crucial mediator of NET progression. Platelet-derived growth factor contributes to the angiogenic process by stimulating the recruitment of pericytes and the resulting vessel coverage (35). Expression of PDGFR-α and PDGFR-β has been described in approximately 75% and 60% of GEP-NETs respectively (36, 37). In particular, while PDGFR-α is predominantly expressed by tumor cells, PDGFR-β is mainly expressed by pericytes and stromal cells. A positive association between PDGFR-α expression and tumor grade as well as between PDGFR-β expression and tumor microvascular density has been documented (36, 37), and the paracrine secretion of PDGF-DD by endothelial cells has been shown to stimulate NET proliferation (38). In this context, experiments in the RIP1-Tag2-PDGFD knockout model demonstrate that the disruption of the PDGF-DD signaling significantly delays panNET growth (7).

Mounting evidence indicates that semaphorins and angiopoietins contribute to neoangiogenesis in NETs. Semaphorins have shown both pro- and anti-angiogenic effects in NETs, and their activity is the result of the interaction with neuropilin and plexin receptors (7). Neuropilin receptors have been found in both pancreatic, intestinal and pulmonary NETs (39–41), while data on the expression of plexin receptors in NETs

are currently lacking. Experiments in RIP1-Tag2 mice have shown that the expression of semaphorin 3A (SEMA3A) is progressively lost during tumor progression and that the inhibition of SEMA3A during the angiogenic switch may enhance tumor formation. Of note, re-expression of SEMA3A by viral gene transfer during late stages of pancreatic endocrine tumorigenesis leads to normalization of the tumor vasculature, increased pericyte coverage and inhibition of tumor progression (42, 43). Similar antiangiogenic effects have been also documented for SEMA3F in ileal NETs (44). On the other hand, protumorigenic activities have been attributed to SEMA4D and SEMA5A. In particular, inhibition of SEMA4D has been recently associated with impaired tumor growth *via* pericyte coverage alteration and vascular function modification in RIP1-Tag2 mice (45). SEMA5A can elicit angiogenesis, tumor growth, invasion and metastasis by activating c-met downstream its interaction with Plexin-B3 (46). Angiopoietins and angiopoietins receptors are widely expressed in NETs (47, 48). The overexpression of Angiopoietin-2 (Ang-2) in orthothopic NET xenografts in nude mice drives increased microvascular density and enhanced metastatic spread through lymphatic vessels (49). The blockade of the interaction between Ang-2 and its cognate receptor TIE2 determines regression of the tumor vasculature and inhibition of tumor progression in the RIP1-Tag2 mouse model of pancreatic endocrine cancerogenesis (50).

Molecular mechanisms of resistance to antiangiogenic therapies in NETs

Inhibition of angiogenesis has revealed therapeutic efficacy in NET patients. Nevertheless, resistance to antiangiogenic therapies inevitably occurs, and the biological events leading to such a phenomenon have been only partly elucidated. While primary resistance refers to an intrinsic unresponsiveness to antiangiogenic treatments, secondary (or acquired) resistance stems from tumor adaption to therapy, mostly as result of the activation of alternative proangiogenic circuits (51). Figure 2 depicts the main biological events driving resistance to antiangiogenic therapies in NETs.

An established cause of resistance to antiangiogenic agents primarily acting through VEGF suppression is tumor hypoxia. Tumor hypoxia can stimulate HIF-1 activation, thus triggering neoangiogenesis through VEGF-independent mechanisms involving FGF, angiopoietins, and ephrins (33, 52). Evidence from the RIP1-Tag2 model demonstrates that inhibition of both VEGF and FGF signaling at the time of VEGF-independent tumor revascularization attenuates both revascularization and tumor growth (33). In this context, brivanib, a first-in-class, dual FGF-VEGF inhibitor has shown superior preclinical antitumor activity against panNETs when compared with single VEGF suppression or single FGF inhibition (34). A marked upregulation of Ang-2 and TIE2 has been observed in tumors from late-stage RIP1-Tag2 mice resistant to VEGFR blockade (53). In this context, dual Ang-2/VEGFR inhibition was shown to suppress tumor revascularization and progression, suggesting that the adaptive enforcement of Ang2-TIE2 signaling plays a key role in the establishment of evasive tumor resistance to anti-VEGF therapy. The upregulation of c-Met is another consequence of the chronic HIF-1 activation induced by tumor hypoxia. In RIP1-Tag2 mice, VEGFR blockade results in c-Met overexpression, leading to increased tumor growth, proliferation and invasion (54). Concurrent inhibition of VEGF and c-Met signaling is able to revert such effects *in vivo* (55).

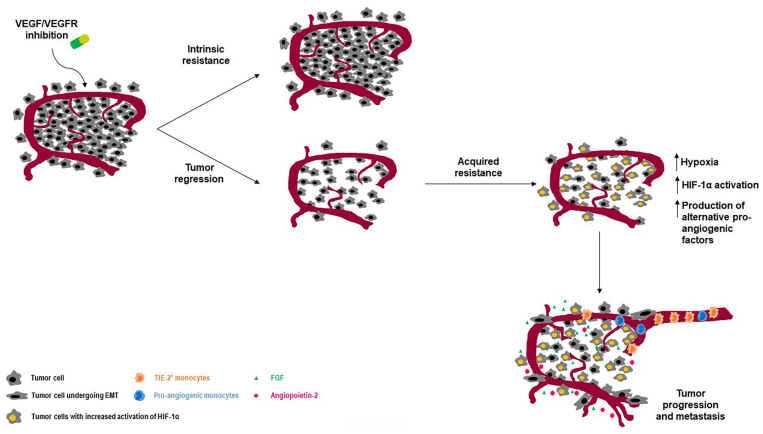

FIGURE 2
Intrinsic and acquired resistance to angiogenesis blockade: an overview on molecular determinants. While some tumors may show primary resistance to antiangiogenic agents, other may develop resistance upon blockade of the VEGF/VEGFR pathway. One of the main mechanisms leading to secondary resistance is the activation of HIF-1 as result of antiangiogenesis-induced hypoxia. Among other important events, there are the recruitment and/or activation of pro-angiogenic cells including TIE-2 expressing macrophages and the activation of the epithelial-to-mesenchymal transcriptional program in NEN cells.

The recruitment of bone marrow derived cells such as endothelial progenitor cells, pro-angiogenic monocytic cells or TIE2-expressing macrophages has been described in panNETs as a result of the hypoxic environment generated by VEGFR inhibition (13, 53). These cells promote the sprouting of new vessels, maintaining the high-demanded blood supply of tumor cells, while concurring to the generation of new premetastatic niches. A progressive increase in the number of tumor-associated macrophages has been described during the sequential progression from hyperplastic islets to angiogenic islets and ultimately invasive tumors in the RIP1-Tag2 model (56). A possible involvement of these cells in the establishment of adaptive resistance to VEGFR blockade through the exploitation of alternative proangiogenic pathways has been inferred.

An increased pericyte coverage has been detected in murine panNETs resistant to VEGFR2 inhibition as compared to those responsive to antiangiogenic therapy (57). Such a phenomenon has been related to a non-angiogenic mechanism of tumor vascularization named vascular co-option. When vascular co-option is activated, cancer cells grow around normal vessels pre-existing in the adjacent "normal" tissue, without the need of generating new vessels. Evidence demonstrates that this process contributes to the emergence of resistance to VEGFR inhibition in the RIP1-Tag2 model (33). Another non-angiogenic mechanism driving resistance to antiangiogenic therapy in panNETs is named "vascular mimicry", and consists of cancer cells forming vascular channels to autonomously sustain their growth (58). An increased expression of Snail, vimentin and N-cadherin as well as a concurrent downregulation of E-cadherin has been observed in tumors treated with sunitinib, and the hypoxia-driven epithelial-to-mesenchymal transition can be therefore listed as an additional mechanism of resistance to angiogenesis blockade and tumor aggressiveness (43, 59).

Ion trapping and degradation of hydrophobic TKIs within the acidic lysosomal compartment is another mechanism leading to resistance to antiangiogenic therapy. Chloroquine, an agent able to permeabilize the lysosomal membrane, has been shown to enhance the antitumor activity of sunitinib in murine models of pancreatic endocrine carcinogenesis by stimulating the release of the TKI in the cytoplasm (60).

Targeting angiogenesis in NETs: established and investigational agents

Proangiogenic pathways can be blocked at different levels in NETs. Both direct suppression of proangiogenic molecules such as VEGF and inhibition of receptors tyrosine kinase including VEGFR and FGFR through TKIs (i.e., sunitinib) or mAb (i.e., ramucirumab) have been exploited in clinical trials. Table 1 provides an overview of the clinical investigations of antiangiogenic agents in patients with NETs. Being the targets of TKIs usually multiple, it is currently difficult to precisely determine to what extent their therapeutic effects are related to anti-angiogenesis, antiproliferative activity against tumor cells *per se*, or interference in the mechanisms of cross-talk between tumor cells and their microenvironment.

VEGF/VEGFR-targeting agents

Bevacizumab is a mAb against VEGF and preliminary evidence suggested the antitumor activity of the drug in patients with NETs (69, 72, 73). However, no benefit in PFS was recorded in a randomized phase 3 trial comparing bevacizumab plus octreotide versus interferon plus octreotide in 427 patients who had high-risk NETs (74). In recent years, bevacizumab has been investigated in combination with chemotherapy, targeted agents or immunotherapy. In 2 separate phase 2 studies, bevacizumab has been tested in association with the capecitabine-oxaliplatin or FOLFOX regimens (75) in 40 and 36 patients with progressive NETs or NECs. Neither study met its primary endpoint, leading to objective responses in 18% and 25% of patients respectively. Another phase 2 trial investigated bevacizumab with 5-FU and streptozocin in 34 patients with progressive, well-differentiated panNETs. A median PFS of 23.7 months was observed, in the presence of an ORR of 56% (76). In a phase 2 trial, 150 patients with advanced panNETs were randomized to receive the mTOR inhibitor everolimus plus octreotide with or without bevacizumab (70). Of note, preclinical evidence suggests that the antitumor activity of mTOR inhibition in NETs results from a combination of antiproliferative and antiangiogenic effects (77). The treatment arm containing the antiangiogenic agent resulted in improved PFS compared to the control arm (16.7 months compared to 14 months; hazard ratio, 0.8; p=0.10), with objective responses seen in 31% and 12% of patients treated with or without bevacizumab respectively (p=0.005). Despite the encouraging efficacy outcomes, the higher rate of treatment-related toxicities observed in the investigational arm may limit further investigations on this combination. Similar results were achieved in a phase II study evaluating the combination of bevacizumab and sorafenib in 44 patients with advanced NETs (78). Despite a median PFS of 12.4 months, grade 3/4 toxicities were described in the 63% of the enrolled patients. Based on the evidence that anti-angiogenic agents such as bevacizumab may modulate the tumor immune microenvironment and decrease the expression of regulatory checkpoints on tumor-infiltrating lymphocytes (79), a single-arm, open-label, nonrandomized study has recently evaluated the association of bevacizumab with the anti-PD-L1 Ab atezolizumab in patients with advanced, progressive, well-differentiated NETs (71). Overall, 20 patients with panNETs and 20 patients with extra-pancreatic NETs have

TABLE 1 An overview of completed studies with antiangiogenic agents in patients with NENs.

Study agent (s)	Main molecular targets	Study design and phase	Patient population	Enrolled patients	mPFS of the investigational agent/ combination	Grade 3/4 AEs (frequency)
Sunitinib (9)	VEGFR-1,-2,-3, PDGFR	Double-blind, placebo-controlled, randomized phase 3 study	G1/G2 advanced progressive panNENs	171	11.1 months	Neutropenia (12%) Hypertension (10%) Palmar-plantar erythrodysesthesia Ashtenia (5%) Diarrhea (5%)
Surufatinib (61, 62)	VEGFR-1,-2,-3 FGFR-1 CSF-1R	Double-blind placebo- controlled, randomized phase 3 studies	Well-differentiated advanced progressive pancreatic (SANET-p) and extra pancreatic (SANET-ep) NENs	SANET-p trial: 172 SANET-ep trial: 198	SANET-p trial: 10.9 months SANET-ep trial: 9.2 months	SANET-p trial: Hypertension (38%) Proteinuria (10%) Hypertrigliceridemia (7%) SANET-ep trial: Hypertension (36%) Proteinuria (19%) Anemia (7%)
Lenvatinib (63)	VEGFR-1,-2,-3 FGFR-1,-2,-3,-4 PDGFRα c-KIT RET	Open-label phase 2 study	Advanced progressive panNENs and gastrointestinal NENs	PanNENs: 55 Gastrointestinal NENs: 56	panNENs: 15 months Gastrointestinal NENs: 15 months	Hypertension (22%) Fatigue (11%) Diarrhea (11%)
Axitinib (64)	VEGFR-1,-2,-3	Open-label phase 2 study	G1/G2 advanced progressive extrapancreatic NENs	30	27 months	Hypertension (63%)
Axitinib + Octreotide LAR (65)	VEGFR-1,-2,-3	Double-blind randomized phase 2/3 study	G1/G2 advanced progressive extrapancreatic NENs	256	17.2 months	Hypertension (21%) Cardiac disorders (3%) Fatigue (9%) Diarrhea (13%) Nausea (2%)
Cabozantinib (66)	MET VEGFR2 c-KIT RET AXL TIE2 FLT3	Open-label phase 2 study	Well-differentiated advanced progressive pancreatic and extrapancreatic NENs	PanNENs: 20 ExtrapancreaticNENs: 41	PanNENs: 21.8 months Extrapancreatic NENs: 31.4 months	Hypertension (13%) Hypophosphatemia (10%) Diarrhea (10%) Fatigue (5%)
Pazopanib (67)	VEGFR-1,-2,-3 FGFR-1,-3,-4 PDGFR-α, -β c-KIT	Randomized, placebo-controlled phase 2 study	Well-differentiated advanced progressive extrapancreatic NENs	171	12 months	Diarrhea (5%) Fatigue (8%) Nausea (5%) Hypertension (27%) Transaminase elevation (18%)
Belzutifan (68)	HIF-2α	Open-label phase 2 study	Advanced panNENs arising in the context of VHL syndrome	22	–	Anemia (8%)* Hypertension (8%)* Fatigue (5%)*
Evofosfasmide + Sunitinib (69)	DNA cross links VEGFR-1,-2,-3, PDGFR	Open-label, phase 2 study	Advanced progressive panNENs	17	10.4 months	Neutropenia (35%) Fatigue (18%) Thrombocytopenia (12%)
Everolimus ± Bevacizumab (70)	mTOR VEGF	Randomized phase 2 study	G1/G2 advanced progressive panNENs	150	16.7 months	Diarrhea (14%) Hyponatremia (12%) Hypophosphatemia (11%) Proteinuria (16%) Hypertension (41%)

(Continued)

TABLE 1 Continued

Study agent (s)	Main molecular targets	Study design and phase	Patient population	Enrolled patients	mPFS of the investigational agent/ combination	Grade 3/4 AEs (frequency)
Atezolizumab + Bevacizumab (71)	PD-L1 VEGF	Open-label, phase 2 study	G1/G2 advanced progressive pancreatic and extrapancreatic NENs	PanNENs: 20 Extrapancreatic NENs: 20	PanNENs: 14.9 months Extrapancreatic NENs: 14.2 months	Hypertension (20%) Proteinuria (8%)

*In the safety cohort (n=61 patients).

been enrolled, and an ORR of 20% and 15% has been recorded in the two cohorts. A median PFS of 14.9 months and 14.2 months has been reported in the pancreatic and extra-pancreatic cohort, suggesting the potential efficacy of this combination. Hypertension and proteinuria have been described as the most common treatment-emergent toxicities. Bevacizumab is currently being investigated in combination with chemotherapy or chemo-immunotherapy in multiple trials of patients with extrapulmonary NECs (80).

Ramucirumab, a humanized mAb targeting VEGFR2, has demonstrated preliminary evidence of efficacy when used in combination with chemotherapy in patients with gastric NEC (81). A prospective, multicenter, single-arm study is currently investigating ramucirumab plus dacarbazine in patients with advanced, progressive, well-differentiated panNETs (82).

The VEGF trap aflibercept has been investigated in a phase II, single-arm trial of 21 patients with advanced panNETs (83). An ORR of 9% has been reported, a finding consistent with other antiangiogenic agents in panNETs.

Sunitinib

Sunitinib is the only antiangiogenic agent currently approved for the treatment of NETs. Sunitinib is an oral TKI targeting, among a number of different kinases, VEGFR-1, -2, -3 and PDFGR, and has demonstrated efficacy in the treatment of advanced, progressive panNETs. A double-blind, placebo-controlled, phase 3 study evaluated sunitinib 37.5 mg daily in 171 patients with low-to-intermediate grade, progressive panNETs (9). The trial demonstrated a statistically significant improvement in median PFS from 5.5 months on the placebo arm to 11.1 months on the sunitinib arm (hazard ratio, 0.42; $p<0.001$). A nonsignificant overall survival (OS) improvement of approximately 10 months was observed in the sunitinib arm compared with the placebo arm (84). Nausea, diarrhea, fatigue, cytopenia, hypertension and palmar-plantar erythrodydesthesia were the main treatment-related toxicities. A similar toxicity profile has been described in a recent phase IV study (85).

Other multikinase inhibitors

Dual inhibitors of the VEGF/FGF signaling carry the promise of overcoming the mechanisms leading to adaptive resistance to sunitinib, and recent clinical research has focused on agents including surufatinib, lenvatinib, axitinib, cabozantinib and pazopanib. Surufatinib is an oral, selective inhibitor of VEGFR-1, -2, -3, FGFR-1 and colony stimulating factor-1 receptor (CSF-1R). The TKI has been tested at a dosage of 300 mg daily in a single-arm, multicenter, phase 1b/2 trial of 81 patients with low-to-intermediate grade advanced NETs (86). A median PFS of 21.2 months and 13.4 months was reported in 42 patients with panNETs and 39 patients with extrapancreatic NETs respectively. Two randomized, double-blind, placebo-controlled, phase 3 studies have recently investigated the safety and efficacy of surufatinib in Chinese patients with well-differentiated, progressive, advanced pancreatic (SANET-p trial) and extrapancreatic NETs (SANET-ep trial). The SANET-p trial (61) randomized 172 patients with panNETs to receive surufatinib or placebo in a 2:1 ratio. The investigator-assessed median PFS was 10.9 months for surufatinib versus 3.7 months for placebo (hazard ratio: 0.49; $p=0.001$), with an investigator-assessed overall response rate (ORR) of 19% in the investigational arm. The SANET-ep trial (62) randomized 198 patients with extrapancreatic NETs to receive surufatinib or placebo in a 2:1 ratio. The investigator-assessed median PFS was 9.2 and 3.8 months in the surufatinib and placebo arms respectively (hazard ratio: 0.33; $p<0.0001$). The ORR was 15%, and the majority of enrolled patients (84%) had G2 tumors. Overall, hypertension, proteinuria, hypertriglyceridemia and diarrhea were reported as the most frequent treatment-related grade 3 or worse adverse events. The occurrence of treatment-related adverse events including hypertension, proteinuria and hemorrhage in the first 4 weeks of treatment has been recently described to predict the antitumor efficacy of surufatinib (87). The efficacy and safety of surufatinib are being currently evaluated in two ongoing trials in the US (NCT02549937) and Europe (NCT04579679), and their results might lead to the approval of the drug in Western countries. It remains currently

unclear whether surufatinib may be active in patients progressing to prior antiangiogenic therapy, and current investigations exclude from enrollment patients who have received prior VEGF/VEGFR targeted therapy.

Lenvatinib is an oral TKI targeting VEGFR-1, -2, -3, FGFR-1, -2, -3, -4, platelet-derived growth factor receptor α (PDGFRα), KIT and RET. The drug has been recently investigated in the phase 2 TALENT study at a dosage of 24 mg daily (63). A total of 55 patients with advanced panNETs and 56 patients with advanced gastrointestinal NETs have been enrolled. All patients had progressive disease according to RECIST criteria, and prior therapy with targeted agents was mandatory for inclusion in the panNET cohort. By central radiology review, the ORR was 44% and 16% for panNETs and gastrointestinal NETs respectively, and the median duration of response was 20 and 34 months in the two cohorts respectively. After a median follow-up of 23 months, the median PFS was 15 months for either panNETs and gastrointestinal NETs. Hypertension, fatigue and diarrhea were the most frequent G3/4 treatment-emergent adverse events. Dose reductions or interruptions were required in the 94% of patients. Although the ORR observed in the TALENT study is the highest reported to date with a TKI in advanced NETs, further clinical investigations of this agent in NETs have not been planned so far.

Axitinib is a TKI that selectively targets VEGFR-1, -2 and -3. In an open-label phase 2 study, axitinib 5 mg twice daily was investigated in 30 patients with progressive, advanced, low-to-intermediate grade NETs of extra-pancreatic origin (64). After a median follow-up of 29 months, a median PFS of 27 months was observed. Grade 3/4 hypertension was recorded in the 63% of the cohort, leading to treatment discontinuation in one fifth of enrolled patients. The double-blind, phase 2/3 AXINET trial has recently randomized 256 patients with advanced, low-to-intermediate grade, progressive, extra-pancreatic NETs to receive axitinib plus octreotide LAR or placebo plus octreotide LAR (65). Per blinded independent central review, the median PFS was 16.6 and 9.9 months in the axitinib and placebo arms respectively (HR: 0.69; p=0.01). An ORR of 13% and 3% has been reported in the investigational and control group respectively (p=0.004). Grade 3 or worse adverse events occurred in the 52% of the enrolled patients and included hypertension, cardiac disorders, fatigue, diarrhea and nausea/vomiting. One treatment-emergent death was reported in the axitinib arm. Overall, axitinib appears a promising candidate for future regulatory approval in patients with NETs.

Cabozantinib is an oral, potent inhibitor of MET, VEGFR2, KIT, RET, AXL, TIE2 and FLT3. The TKI has been tested at 60 mg daily in a two-cohort, phase 2 study enrolling 20 patients with panNETs and 41 patients with extra-pancreatic NETs (66). All patients had well-differentiated tumors and progressive disease according to RECIST 1.1 criteria. The ORR was 15% in either cohort, while a median PFS of 21.8 and 31.4 months was recorded in patients with pancreatic and extra-pancreatic neoplasms respectively. Hypertension, hypophosphatemia, diarrhea and fatigue were among the most common grade 3/4 adverse events. Dose reductions were required in the 80% of patients. The phase 3 CABINET trial (NCT03375320) is currently randomizing patients with well-differentiated, advanced, progressive, pancreatic or extra-pancreatic NET to receive cabozantinib 60 mg daily or placebo. Combinations of cabozantinib plus temozolomide (NCT04893785), lanreotide (NCT04427787) or ^{177}Lu-DOTATATE (NCT05249114) are presently under scrutiny in phase 2 studies.

Pazopanib is an oral TKI targeting VEGFR -1, -2, -3, FGFR-1, -3, -4, PDGFR-α and -β and c-KIT. The drug has been investigated in the open-label, phase 2 PAZONET trial (88). In 44 patients with advanced, progressive, well-differentiated NETs, the TKI was associated with a median PFS of 9 months. The most common grade 3/4 toxicities of pazopanib included diarrhea, fatigue and hypertension, and drug dosage reductions were required in approximately one fifth of enrolled patients. More recently, pazopanib has been tested at 800 mg daily in the phase 2 Alliance A021202 study (67). The trial randomized 171 patients with well-differentiated, progressive, extrapancreatic NETs to receive pazopanib or placebo. After a median follow-up of 31 months, a median PFS of 12 and 8 months was recorded in patients treated with pazopanib or placebo respectively (HR: 0.53; p=0.0005). Pazopanib was associated with an ORR of only 2%. Among pazopanib-treated patients, treatment-related grade 3/4 adverse events were reported in 61% of cases, and hypertension, fatigue, nausea, diarrhea and transaminases elevation were the most common toxicities. Pazopanib has also demonstrated clinical activity against panNETs arising in the context of von Hippel-Lindau syndrome. In a single-arm study enrolling 31 patients with this inherited syndrome, the drug induced objective responses in 53% of 17 pancreatic lesions (89).

Nintedanib is an oral inhibitor of FGFR1-3, VEGFR1-3 and PDGFR. The drug has been tested in a phase II study of 32 patients with extra-pancreatic NETs on a stable dose of somatostatin analog (90). A median PFS of 11 months has been observed, and toxicities were manageable.

HIF inhibitors and hypoxia-activated prodrugs

Novel antiangiogenic compounds investigated in patients with NETs comprise the HIF-2α inhibitor belzutifan and the hypoxia-activated prodrug evofosfamide. Belzutifan has been recently tested at 120 mg daily in an open-label, phase 2 trial of

61 patients with von Hippel-Lindau syndrome. Among 22 patients harboring a panNET, objective responses were seen in 90% of cases, with complete responses in 14% of the cohort (68). Anemia and fatigue were the most common adverse events, being reported in 90% and 66% of patients respectively. On this basis, belzutifan has received regulatory approval for the treatment of tumors arising in the context of Von-Hippel Lindau syndrome. An international phase 2 study of belzutifan in patients with sporadic panNETs is currently ongoing (NCT04924075). Evofosfamide is a prodrug of the alkylating agent bromoisophosphoramide mustard. The release of the active drug occurs exclusively under hypoxic conditions, and results in intra- and inter-strand DNA cross links in tumor cells. Given the well-known ability of sunitinib in inducing intratumor hypoxia, evofosfamide has been recently investigated in combination with sunitinib in the open-label, Simon's two-stage design, phase II SUNEVO trial (69). The study enrolled 17 patients with advanced panNETs, and only prior therapy with somatostatin analogs was permitted. After a median follow-up of 16 months, three objective responses were recorded, in the presence of a median PFS of 10.4 months. Grade 3 or worse treatment-related adverse events were reported in the 65% of the cohort, the most frequent being neutropenia, fatigue and thrombocytopenia. Overall, treatment discontinuation due to toxicity was required in 88% of the patients. In light of the unfavorable safety profile and the modest efficacy shown by sunitinib and evofosfamide in this study, further clinical investigations of this combination have not been planned.

Future directions for angiogenesis blockade in NETs

Developing new antiangiogenic agents, testing new combinations of antiangiogenic agents with targeted drugs or immunotherapy and defining the correct positioning of antiangiogenic therapies in the context of treatment sequences are among the main priorities for future research on angiogenesis blockade in NETs.

Angiogenesis is a complex process involving distinct biological mechanisms. Mechanistically, endothelial cell proliferation, vessel guidance, vessel maturation, stabilization and quiescence are driven by different families of molecular cues, and angiogenic processes can be thereby inhibited at different levels. There is a need to identify and characterize additional molecular regulators of angiogenesis in NETs in order to develop the next generation of antiangiogenic drugs to be tested (alone or in combination) in clinical trials. Moreover, since evidence demonstrates that different angiogenic molecules may be expressed differently during tumor progression, a precise understanding of the molecular events driving neoangiogenesis during NET evolution might be instrumental to provide molecular-level guidance on the correct positioning of angiogenesis blockade throughout the treatment journey of NET patients. Combinatorial strategies aimed at concurrently disrupting key pathways operating in NET progression (i.e., concurrent inhibition of angiogenesis and mTOR signaling) have been only partially investigated. In a phase II study of bevacizumab and temsirolimus, an ORR of 41% and a median PFS of 13.2 months were observed among 58 patients with panNETs, in the presence of toxicities leading to treatment discontinuation in approximately one third of the cohort (91). While the efficacy/toxicity ratio of combinatorial treatments should be always carefully scrutinized in relatively indolent tumors such as NETs, clinical trials should explore the impact of targeted agent combinations in disease settings where tumor shrinkage is the goal of treatment (i.e., neoadjuvant setting). Accumulating evidence indicates that a tight link exists between aberrant tumor angiogenesis and the immune microenvironment, and antiangiogenic agents have been shown to synergize with immune checkpoint inhibitors in malignancies including renal cell carcinoma, endometrial cancer and hepatocellular carcinoma (92). Future studies should assess the potential of antiangiogenic agents in tuning the microenvironment of NETs from an immune-suppressive to an immune-supportive one, thus enhancing the efficacy of immunotherapy.

Conclusions

The concept of angiogenesis inhibition as a potential weapon against cancer was first proposed by Folkman in the 70s (93). After the initial skepticism of the scientific community, multiple lines of evidence have demonstrated that antiangiogenic agents can be clinically effective in controlling tumor growth. Sunitinib is the only antiangiogenic drug approved for the treatment of NETs, and its use is restricted to pancreatic primaries. Newer TKIs including surufatinib, cabozantinib, axitinib and lenvatinib seem to possess more potent antitumor activity, probably as result of their multi-targeting potential, and might be utilized as monotherapy or as backbone for combinatorial treatment of both pancreatic and extra-pancreatic NETs in the near future. While it is currently unclear whether combinations of antiangiogenic agents with chemotherapeutics, targeted agents or immunotherapy are more effective than the monotherapy, the results of the first study comparing the efficacy of sunitinib versus PRRT in patients with progressive panNETs are awaited soon (NCT02230176). In the absence of predictors of response and given the lack of high-level evidence on optimal treatment sequences, clinical wisdom continues to be critical in defining the timing of antiangiogenic therapy in patients with NETs.

Author contributions

All authors contributed to literature review and to the writing the manuscript. All authors approved the final version of the manuscript.

Funding

This work was supported by the Associazione Italiana per la Ricerca sul Cancro [MFAG #23583] and Associazione per la Ricerca Biomolecolare Onlus, Acquaviva, Italy [2020].

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Oncology

TYPE Review
PUBLISHED 23 September 2022
DOI 10.3389/fonc.2022.993573

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

RECEIVED 13 July 2022
ACCEPTED 23 August 2022
PUBLISHED 23 September 2022

CITATION
Salati M, Caputo F, Bocconi A, Cerri S, Baldessari C, Piacentini F, Dominici M and Gelsomino F (2022) Successes and failures of angiogenesis blockade in gastric and gastro-esophageal junction adenocarcinoma. *Front. Oncol.* 12:993573. doi: 10.3389/fonc.2022.993573

Successes and failures of angiogenesis blockade in gastric and gastro-esophageal junction adenocarcinoma

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Gastric and gastro-esophageal junction adenocarcinoma (GEA) remains a considerable major public health problem worldwide, being the fifth most common cancer with a fatality-to-case ratio that stands still at 70%. Angiogenesis, which is a well-established cancer hallmark, exerts a fundamental role in cancer initiation and progression and its targeting has been actively pursued as a promising therapeutic strategy in GEA. A wealth of clinical trials has been conducted, investigating anti-angiogenic agents including VEGF-directed monoclonal antibodies, small molecules tyrosine kinase inhibitors and VEGF-Trap agents both in the resectable and advanced setting, reporting controversial results. While phase III randomized trials testing the anti-VEGFR-2 antibody Ramucirumab and the selective VEGFR-2 tyrosine kinase inhibitor Apatinib demonstrated a significant survival benefit in later lines, the shift of angiogenesis inhibitors in the perioperative and first-line setting failed to improve patients' outcome in GEAs. The molecular landscape of disease, together with novel combinatorial strategies and biomarker-selected approaches are under investigation as key elements to the success of angiogenesis blockade in GEA. In this article, we critically review the existing literature on the biological rationale and clinical development of antiangiogenic agents in GEA, discussing major achievements, limitations and future developments, aiming at fully realizing the potential of this therapeutic approach.

KEYWORDS

gastric cancer, angiogenesis, targeted therapy, precision medicine, clinical trials, gastro-esophageal adenocarcinoma

Introduction

Gastric and gastroesophageal junction adenocarcinoma (GEA) remains a considerable health problem, ranking fourth as the leading cause of cancer-related death globally, with more than 700,000 deaths in 2020 (1). Therapeutic advances occurred over the recent years have led to incremental improvements in patient outcomes, and the advent of next-generation sequencing technologies have improved our biological understanding of GEA, unravelling profound molecular heterogeneity and potential vulnerabilities (2). For patients with resectable GEA, taxane-based triplet has outperformed anthracycline-based therapy as the reference regimen in the perioperative setting, leading to a 10%-increase in the curability rate (3). In the advanced disease setting, the growing number of novel effective drugs, including cytotoxics, targeted agents and checkpoint inhibitors and the improved patients and molecular selection are making the continuum of care a reality in GEA, with some patients experiencing an unexpected survival (4–10).

Among the most successfully exploited targets in GEA is angiogenesis with the VEGFR2-directed antibody ramucirumab being the second targeted agent to be approved, either alone or combined with paclitaxel, for advanced GEA failing prior platinum and fluoropyrimidine chemotherapy (11). Angiogenesis is a well-established cancer hallmark and angiogenesis-related factors, among which the VEGF/VEGFR axis is the most extensively studied, therapeutically validated and characterized, are overexpressed in many cancers, including GEA, and correlate with a poor prognosis (12–14). This has soon made angiogenesis an attractive target for therapeutic interventions and antiangiogenic therapy a component of standard treatment across a variety of cancer types. However, aside from the established role for ramucirumab in the second-line setting, the targeting of angiogenesis has provided controversial results in GEA. While early phase trials displayed promising antitumor activity, the near totality of them failed to prolong survival in randomized-controlled phase III trials in both resectable and advanced disease (15). Moreover, the lack of reliable predictive biomarkers has further hindered the optimization of angiogenesis blockade in GEA. Here, we extensively reviewed the biological rationale, the successes and failures from clinical trials as well as future developments for angiogenesis blockade in GEA.

The biological rationale for angiogenesis targeting in GEA

Angiogenesis enables the generation of a neovasculature from pre-existing blood vessels sustaining tumor progression and invasion since the early stages of cancer development (13). During this multistage process of invasive cancers indeed, malignant cells acquire the capability of activating an "angiogenic switch" through the unbalance in favor of pro-angiogenic factors. Compelling evidence showed that this "angiogenic switch" is regulated by stimulatory and inhibitory factors, which act through signaling proteins and transmembrane receptors on the surface of vascular endothelial cells. The most extensively characterized angiogenic regulator is the vascular endothelial growth factor (VEGF) together with its receptors (VEGFR) (14). The VEGF family consists of six protein isoforms called VEGF-A, -B, -C, -D, -E, -F, and placental growth factors -1 and -2 (PIGF). VEGF binds to transmembrane tyrosine kinase receptors VEGFR in three members VEGFR-1 and VEGFR-2 that present high affinity for VEGF-A, and VEGFR-3, specific for VEGF-C e VEGF-D (16). Upon VEGF binding to the extracellular domain of VEGFR, the receptor undergoes a dimerization process, phosphorylation of the tyrosine kinase domain, and subsequent activation of an intracellular pathway that in turn results in changes in cell morphology, cytoskeleton alteration, and stimulation of endothelial cell survival, proliferation and migration (13). While the role of VEGFR-1 in the angiogenesis process remains unclear, with some studies suggesting it as a negative regulator of VEGF activity and others showing an active role in the recruitment of endothelial cell progenitors, the VEGFR-2 represent the main actor in this process (14). Indeed, thanks to the binding with the ligand VEGF-A, VEGFR-2 mediates proliferation, invasion, migration and survival of endothelial cells via the MAP-kinase signalling pathway (17, 18). Instead, the VEGFR-3, which is expressed only in lymphatic endothelial cells in adults, preferentially binds VEGF-C and VEGF-D and its activation and up-regulation enhance tumor lymphangiogenesis and lymph node metastasis (19). In addition to causing the sprouting of new vessels, the VEGF/VEGFR axis induces increased vascular permeability, which results in the leakage of plasma protein, fibrin deposition and ultimately in maintaining the proangiogenic make-up of tumor microenvironment (13, 14). Notably, the aberrant neovasculature resulting from cancer angiogenesis is also responsible for the malignant ascites typically shown in advanced-stage GEA (20, 21). These effects are also dependent on the influence of the VEGF/VEGFR signalling system on the proteins expressed on tight and adherens junctions, as VE-cadherin, occludin, and claudin 5. During angiogenesis, after interaction between VEGF and its receptor, a process including the phosphorylation of VE-Cadherin and its internalization into clathrin-coated pits leads to the disruption of the architecture of endothelial junctions, allowing for the passage of molecules and cells (22). Similarly, VEGF induces phosphorylation of tight junction proteins occludin and Zonula Occludens 1, increasing vasculary permeability, as shown both in pathological brain and ocular conditions, as well as in cancers (23, 24).

In recent years, lights have been shed on the molecular mechanisms underpinning VEGF/VEGFR-mediated angiogenesis,

among which the hypoxia-inducible factor-1 (HIF-1) activation through the hypoxic neoplastic milieu is the best-established one. Following its translocation to the cellular nucleus, HIF-1 binds the VEGF promoter and leads to an increase VEGF transcription (25, 26). Additionally, the EGFR/HER2 system has been shown to induce neuropilin-1 and VEGFR expression in several cancer types, including GEA (27). On the other hand, it has been shown that Notch is a negative regulator of the VEGF/VEGFR axis (28, 29) activity as an increased in VEGFR-2 and VEGFR-3 expression is seen in stalk cells upon abrogation of Notch signalling. Wnt5a may suppress angiogenesis via non-canonical signaling by reducing both EC proliferation and capillary length (30).

Another signaling pathway implicated in angiogenesis is represented by the angiopoietin/tie cascade, which is made of four different angiopoietins (Ang-1,-2,-3,-4), which bind the tyrosine kinase receptor Tie-2. Ang-2, which is involved in vessel maturation, migration, adhesion and survival of endothelial cells, has a crucial role in tumor angiogenesis (31). Again, the FGF (fibroblast growth factor)/FGFR pathway, which controls tumor angiogenesis through the activation of the AKT-pathway, deserves to be mentioned (32). An additional player described in GEA is the non-classical activator tryptase that activates proteinase-activated receptor-2 (PAR-2) and stimulates VEGF production (32, 33). The role of this tryptase and the potential usefulness of anti-angiogenic therapy in gastric cancer is demonstrated by the evidence that infiltrating mast cells, that released tryptase, was related to microvascular density (34).

Taken together, these findings have demonstrated that GEA displays a high angiogenic potential enabled mostly by the VEGF/VEGFR axis and have highlighted a robust and consistent biological rationale for the targeting of angiogenesis in GEA. One of the first proof-of-concept experiences on the targeting of VEGF-driven angiogenesis was reported by Yuan et al. showing time-dependent vascular endothelial regression and changes in vessels permeability in human tumor xenograft treated with neutralizing anti-VEGF antibodies (35). Consistent with that, the anti-VEGF bevacizumab showed to reduce cell growth and tumor size in both in vitro and in vivo experimental models of GEA (36, 37). Analogously, the blockade of VEGFR-2 by means of a targeted monoclonal antibody has resulted in inhibited proliferation and increased apoptosis in preclinical models of GEA (38, 39). These effects were even more pronounced when the anti-VEGF agent was combined with HER2 inhibitors in mice (40). More interestingly, an attractive strategy was suggested by the inhibition of VEGFR-1 or VEGFR-2, which has been reported to enhance paclitaxel sensitivity by decreasing the chemoresistance-conferring elements HIF-1α and TUBB3 (41). Based on this promising preclinical evidence, the targeting of GEA-induced angiogenesis has rapidly advanced to clinical development with controversial study results, which are reviewed in the next chapter.

Clinical development of antiangiogenic agents in GEA

The crucial role played by angiogenesis in cancer progression and invasion, together with the promising results achieved through its pharmacological inhibition in preclinical models, have rapidly advanced the development of this therapeutic strategy to the clinical stage across a wide array of malignancies, including GEA. Although from one hand, the blockade of angiogenesis was the second success of precision medicine in GEA following HER2 inhibition, on the other hand the clinical development process of anti-angiogenic agents is studded with disappointing results. In this section, we discuss major positive and negative trials that have attempted angiogenesis blockade in GEA (Figure 1).

Positive trials

Ramucirumab is a fully human IgG1 anti-VEGFR-2 monoclonal antibody, that exerts its action by preventing the binding of VEGF (A, C, D) to the receptor and blocking signaling activation in endothelial cells. Two pivotal trials demonstrated overall survival (OS) prolongation, leading to the approval of the first target agent in the setting of pre-treated GEA (6, 7). The REGARD study is a phase III randomized trial, in which advanced GEA patients whose disease progressed after first-line platinum/fluoropyrimidine-containing chemotherapy were randomly allocated (2:1) to either ramucirumab 8 mg/kg intravenously once every 2 weeks plus best supportive care (BSC) or placebo. The median OS was 5.2 months in the ramucirumab group and 3.8 months in the placebo group (hazard ratio [HR] =0.77, 95% CI 0.60-0.99; p=0.047) (6). Regarding treatment-related adverse events (AEs), the occurrence of hypertension was higher in the investigational arm than in the control arm (16% vs 8%), whereas rates of other AEs were similar between treatment groups (94% vs 88%). In the phase III RAINBOW trial patients with advanced GEA progressing on or within 4 months after upfront chemotherapy (platinum/fluoropyrimidine with or without anthracycline) were randomized 1:1 to receive ramucirumab 8 mg/kg intravenously once every 2 weeks or placebo, plus paclitaxel 80 mg/mq intravenously on days 1, 8, and 15 of a four-week cycle (7). The OS was significantly prolonged in the experimental arm compared to the control arm (median OS 9.6 months vs 7.4 months, HR=0.80, 95%CI 0.67-0.96, p=0.017). The most common grade 3 or higher AEs (occurring in more than 5% of patients) in the ramucirumab plus paclitaxel arm versus the paclitaxel arm were neutropenia, leucopenia (17% vs 7%), hypertension (14% vs 2%), fatigue (12% vs 5%), anemia (9% vs 10%), and abdominal pain (6% vs 3%). Given the results of these two phase III trials, ramucirumab has been approved by regulatory

10.3389/fonc.2022.993573

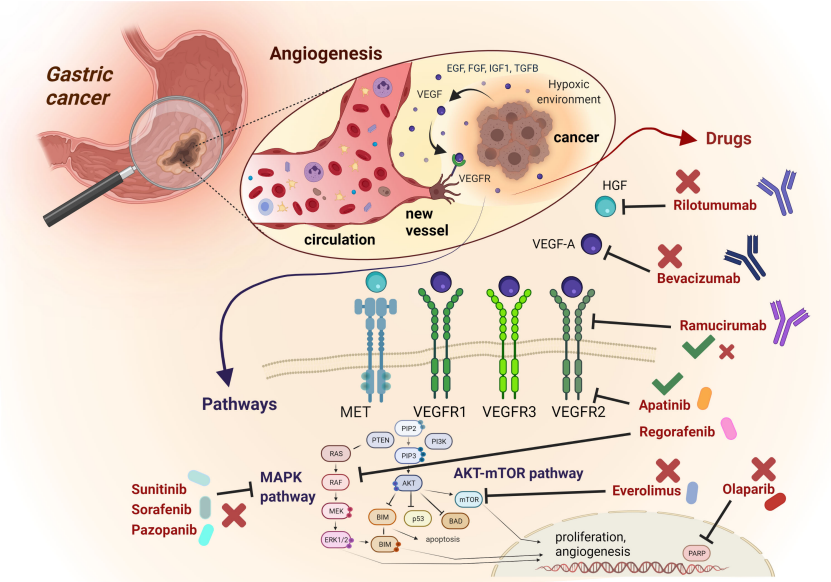

FIGURE 1
Schematic representation of most relevant angiogenic signalling pathways and related targeted compounds in GEA. The figure describes an enlargement of the gastric wall, with the most relevant mechanisms involved in angiogenesis, The anti-angiogenic drugs mentioned in the text are reported, with their targets and their related positive (green thick) or negative (red cross) results in clinical trials concerning GEA patients.

agencies either in monotherapy or in combination with paclitaxel for the second-line treatment of GEA.

Apatinib is an oral small molecule tyrosine kinase inhibitor that selectively targets VEGFR-2. In a phase III randomized-controlled trial, Chinese patients with advanced GEA failing two or more prior lines of chemotherapy, were assigned to apatinib 850 mg once daily or placebo (42). The median OS was significantly improved in the apatinib arm compared with the placebo arm (6.5 months vs 4.7 months, HR=0.709, 95%CI, 0.53-0.93, p=0.0156). The most frequently reported grade 3 to 4 non-hematologic AEs included hand-foot syndrome, proteinuria and hypertension. Based on these results, in 2014 Apatinib was approved in China in this setting. However, the subsequent phase III ANGEL trial failed to confirm the efficacy of apatinib in a global population (43) (Table 1).

Negative trials

The anti-VEGF-A humanized IgG1 monoclonal antibody bevacizumab, which is an evidence-based option in numerous cancer types including, among others, metastatic colorectal

TABLE 1 Major clinical trials reporting positive results for angiogenesis blockade in GEA.

Trial (Authors)	Phase	Line	Target	Treatment arms	Overall Survival[1], months	Safety profile[2]
REGARD (Fuchs et al.)	III	II	VEGFR-2	Ramucirumab (n=238) vs Placebo (n=117)	5,2 vs 3,8 HR=0,776 (0,603-0,998) p=0,042	Hypertension (16% vs 8%)
RAINBOW (Wilke et al.)	III	II	VEGFR-2	Ramucirumab + Paclitaxel (n=330) vs Paclitaxel + Placebo (n=330)	9,6 vs 7,4 HR=0,807 (0,678-0,962) p=0,017	Neutropenia(41% vs 19%); hypertension (14% vs 2%); fatigue (12% vs 5%)
Li et al.	III	≥ III	VEGFR-2	Apatinib (n=176) vs Placebo (n=91)	6,5 vs 4,7 HR=0,709 (0,537-0,937) P=0,0156	HFS (8.5% vs 0%); Proteinuria (2.3% vs 0%); Hypertension (4.5% vs 0%)

[1]Investigational arm vs control arm.
[2]Grade 3-4 adverse events.

cancer, has been the most extensively investigated anti-angiogenic drug in GEA. Early phase II trials showed an encouraging activity for bevacizumab in combination with cytotoxic agents such as fluoropyrimidines, platinum derivatives, irinotecan and taxanes in metastatic GEA, with an overall response rate (ORR) in the range of 42-74%, a median progression-free survival (PFS) of 6-12 months and a median OS of 10.8-17.9 months (44–48). Despite this encouraging activity, the phase III advancement of bevacizumab to the first-line setting proved unsuccessful results in both the western and Asian patient population. In fact, in the Avastin in Gastric Cancer trial (AVAGAST), the addition of bevacizumab to standard first-line combination capecitabine-cisplatin improved PFS and ORR, yet this did not translate into an OS advantage (primary endpoint) (12.1 vs 10.2 months, HR=0.87; p=0.1002) in 774 patients from around the world. Among factors advocated to explain the failure of this large-scale trial is the heterogeneity of the enrolled population, as supported by the differential treatment efficacy recorded in Pan-American as opposed to Asian patients (HR for OS 0.63 vs 0.97, respectively) (49). Accordingly, the AVATAR trial conducted with the same study design in 202 Chinese patients, demonstrated a superimposable OS between the experimental and the control group (HR=1.11; p= 0.5567) (50). Although a biomarker analysis of the AVAGAST trial suggested a positive predictive value for high plasma VEFG-A and neuropilin-1 levels in the non-Asian subgroup, these preliminary findings have been neither validated nor developed further (51). Likewise, bevacizumab failed to prove its efficacy in the setting of resectable GEA. In fact, in the ST03 phase II-III trial, more than 1000 resectable GEA patients were randomly allocated to standard perioperative chemotherapy consisting of epirubicin, cisplatin and capecitabine plus or minus bevacizumab. The 3-year OS was 50.3% in the chemotherapy only arm and 48.1% in the chemotherapy plus bevacizumab arm (HR=1.08, 95%CI 0.91-1.29; p=0.36), with an increased risk of impaired wound healing in the bevacizumab arm (52).

Furthermore, despite ramucirumab having demonstrated its clinical activity in monotherapy or combined with paclitaxel in the second-line setting, the results of 3 randomized trials, including 2 phase II and one phase III, in the front-line treatment of GEA were surprisingly disappointing (53–55). In particular, RAINFALL study was an international, phase 3 trial in which patients with metastatic, HER2-negative GEA were randomized 1:1 to cisplatin plus capecitabine (or 5-FU) and either ramucirumab or placebo. The investigator-assessed PFS (primary endpoint) was significantly extended in the experimental arm as compared to the control arm (HR=0.75, 95%CI 0.60-0.93, p=0.0106; median PFS 5.7 vs 5.4 months). However, a sensitivity analysis based on a central radiological review did not confirm the investigator-assessed difference in PFS nor showed any difference in terms of OS between the two groups (55). This study raised some major concerns. Firstly, as previously reported, PFS does not appear to be a good surrogate endpoint for OS in advanced GEA (56). Secondly, the high rate of post-progression treatments (46% and 50%, in the ramucirumab and in the placebo arm, respectively) might have diluted the difference between the two groups in terms of OS. Thirdly, since ramucirumab has proved its efficacy when paired with a taxane for second-line treatment, it is unknown whether the addition of a taxane instead of the combination of cisplatin and a fluoropyrimidine in the first-line setting could have improved outcomes.

Ziv-aflibercept is a recombinant fusion protein that functions as a decoy receptor to bind VEGF-A, VEGF-B, and PIGF with a high affinity (57), which has been approved in combination with the FOLFIRI regimen in patients with colorectal cancer who were pre-treated with an oxaliplatin-contaning regimen. In a phase II randomized trial in patients with treatment-naive metastatic GEA, the addition of ziv-aflibercept to mFOLFOX6 did not demonstrate an advantage in terms of 6-month PFS (primary endpoint), RR and OS (58, 59).

Sunitinib is a multikinase inhibitor targeting multiple RTKs, including VEGFR1-3, PDGFR, KIT, RET, colony-stimulating factor 1 and FLT3 (60), which are involved in different malignancies, that showed poor efficacy in terms of ORR (less than 5%) in two phase II trials (61, 62) in pretreated patients with advanced GEA. Moreover, other studies testing the addition of sunitinib to chemotherapy in different treatment lines did not demonstrate any advantage in terms of PFS and OS (63, 64).

Similarly, other multikinase inhibitors, such as Sorafenib and Pazopanib, showed modest activity in the treatment of advanced GEA (65–69) (Table 2).

Rilotumumab is a fully human monoclonal antibody selectively targeting the hepatocyte growth factor (HGF). This pathway plays an important role in angiogenesis and tumor growth, with a synergistic action by HGF and VEGF on endothelial cells and also an increasing expression of angiogenic factors mediated by HGF (70, 71). For these reasons, inhibitors of HGF/c-Met signaling pathway are being developed to inhibit angiogenesis. Rilotumumab was investigated in phase III RILOMET-1 trial, in which more than 600 patients with untreated unresectable locally advanced or metastatic MET-positive GEA (defined as ≥25% of cancer cells with a membrane staining intensity of ≥1+) were randomly allocated to receive rilotumumab or placebo combined with antracycline-based chemotherapy (i.e. epirubicin, cisplatin and capecitabine). The investigational treatment was stopped prematurely after a reporting by the independent data monitoring committee of an increased number of deaths in the rilotumumab arm. The median OS was 8.8 months in the rilotumumab group compared with 10.7 months in the placebo group (stratified HR=1.34, 95%CI 1.10-1.63; p=0.003) (72).

10.3389/fonc.2022.993573

TABLE 2 Major clinical trials reporting negative results for angiogenesis blockade in GEA.

Trial(Authors)	Phase	Line	Target	Treatment arms	Overall survival[1], months	Safety profile[2]
AVAGAST (Ohtsu A. et al.)	III	I	VEGF	Bevacizumab + chemo (n=387) vs chemo + placebo (n=387)	12,1 vs 10.1 HR=0,87 (0.73-1,03) p=0,1002	Neutropenia (35 vs 37%); anemia (10 vs 14%)
RAINFALL (Fuchs C et al.)	III	I	VEGFR-2	Ramucirumab + chemo (n=326) vs chemo + placebo (n=319)	11,2 vs 10,7 HR=0,96 (0,80-1,15) p=0,74	Neutropenia (26% vs 27%); Hypertension (10% vs 2%)
RILOMET-1 (Catenacci D. et al.)	III	I	HGF	Rilotumumab + chemo (n=304) vs chemo + placebo (n=305)	8,8 vs 10,7 HR=1,34 (1,10-1,63) p=0,003	Neutropenia (29% vs 32%); anemia (12 vs 14%); fatigue (10% vs 12%)
GOLD (Bang YJ et al.)	III	II	PARP	Olaparib + paclitaxel (n=263) vs paclitaxel + placebo (n=262)	8.8 vs 6,9 HR=0,79 (0,63-1,00) p=0,026	Neutropenia (30% vs 23%)
GRANITE 1 (Ohtsu A. et al.)	III	III	mTOR	Everolimus (n=439) vs Placebo (n=217)	5.4 vs 4.3 HR=0,90 (0,75-1,08) p= 0,124	Anemia (16% vs 13%) Decresead appetite (11% vs 6%); Fatigue (8% vs 5%)

[1]Investigational arm vs control arm.
[2]Grade 3-4 adverse events.

Everolimus is an oral mTOR inhibitor, which demonstrated encouraging antitumor activity in a phase II study of previously-treated advanced GEA (73). mTOR is part of the phosphatidylinositol 3-kinase/Akt/mTOR signaling pathway, which is implicated in tumor angiogenesis (74). This pathway is involved in the regulation of HIF-1α, VEGF, as well as in the endothelial cells function, thus regulating tumor vascularization (75). In the phase III GRANITE-1 trial patients progressing after one or two chemotherapy lines were randomly assigned 2:1 to everolimus 10 mg/die or placebo, both given in combination with BSC. In this study, everolimus did not significantly prolong OS as compared to placebo, with a median OS of 5.4 months in the experimental arm and 4.3 months in the placebo arm (HR=0.90; 95%CI, 0.75-1.08; p=0.124) (76).

Interestingly, the PARP inhibitor olaparib has shown a significant improvement in OS when added to paclitaxel as second-line therapy in a phase II study enrolling Asian patients with advanced GEA, particularly in the molecular subset with ataxia-telangiectasia mutated protein (ATM)-negative tumors (77). In fact, PARP inhibitors have shown to have anti-angiogenic effects by inhibiting VEGF action (78).

Therefore, in the phase III GOLD study Asian patients with advanced GEA progressing after or during first-line chemotherapy were randomized 1:1 to oral olaparib plus paclitaxel or placebo plus paclitaxel. The OS did not differ between treatment groups neither in the overall patient population (median OS 8.8 months vs 6.9 months in the olaparib and placebo group, respectively, HR=0.79, 95%CI 0.63-1.00, p=0.026) nor in the ATM-negative population (12.0 months vs 10.0 months, HR=0.73, p=0.25) (77, 79).

Ongoing studies

Regorafenib is an oral multikinase inhibitor that blocks several receptor tyrosine kinases implicated in angiogenic, oncogenic and stromal signalling. On the basis of the very promising results of the randomized phase II INTEGRATE trial, in which regorafenib proved to be effective in prolonging PFS in advanced, refractory GEA (80), an international phase III trial (INTEGRATE II, NCT02773524) is currently enrolling patients. Furthermore, two phase I/II studies are testing the combination of regorafenib with immune checkpoint inhibitors, including avelumab (REGOMUNE, NCT03475953) and nivolumab (REGONIVO, NCT03406871). Various ongoing clinical trials are currently evaluating the potential of ramucirumab in other settings or in combination with other drugs. RAMSES trial (NCT02661971) is a randomized phase III trial of perioperative FLOT plus or minus ramucirumab in resectable GEA, while RAMIRIS (NCT03081143) is comparing FOLFIRI plus ramucirumab versus paclitaxel plus ramucirumab. The ARMANI trial (NCT02934464) is a phase III trial comparing the first-line treatment continuation versus a switch maintenance strategy with paclitaxel plus ramucirumab. Fruquintinib is a selective oral inhibitor of VEGFR-1-3, which is currently under investigation in numerous clinical trials. FRUTIGA (NCT03223376) is a phase III study comparing paclitaxel plus Fruquintinib versus paclitaxel alone in the second-line setting.

Table 3 summarizes the most promising ongoing clinical trials with anti-angiogenic in the treatment of GEA.

The challenge of biomarkers discovery

In light of the conflicting results achieved so far by the targeting of angiogenesis in GEA, the search for biomarkers predictive of response/resistance has long been pursued to identify patients more likely to benefit from this approach.

Until now, only a few angiogenic factors have been linked to the prognosis of GEA, among which the potential role of VEGF isoforms has been analyzed in many studies. As such, Wang et al. reported that preoperatively VEGF-C levels in serum are significantly higher in patients who develop lymph nodes and distant metastases (81). Then, in a study by Tsirlis et al. (82) it has been shown that in resectable gastric cancer, presurgical VEGF-C levels were lower and VEGF-D levels were higher compared with healthy controls. After surgical resection, an increase in VEGF-C levels and a simultaneous decrease in VEGF-D levels was seen as compared to the preoperative scenario. Notably, both serum VEGF-C and VEGF-D levels were independent predictors of the presence of gastric cancer, with an optimal predictive model of 88% sensitivity and 83% specificity. Therefore, the VEGF-C/VEGF-D ratio was identified as the most robust predictor of malignancy with 88% sensitivity and 75% specificity, by using a cut-off value of <2.7. The authors also identified a predictive model that included VEGF-D, Ca19-9, and demographic parameters (sex, age) for the presence of lymph node metastases with 85% accuracy. Similar results were reported in another study (83), where patients displaying the expression of VEGF-C had a poorer mean survival than those without the expression of the marker (33.8 ± 13.3 vs 42.6 ± 7.4 months, p<0.001). Taken together, these findings suggest that a high expression of VEGF-C in the serum of healthy individuals is a promising diagnostic marker for predicting GEA risk, while in patients with a known diagnosis of GEA, VEGF-C levels could predict an unfavourable prognosis after surgery.

In a further study, Kikuchi et al. (84) showed that VEGF serum levels were significantly higher in patients with cancer than in healthy controls, while the levels of VEGFR-1 and VEGFR-2 were lower in the former versus latter group. VEGF was particularly sensitive and specific as a marker associated with intestinal-type cancer. Indeed, VEGFR-1 had the highest sensitivity and specificity for early gastric cancer and VEGFR-2 for diffuse-type and advanced cancer. According to the authors, a possible reason of the reduced VEGFR serum levels was that the antibody used for ELISA analysis could recognize the same or a near region as the ligand binds. High VEGF levels may bind to these receptors, thus reducing the VEGFR-1 and -2 serum levels. As vascularization promotes cancer progression, soluble VEGFR-1 and -2 could act as decoys impeding VEGF-VEGFR-2 binding on the surface of target cells.

Of interest, the circulating levels of the isoform VEGF-A were significantly higher in both serum and plasma from GEA patients. This value has been shown to decline upon curative-intent surgery, suggesting its secretion by the tumor mass (85). This study highlights a potential role of this biomarker in assessing the completeness of surgical excision.

In another study, Park and colleagues looked at ethnical differences regarding GEA characteristics and VEGF-A expression between Caucasian and Asian patients (86). They were able to find that serum levels of VEGF-A were twice as high in the former than in the latter patients' group and that VEGF-A was an independent prognostic factor for OS, though only among non-Asian patients: the 5-year OS for VEGF-A-high vs –low patients was 72 vs 43% among Caucasians (p=0.001) and 86 vs 77% among Asians (p=0.236).

In the advanced disease setting, a correlation between serum VEGF and platelet counts has been reported, together with worse outcome in patients expressing high serum VEGF per platelet count (87). In the study of Seo et al., the OS was poorer in patients harboring high VEGF per platelet count (p=0.0432), as was the mPFS (4.5 vs. 8.9 months; P = 0.0116). This comes with no surprise, as peripheral blood cells, such as platelets, granulocytes, and lymphocytes normally express VEGF and this

TABLE 3 Selected ongoing trials investigating antiangiogenic strategies in GEA.

Study name	Phase	Disease Setting	Investigational arm	Planned accrual	ClinicalTrials.gov Identifier	Status
ARMANI*	III	First-line, HER-2 negative GEA	Paclitaxel + Ramucirumab	280 patients	NCT02934464	Recruiting
REGONIVO*	III	Third-line, refractory GEA	Regorafenib[1] + Nivolumab[2]	450 patients	NCT04879368	Recruiting
INTEGRATE II*	III	Third-line, refractory GEA	Regorafenib[3]	250 patients	NCT02773524	Active, not recruiting
REGOMUNE*	I/II	Solid tumors (cohort C: GEA)	Regorafenib[4] + Avelumab[5]	482 participants	NCT03475953	Recruiting

[1]Orally at a dose of 90mg (3x30mg) qd, d1-21, q4 weeks 28-day.
[2]Intravenously at a dose of 240 mg d1 q2 weeks; after 2 months, patients whose disease is controlled may have nivolumab administered 480 mg q4 weeks.
[3]Orally at a dose of 160 mg (4x40mg) qd, d1-21, q4 weeks.
[4]Orally at a dose of 160 mg (4x40mg) qd, d1-21, q4 weeks.
[5]Intravenously at a dose of 10 mg/kg every 2 weeks, starting at Cycle 1 Day 15.
*Database accessed on 29th July.

is released during clotting upon platelet activation. However, in the multivariate analysis for predictors of OS and PFS, a statistically significant role for VEGF per platelet was confirmed only on PFS.

Other experiences have been reported on the potential biomarker role of Ang 1-4 and Endostatin, which act as negative regulators of angiogenesis. In a review of Wang et al. (88), an increased level of endostatin in patients with gastric cancer, especially in those with lymph node invasion, as compared to healthy controls, and a lower serum level in low-grade tumors was found. These results suggest the link between serum Endostatin levels and the biological aggressiveness of cancer. Endostatin has also been shown to contribute to lymph node dissemination and to represent a prognostic biomarker in patients with metastatic GEA. Other studies showed a possible correlation between Endostatin serum levels and the histologic intestinal type of gastric tumors (89) and a significant correlation with the presence of distant metastases (90).

On the other hand, Engin et al. (91) found that the concentrations of Ang-2 and Tie-2 were significantly more elevated in patients with gastric cancer as compared to controls, even if this difference was not statistically significant across cancer stages. Jo et al. (92) confirmed this result, also showing that Ang levels were strongly associated with the presence of lymph node metastasis. Similarly, the Ang-like family protein has been explored. The Ang-like 2 (ANGPTL2), which regulates angiogenesis, has been found at higher levels in patients with gastric cancer than in controls (93). Its expression has also been correlated with tumor progression, early recurrence, and poor prognosis (94).

In the study of Oh et al. (95), both p53 and HIF-1α proteins were positively linked to depth of invasion, and p53 and VEGF levels were associated with lymph node involvement. HIF-1α was also identified as a negative prognostic factor for disease recurrence and progression.

Finally, the well-known role of some Interleukins (IL) as pro-angiogenic chemokines has led to the identification of IL-8 as a potential biomarker of angiogenesis in gastric cancer by inducing the overexpression of the VEGF-A, and the receptor isoforms VEGFR-1, and VEGFR-2 (96).

However, to date, these biomarkers have been not been validated for use in clinical practice. Furthermore, there are no predictive biomarkers also for antiangiogenic agents, even if some retrospective studies provided interesting data. In the REGARD exploratory biomarkers analysis, the authors evaluated patients' serum and tumor samples, aiming at correlating ramucirumab efficacy with biomarker expression, including VEGFR family, HER2, VEGF-C and -D. Even if high VEGFR-2 endothelial levels were correlated to a shorter PFS (high vs low HR = 1.65) and ramucirumab treatment was associated with an improved OS in both high (HR = 0.69) and low (HR = 0.73) VEGFR-2 subgroups, none of these and other correlations reached the level of statistical significance (97). A similar conclusion was given by Van Cutsem et al. in the biomarker analyses from the RAINBOW trial (98). However, in this analysis, some pharmacodynamic and prognostic relationships were found, such as the increase of VEGF-D and PlGF plasma levels from baseline levels during treatment and their decrease after treatment discontinuation. In contrast, Angiopoietin-2 exhibited a decrease during treatment, then increased after treatment discontinuation. In this setting, also in a retrospective Korean study (99) an exploratory analysis was conducted to identify potential biomarkers predictive of response to ramucirumab plus paclitaxel in GEA patients. The authors investigated both molecular characteristics on tumor tissue (e.g. EBV, MMR, EGFR, HER2, C-MET) and circulating biomarkers (VEGF, VEGFR-2, neuropillin-1, IL-8, and PIGF) before and after treatment in a subset of patients 44 and 14 patients, respectively. They found a longer PFS in patients with higher pretreatment serum levels of VEGFR-2 (4.1 vs. 2.4 months, p=0.01) and lower pretreatment serum levels of neuropillin-1 (5.8 vs. 2.4 months, p< 0.01), suggesting that circulating angiogenesis-related biomarkers may predict prolonged response to Ramucirumab. Finally, Natsume et al. identified PIGF as a significant gene with an aberrant expression between Ramucirumab responders and non-responders (100). In fact, both the OS and PFS were significantly shorter in the PlGF-high compared with the PlGF-low group, with an ORR of 50% in the latter versus 0% in the former. Furthermore, the authors showed that genetic silencing of PIGF significantly enhanced the inhibitory effect of ramucirumab cell lines of gastric cancer co-cultured with endothelial cells. Moreover, Hacker et al. evaluated the role of Ang-2 as a prognostic and/orpredictive biomarker of bevacizumab efficacy from the AVAGAST trial (101). The median baseline plasma Ang-2 level was lower in Asian vs non-Asian patients (P<0.0001), and was identified as an independent prognostic marker for OS and correlated with the frequency of liver metastasis. However, it did not predict bevacizumab efficacy neither alone nor in combined with baseline VEGF.

Novel developments and future perspectives

As reported before, considering the great efficacy of the combinations of anti-angiogenic drugs with immunotherapy in different cancer entities like non-small-cell lung cancer, hepatocarcinoma, and renal cell cancer, this approach is being evaluating also in gastric cancer. In the REGONIVO study, the combination of regorafenib and nivolumab showed great activity (ORR = 44%) in patients with gastric cancer progressed to prior therapy. Furthermore, a phase I trial showed encouraging ORR and DCR in patients treated with ramucirumab plus pembrolizumab (102).

In the EPOC1706 trial, patients with advanced GEA received lenvatinib in combination with pembrolizumab as first- or second-line therapy. Objective remission was reached in 20 patients out of 29, with an ORR of 69%, mPFS of 7.1 months (95% CI: 5.4-13.7), and mOS not achieved (95% CI: 8-11 months not achieved) (103). The combination of ramucirumab with pembrolizumab was explored in a multi-cohort phase 1B trial of GEA patients; it showed an ORR of 7% and a DCR of 44% (104). In a similar cohort, the mPFS was 2.6 months and OS was 12.4 months in patients treated with ramucirumab/durvalumab in the unselected population, with enhanced activity observed in patients with high PD-L1 expression (105). Finally, another phase I/II clinical trial is evaluating the combination cabozantinib plus durvalumab in pretreated patients with advanced GEA. The authors reported an mPFS of 3.8 months (95% CI: 3.4–6.3), a mOS of 9.1 months (95% CI: 5.8–21.8), and a 6-month PFS rate of 34.5% (95% CI: 17.9–54.3) (104).

One of the most actively investigated subjects in cancer research is represented by microRNAs (miRNA). They consist of a group of small non-coding transcripts, 18-24 nucleotides in length, that regulate gene expression at the translational level, being involved in a plethora of biological functions including proliferation, apoptosis, embryonic stem cell advancement regulation, and cancer cell invasion (106, 107). In particular, a growing body of evidence has been showing that miRNAs can affect tumor angiogenesis through the targeting of both pro- and anti-angiogenic factors, including, among others, RTK signalling proteins, HIF, VEGF, and epidermal growth factor (EGF) (108). To this end, MiR-616-3p has been demonstrated to promote angiogenesis in many cancers, including gastric cancer, via the PTEN/AKT/mTOR pathway (109). In a study performed on patients who underwent upfront surgery, the authors investigated miR-616-3p expression in tumor/normal tissues using real-time PCR and found a significant miR-616-3p up-regulation in cancerous tissues compared with their normal counterparts. Furthermore, miR-616-3p was associated with poor relapse-free survival (RFS) and OS in gastric cancer patients, suggesting its role as a potential prognostic marker in this cancer. Finally, they showed that miR-616-3p promoted epithelial-to-mesenchymal transition (EMT) and angiogenesis in vitro, by modulating VEGF-A and VEGFR-2 expression, and down-regulating PTEN, which resulted in the activation of the AKT/mTOR pathway. In contrast, the reduced expression of miRNA-126 facilitates angiogenesis through its interaction with VEGF-A in gastric cancer (110). Similarly, the diagnostic, prognostic and predictive value of other miRNAs in gastric cancer has been described (111). In a comprehensive review, the authors identified five groups of miRNAs, according to their referral pathway: 1) miRNAs involved in the VEGF pathway, as the previously mentioned miR-616-3p and miR-126, but also miR-1, whose overexpression significantly decreases VEGF-A and endothelin-1 expression, miR-27b, miR-101 and miR-128, that downregulate VEGF-C expression. 2) miRNAs involved in the HIF pathway, such as miR-574-5p and miR-210, are upregulated in hypoxic conditions in gastric cancer. 3) miRNAs related to HGF/c-MET signaling, such as miR-26a/b, are downregulated both in vitro and in vivo in gastric cancer, when HGF is upregulated. 4) miRNAs involved in the PI3K pathway, with different targets, such as PTEN (miR-23°, miR-616-3p, miR-718 and miR-382), FOXO (miR-155 and miR-135b), mTOR (miR-18°, miR-17-92, and miR-101-2), and NF-kB (miR-532-5p). 5) miRNAs related to STAT-3 signaling, such as miR-874, whose upregulation inhibits STAT-3 gene expression, resulting in the inhibition of VEGF-A expression and reduction in tumor growth and angiogenesis in gastric cancer.

With the development of liquid biopsy technology and exosome research, researchers have increasingly investigated the link between exosomes and circular RNAs (circRNA) in tumors. These circRNAs in exosomes could represent biomarkers for tumor diagnosis (112). Furthermore, exosomes are essential mediators of metastasis and angiogenesis in the tumor microenvironment. Some of them are deeply associated with VEGF. In fact, Xie et al. found that exosomal circSHKBP1 can promote gastric cancer cell proliferation, invasion, migration and angiogenesis by regulating the miR-582-3p/HUR/VEGF pathway (113).

Owing to the relevant biological role played by angiogenesis-related ncRNAs, they represent a promising cancer biomarker. Furthermore, some of these miRNAs may be associated with the benefit and toxicity of antiangiogenic agents, even if a prospective validation is needed to confirm these data.

More recently, several studies concentrated efforts on optimizing antiangiogenic therapeutic strategies. Shu et al. investigated the role of branched-chain amino acid transaminase 1 (BCAT1) in the pathogenesis of gastric cancer, particularly in angiogenesis (114). BCAT1 is the predominant isoform of BCAT that initiates the catabolism of branched-chain amino acids and has been involved in tumor pathogenesis, including breast cancer (115), leukaemia (116), ovarian cancer (117), glioma (118), and nasopharyngeal carcinoma (119). BCAT1, which act as an oncogenic factor, has been implicated in proliferation, invasion, and angiogenesis via the activation of the PI3K/AKT/mTOR pathway, The authors discovered that the overexpression of BCAT1 in gastric cancer patients was associated with a poor prognosis. These findings could represent the basis for a possible therapeutic target in this cancer, optimizing antiangiogenesis strategies.

Novel approaches focus on inhibiting gastric cancer vasculogenic mimicry (VM), which plays an essential role in regulating the progression and metastasis (120). The mechanism underlying VM formation is unclear, so there is a need for targeted therapies. Two recent works have recently been published on this topic. In the first, the role of Tenascin-c (TNC) has been explored (121). It is an extracellular matrix protein that induces VM in glioma (122), melanoma (123), and

gastric cancer, which is strongly expressed and associated with poor prognosis (124). Qi et al. showed that gastric cancer patients displaying higher levels of TNC experienced shorter OS and RFS compared to those with low expression. They also found that the TNC knockdown inhibited the VM formation both in vitro and in vivo and suppressed the proliferation of gastric cancer cell lines through the induction of cell cycle arrest in the G0/G1 phase. Then, they discovered a strong association between TNC and EMT, which is involved in VM formation through MAPK/ERK pathway (125, 126). For this reason, the authors stopped the EMT process by blocking ERK phosphorylation and TNC, inhibiting the VM formation simultaneously. Therefore, they pointed out that the combined targeting of TNC and ERK pathways may provide a promising antitumor strategy for inhibiting VM formation and decreasing antiangiogenic therapeutic resistance.

In a second work (127), the authors investigated the role of crocetin, a component of saffron stigma, which has significant therapeutic effects on various diseases, including cancers. They showed that crocetin significantly inhibited angiogenesis and metastasis development in gastric cancer by inhibiting Human Umbilical Vein Endothelial Cells and VM formation of cells. This ability was mediated by the inhibition of the sonic hedgehog signalling pathway, making crocetin a potentially effective therapeutic drug against this cancer.

Author contributions

MS, FG, and FP conceived the topic and MS, FC, AB, SC, CB, FP, MD, and FG wrote this manuscript. MS, FG, MD, and FP revised the manuscript. All the authors contributed to this article and approved the final submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor AP declared a past co-authorship with the authors.

Publisher's note

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frontiers | Frontiers in Oncology

TYPE Review
PUBLISHED 10 October 2022
DOI 10.3389/fonc.2022.1021772

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology

RECEIVED 17 August 2022
ACCEPTED 26 September 2022
PUBLISHED 10 October 2022

CITATION
Saoudi González N, Castet F, Élez E, Macarulla T and Tabernero J (2022) Current and emerging anti-angiogenic therapies in gastrointestinal and hepatobiliary cancers.
Front. Oncol. 12:1021772.
doi: 10.3389/fonc.2022.1021772

Current and emerging anti-angiogenic therapies in gastrointestinal and hepatobiliary cancers

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Gastrointestinal tumours are a heterogeneous group of neoplasms that arise in the gastrointestinal tract and hepatobiliary system. Their incidence is rising globally and they currently represent the leading cause of cancer-related mortality worldwide. Anti-angiogenic agents have been incorporated into the treatment armamentarium of most of these malignancies and have improved survival outcomes, most notably in colorectal cancer and hepatocellular carcinoma. New treatment combinations with immunotherapies and other agents have led to unprecedented benefits and are revolutionising patient care. In this review, we detail the mechanisms of action of anti-angiogenic agents and the preclinical rationale underlying their combinations with immunotherapies. We review the clinical evidence supporting their use across all gastrointestinal tumours, with a particular emphasis on colorectal cancer and hepatocellular carcinoma. We discuss available biomarkers of response to these therapies and their utility in routine clinical practice. Finally, we summarise ongoing clinical trials in distinct settings and highlight the preclinical rationale supporting novel combinations.

KEYWORDS

anti-angiogenic, tyrosine kinase inhibitor, neoangiogenesis, gastrointestinal cancer, hepatobiliary tumour, hepatocellular carcinoma, colorectal cancer

1 Introduction

The process of angiogenesis was identified in 1971 as one of the key steps in cancer progression, and has been considered a hallmark of cancer since 2000 (1, 2). Angiogenesis is a pathway that implies the growth of new capillary blood vessels to maintain oxygen and nutrient supplies during tumour expansion. Cancer cells develop this angiogenic capacity *via* an "angiogenic switch" triggered by the synthesis and delivery of different positive signals that encourage angiogenesis, such as vascular

endothelial growth factor (VEGF) that binds to the VEGF receptor (VEGFR) located on endothelial cells. More molecules participate in this delicate equilibrium, which is a balance between proangiogenic and anti-angiogenic signals crucial for the angiogenic switch (3). Soon after the discovery of the angiogenic pathway, efforts were made to develop treatments to block this process. This included large monoclonal antibodies such as bevacizumab and small tyrosine kinase inhibitors (TKIs) including sorafenib, sunitinib, and regorafenib, that have been approved in different tumour types (4).

Gastrointestinal cancers are drivers of cancer mortality worldwide. Colorectal cancer (CRC) is the second cause of cancer-related deaths, liver cancer the third, and stomach cancer the fourth (5). Therefore, there is a global concern and a need to generate more efficient diagnostic and therapeutic approaches to increase patient survival, with anti-angiogenics representing an attractive target in this setting. VEGF expression is associated with poor prognosis in colorectal, gastric and pancreatic cancer (6–8). The value of testing anti-angiogenic therapy in different gastrointestinal cancers has been established, and today different treatments and combination regimens are available for these tumours (Figure 1), as summarised in this review. Nevertheless, there is an unmet need for a better understanding of the mechanism of resistance as well as of optimal selection of those patients more likely to benefit from VEGF-targeted therapy, thus novel therapeutic strategies are also reviewed.

2 Angiogenesis in gastrointestinal cancer

Angiogenesis is essential for tumour progression and develops following an "angiogenic switch" (9–11). The onset of this event is dictated by the balance of pro- and anti-angiogenic factors (10, 11), which eventually leads to chronic activation of proangiogenic factors favouring the formation of new, morphologically aberrant blood vessels that will sustain tumour development and foster metastatic spread (9). Despite angiogenesis being universal to all cancers, tumours exhibit diverse patterns of neo-vascularisation which may influence response to therapy. An example of this heterogeneity is seen with pancreatic ductal adenocarcinomas (PDAC) that are generally hypovascularised and hypoxic owing to the high desmoplastic microenvironment, which may limit drug delivery (12). Conversely, hepatocellular carcinomas (HCC) are hypervascular tumours with a characteristic radiological pattern and a highly abnormal vessel architecture resulting from the overexpression of VEGF (13, 14). Similarly, neuroendocrine tumours have a rich vascular supply and a dense microvascular network which together constitute one of the most useful diagnostic characteristics of these tumours (15). In addition to the observed between-tumour type heterogeneity, substantial differences exist amongst tumours belonging to the same anatomic location. In CRC, the Consensus Molecular Subtype (CMS) 4 (mesenchymal subtype), which constitutes ~25% of all CRC, is characterised by high stromal infiltration, increased angiogenesis and is associated with a significantly worse prognosis (16). Likewise, gastric cancers classified as genomically stable are enriched in angiogenic pathways (17). These traits may help to explain, at least in part, the different patterns of response to anti-angiogenic therapies in distinct tumour types and amongst patients.

2.1 Signalling pathways in angiogenesis

Different mechanisms may lead to the formation of new blood vessels, such as the proliferation of pre-existing endothelial cells ("sprouting angiogenesis"), recruitment of endothelial progenitor cells to the tumoural microenvironment ("vasculogenesis"), remodelling of pre-existing blood vessels ("intussusceptive angiogenesis") or formation of new vessels by tumoural cells independently of endothelial cells ("vascular mimicry") (18). In addition, tumours may develop close to pre-existing mature blood vessels thus ensuring an adequate blood supply without the need for developing new vessels (referred to as "vessel co-option") (19). While all of these mechanisms are known to contribute to tumour vascularisation in gastrointestinal tumours, sprouting angiogenesis remains the dominant mechanism and is triggered by multiple proangiogenic pathways.

The most potent angiogenic pathway in cancer is the VEGF signalling pathway (20), which is composed of five ligands (VEGF-A, -B, -C, -D and placental growth factor [PlGF]) and three receptors (VEGFR-1, -2 and -3). In gastrointestinal cancers, hypoxia mainly upregulates VEGF-A in tumour cells. This in turn binds to VEGFR-2 that is expressed on endothelial cells, leading to proliferation, vascular permeability and endothelial cell migration (10, 20). The mammalian fibroblast growth factor (FGF) signals through the FGF receptors 1-4 (FGFR 1-4) to mediate multiple functions including angiogenesis, cellular proliferation, invasiveness and enhanced metastasis. In addition to VEGF and FGF, many other pathways have been shown to be highly relevant in regulating angiogenesis in these tumour types, including the platelet-derived growth factor (PDGF) family, and the angiopoeitin family which bind to the tyrosine kinases TIE-1 and TIE-2 (20).

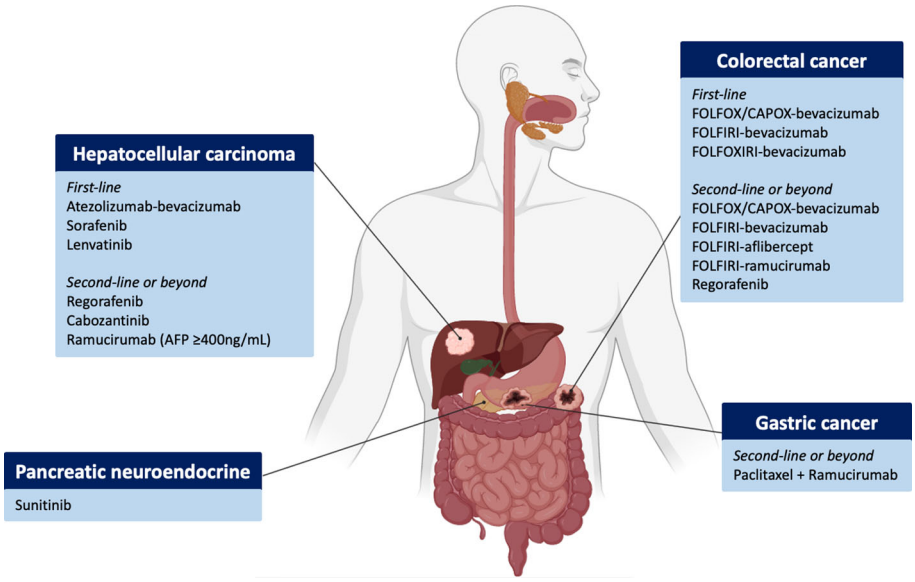

FIGURE 1
Overview of FDA-approved antiangiogenic agents across gastrointestinal and hepatobiliary tumours and disease settings. Figure generated with www.biorender.com.

2.2 Mechanism of action of anti-angiogenic drugs

Anti-angiogenic therapies commonly used in gastrointestinal malignancies can be broadly categorised into monoclonal antibodies and TKIs. The former includes bevacizumab, which binds to VEGF-A, ramucirumab (which inhibits VEGFR-2) and aflibercept, a decoy receptor that binds to all isoforms of VEGF-A. TKIs are small-molecule compounds that inhibit a broad range of protein kinases. They include sorafenib, lenvatinib, regorafenib, cabozantinib and sunitinib, amongst others (Figure 1). The main targets of sorafenib are VEGFR-1, -2, -3, PDGFR, RAF and KIT; regorafenib and sunitinib have a similar inhibitory profile, as does lenvatinib that additionally targets FGFR-1, -2, -3 and -4. Cabozantinib is a potent VEGFR-2 and MET inhibitor.

Anti-angiogenic therapies may mediate antitumour effects in at least 4 different mechanisms (Figure 2). Firstly, the development of these therapies stems from the hypothesis that starving tumours by depleting them of blood vessels will induce necrosis and slow tumour progression (21). However, fostering a hypoxic and nutrient-deprived microenvironment may also result in treatment resistance and insufficient efficacy (22). This has led to the concept of vascular normalisation (23), understood as the resulting effect on the tumour vasculature of a judicious use of anti-angiogenic drugs that may balance the excess of proangiogenic factors and lead to a remodelling and pruning of tumour blood vessels to normalize the tumour vasculature (24). This, in turn, will improve drug delivery and foster a less hostile microenvironment, thus increasing the efficacy of combination partners (22).

Thirdly, increased attention has been placed on the immunomodulatory effects of anti-angiogenic therapies (Figure 2). Angiogenic modulating factors may alter the immune microenvironment through three established paths (25). First, VEGF can directly act on immune cells, leading to $CD8^+$ T cell exhaustion, increased proliferation of T regulatory cells (Tregs), thereby fostering the expansion of myeloid-derived suppressor cells (MDSCs), and inhibiting the differentiation of monocytes to dendritic cells (DC) and decreasing DC maturation (26, 27). Secondly, the endothelium of tumour blood vessels creates a natural barrier for immune cells to infiltrate the microenvironment due to its lack of adhesion proteins (such as ICAM1 and VCAM1), as well as increased expression of proapoptotic molecules (FASL and galectin 1) and immunosuppressive molecules (PD-L1, PD-L2, TIM3 and IDO) (28). Finally, the hypoxic and acidotic tumour microenvironment also favours immunosuppressive changes, including the reprogramming of tumour-associated macrophages (TAMs) from an antitumour M1-like phenotype to a pro-tumoural M2-like phenotype, also decreasing the maturation and proliferation of DCs and increasing the proliferation of Tregs (26). All these factors will lead to a highly immunosuppressed

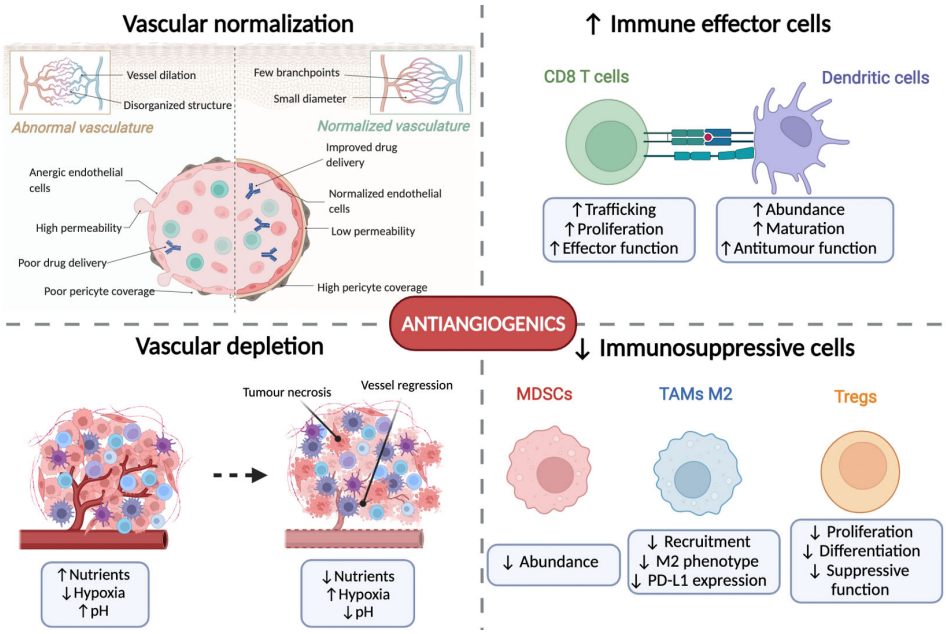

FIGURE 2
Mechanisms of action of antiangiogenic agents. Antiangiogenics exert their anti-tumoural effects *via* four axes. First, they induce normalisation of the tumour vasculature which improves drug delivery and oxygenation. Second, they induce vascular depletion which induces tumour starvation and necrosis. Thirdly, they favour an antitumour immune response by increasing the proliferation and activation of CD8+ T cells and dendritic cells. Finally, they decrease the presence and function of immunosuppressive cells including myeloid-derived suppressor cells (MDSCs), tumour-associated macrophages (TAMs) and T regulatory cells (Tregs). Figure generated with www.biorender.com.

microenvironment that could be potentially reversable with appropriate anti-angiogenic drugs.

These immunomodulatory effects have prompted the development of combinations of anti-angiogenic drugs with immune checkpoint inhibitors (ICIs). The rationale underlying these novel therapies is sound: ICIs increase the recruitment and/or activation of effector $CD8^+$ T cells, DCs and natural killer cells and promote an antitumour M1 macrophage phenotype, while simultaneously decreasing the infiltration and activity of MDSCs, Treg cells and M2 macrophage polarisation (26, 29). Specific combinations in animal models further support this notion. Cabozantinib combined with anti-PD1 antibodies in syngeneic HCC mice models promoted the infiltration of neutrophils (30), and lenvatinib decreased the abundance of tumoural Tregs (31). Sorafenib specifically suppressed the activation of macrophages with an M2-like polarisation (32) and regorafenib favoured the infiltration of activated $CXCR3^+CD8^+$ T cells (33) and an M1-like macrophage polarisation (34). Similarly, sunitinib or antibodies blocking the VEGF/VEGF-R pathway in syngeneic colon mice models decreased the population of Tregs (35). Hence, combining therapies that target angiogenesis with immune stimulating agents represents a promising strategy that is being actively explored in clinical trials in many gastrointestinal, hepatobiliary and pancreatic tumours.

2.3 Mechanisms of resistance to anti-angiogenic drugs

Some tumours may be primarily resistant to anti-angiogenic drugs while others can develop mechanisms of resistance following drug exposure. This process of adaptation may undergo different sequential phases. In an early phase, tumours will upregulate alternative or redundant pro-angiogenic pathways that are not targeted by a specific drug, thus resulting in sustained angiogenesis despite optimal inhibition of the targeted pathway (36). Moreover, some tumours, such as PDAC, rely primarily on signalling pathways other than VEGF, leading to primary resistance to these inhibitors. In a later step, tumours adapt to hypoxia by promoting autophagy, which degrades cell components to promote survival in unfavourable conditions. In HCC, increased activation of mTOR or Akt pathways has been shown to trigger autophagy and cell survival when exposed to sorafenib, and can be overcome by combining sorafenib with autophagy inhibitors (37). The stress induced by antiangiogenic therapies stimulates inflammatory pathways and cytokines that lead to the recruitment of cells that favour angiogenesis, such as bone marrow-derived cells (BMDCs), myeloid-derived suppressor cells (MDSCs), endothelial progenitor cells, pericytes and cancer-associated fibroblasts (38, 39). In a late

adaptation phase following exposure to antiangiogenic agents, tumours adopt different neovascularization modalities, including vessel co-option and vascular mimicry, which in turn may fuel metastatic spread and increase tumour aggressiveness (38).

An area of increasing interest and research is the significant heterogeneity of tumour endothelial cells which may contribute to resistance to anti-angiogenic drugs (40, 41). A single-cell analysis of endothelial cells following angiogenic inhibition has found that these cells can be broadly categorized into tip cells, transition and stalk-like cells. VEGF inhibition reduces all three subpopulations but has a particularly strong inhibitory effect on tip cells. In contrast, blockade of Dll4 promotes endothelial proliferation as well as tip cell markers (41). In liver tumours specifically, endothelial cancer cells show liver-specific gene expression signatures that are independent of the site of the original tumour, display distinct clusters that recapitulate tip-like and stalk-like characteristics and show stronger similarities to venous rather than endothelial cells (42). Furthermore, HCCs present endothelial cells that are reminiscent of fetal development, with a shared onco-fetal programme that is mediated in part by VEGF and NOTCH (43). In lung cancer, 13 different endothelial cell phenotypes have been described, including some subsets potentially involved in immune surveillance. This study further supports the notion that tip endothelial cells are particularly sensitive to antiangiogenic therapies (40).

3 Colorectal cancer

CRC is the third most frequently diagnosed cancer worldwide, and the second cause of cancer-related deaths (5). Unfortunately, approximately 20% of patients with CRC have metastatic spread at diagnosis (mCRC), and almost half of all patients will develop metastases during the course of the disease (44, 45). The incorporation of biological agents targeting two major pathways involved in mCRC such as the epidermal growth factor receptor (EGFR) targeted by panitumumab or cetuximab, and the VEGF signalling pathway suppressed by bevacizumab, aflibercept, ramucirumab and regorafenib have improved median overall survival (OS) to more than 30 months (46–48).

3.1 Clinical overview of anti-angiogenic drugs in mCRC

3.1.1 Advanced disease: First-line setting

To define the optimal strategy treatment for patients with mCRC, is crucial to take into account the Eastern Cooperative Oncology Group (ECOG) performance status of the patient, the sidedness of colon tumour, molecular status of all *RAS* and *BRAF* genes, microsatellite status and resectability of metastatic disease, in addition to the patient's preferences and toxicity of the treatments. According to European Society for Medical Oncology (ESMO) guidelines, combination of biological agents with FOLFIRI or FOLFOX chemotherapy is standard treatment for the first and second line setting in mCRC (45, 49–55).

Bevacizumab is a recombinant humanised monoclonal antibody targeting the VEGF ligand A (VEGF-A) and is approved for use in mCRC patients in the first and second lines of therapy. Over the last 20 years, multiple clinical trials have investigated the combination of bevacizumab with chemotherapy in this setting. The first phase III trial published in 2004 explored the combination of bevacizumab with irinotecan, bolus fluorouracil and leucovorin (IFL) *vs* IFL alone. Median OS was 20.3 months in the group that received IFL plus bevacizumab, compared with 15.6 months in the group given IFL plus placebo, corresponding to an HR for death of 0.66 ($P<0.001$) (56). Since then, the benefit of adding this monoclonal antibody to cytotoxic chemotherapy backbone regimens that contain either oxaliplatin or irinotecan, or both, or a fluoropyrimidine as monotherapy has been explored and demonstrated. Table 1 summarizes the major clinical trials in this setting. In combination with first line oxaliplatin, the NO16966 trial demonstrated a benefit for the addition of bevacizumab to FOLFOX or CAPOX. There was a significant improvement in progression-free survival (PFS) with the addition of bevacizumab; however, the magnitude of benefit was smaller than expected, and neither median OS nor overall response rates (ORRs) were significantly higher in patients who received bevacizumab (53). The BECOME trial randomised patients with *RAS* mutant unresectable, liver-limited mCRC to receive bevacizumab plus mFOLFOX6 *vs* FOLFOX6 alone. This trial demonstrated higher ORR (55% *vs* 37%), median PFS (9.5 *vs* 5.6 months), median OS (25.7 *vs* 20.5 months), and complete (R0) resection rates (22.3% *vs* 5.8%) (57).

For patients who are not suitable for doublet chemotherapy, the combination of fluoropyrimidines plus bevacizumab has demonstrated superiority over fluoropyrimidine monotherapy. The phase III AVEX trial focused on elderly patients with mCRC (70 years-old or older), enrolling patients who were not candidates for a combination of oxaliplatin or irinotecan-based chemotherapy regimens. Patients were randomised to bevacizumab plus capecitabine *vs* capecitabine alone, and this study demonstrated that the combination regimen was well tolerated and significantly improved outcomes, with median PFS of 9.1 *vs* 5.1, respectively (HR 0.53; 95% CI, 0.41–0.69; $P<0.0001$) (58). Median OS was not significantly different between the two groups as the study was not sufficiently powered to detect such differences (20.7 months in the bevacizumab plus capecitabine group *vs* 16.8 months in the capecitabine alone group [HR 0.79, 95% CI 0.57–1.09; $P=0.18$]).

Unlike frail patients, there are also a group of fit patients with metastatic disease who will benefit from a high response

rate, thus more intense chemotherapy backbones have been investigated. Phase II/III trials have explored the combination of FOLFOXIRI with or without bevacizumab *vs* doublet combinations with or without bevacizumab (59–63). A recent individual-patient data meta-analysis of these clinical trials was published, demonstrating that FOLFOXIRI plus bevacizumab significantly and meaningfully improves OS of patients with mCRC compared with bevacizumab-based doublets and offers

TABLE 1 Overview of selected phase III trials testing anti-angiogenic agents in mCRC.

Trial	Population	Treatment arms	N-patients	OS		PFS		ORR (%)	DCR (%)	Grade 3-4 TRAEs (%)
				Median (mo)	HR (95% CI)	Median (mo)	HR (95% CI)			
Bevacizumab in first line										
Hurwitz et al. 2004	pre*RAS*	IFL + bevacizumab	402	20.3	0.66 (NA)*	10.6	0.54* (NA)	44.8*	NA	85
		IFL + placebo	411	15.6		6.2		34.8*		74
NO16966	pre*RAS*	XELOX/FOLFOX4 + bevacizumab	699	21.3	0.89 (0.76-1.03)	9.4	0.83 (0.72-0.95)*	47	NA	80
		XELOX/FOLFOX4 + placebo	701	19.9		8.0		49		75
BICC-C	pre*RAS*	FOLFIRI + bevacizumab	57	28	NA	11.2	NA	57.9	NA	NA
		FOLFIRI	144	23.1		7.6		47.2		
Bevacizumab in first line frail patients										
AVEX	≥70 years old	Capecitabine + bevacizumab	140	20.7	0.79 (0.57-1.09)	9.1	0.53 (0.41-0.69)*	19*	74*	40
		Capecitabine	140	16.8		5.1		10*	58*	22
Bevacizumab vs anti-EGFR treatment first line										
CALGB/SWOG 80405	*KRAS* wt (initially all *RAS*)	CT + cetuximab	578	30	0.88 (0.77-1.01)	10.5	0.95 (0.84-1.08)	59.6	NA	NA
		CT + bevacizumab	559	29		10.6		55.2	NA	NA
FIRE-3	*KRAS* exon 2 wt	FOLFIRI + cetuximab	297	28.7	0.77 (0.62-0.96)*	10	1.06 (0.88-1.26)	62	80	64
		FOLFIRI + bevacizumab	295	25		10.3		58	87	71
PARADIGM	*RAS*/*BRAF* wt left/right colon	mFOLFOX6 + panitumumab	312 (left)	37.9	0.82 (0.68-0.99)*	13.7	0.98 (0.82-1.17)	80.2	NA	NA
		mFOLFOX6 + bevacizumab	292 (left)	34.3		13.2		68.6		
TRIBE	Independent status *RAS*/*BRAF*	FOLFOXIRI + bevacizumab	256	29.8	0.8 (0.65-0.98)*	12.3 9.7	0.77 (0.64-0.94)*	65*	90	NA
		FOLFIRI + bevacizumab	252	25.8				53*	86	
Bevacizumab second line										
ECOG E3200	pre*RAS* PD after 1st L CT FU + irinotecan	FOLFOX4 + bevacizumab	286	12.9	0.75 (NA)*	7.3	0.61 (NA)*	22.7	NA	75
		FOLFOX4	291	10.8		4.7		8.6	61	
		Bevacizumab	243	10.2	NA	2.7	NA	3.3	NA	
ML18147	Independent status *KRAS* 1stL CT + Bevacizumab	CT + bevacizumab	409	11.2	0.81 (0.69-0.94)*	5.7	0.68 (0.59-0.78)*	6	69	64
		CT	411	9.8		4.1		4	54	57
Aflibercept second line										
VELOUR	Advanced – 2nd line	Independent status *KRAS* PD 1stL oxaliplatin based CT	FOLFIRI + Aflibercept	13.5	0.82 (0.71-0.94)*	6.9	0.76 (0.66-0.87)	19.8*	86	83.5
		Placebo	FOLFIRI + placebo	12.06		4.7		11.1*	65	62.5
Ramucirumab second line										

(Continued)

TABLE 1 Continued

Trial	Population	Treatment arms	N-patients	OS		PFS		ORR (%)	DCR (%)	Grade 3-4 TRAEs (%)
				Median (mo)	HR (95% CI)	Median (mo)	HR (95% CI)			
RAISE	Independent status *KRAS* PD 1stL Oxaliplatin + FU + Bevacizumab	FOLFIRI + ramucirumab	536	13.3	0.84 (0.73-0.98)*	5.7	0.79 (0.70-0.90)*	13.4	NA	79
		FOLFIRI + placebo	536	11.7		4.5		12.5	62	
Regorafenib in refractory setting										
CORRECT	Independent status *KRAS*; refractory setting	Regorafenib	505	6.4	0.77 (0.64-0.94)*	1.9	0.49 (0.42-0.58)*	1	41	54
		Placebo	255	5		1.7		0.4	15	14

5-FU, fluorouracil; CI, confidence interval; CT, chemotherapy; DCR, disease control rate; HR, hazard ratio; IFL, irinotecan, bolus fluorouracil, leucovorin; L, line; mo, months; NA, not available; ORR, overall response rate; OS, overall survival; PD, progressive disease; PFS, progression-free survival; TRAEs, treatment-related adverse events. *Indicates statistically significant differences.

advantages in PFS, ORR and R0 resection rate, albeit at the price of increased toxicity. In contrast to initial observations from the subgroup analysis of the TRIBE trial, no increased benefit was observed among patients with *BRAF*V600-mutant tumours in this meta-analysis (61). Thus, the use of FOLFOXIRI–bevacizumab should no longer be regarded as the first choice for patients with a *BRAF*V600E mutation, in whom the use of FOLFOX–bevacizumab is currently the upfront treatment option of choice.

The question of which biologic is preferable in first line treatment for all *RAS* wild-type (wt) mCRC was addressed in several phase III trials. In the phase III trial FIRE-3, patients with previously untreated *KRAS* wt mCRC (initially this trial recruited allcomers, however due to the emerging evidence for the negative predictive value of *KRAS* exon 2 mutations, a protocol amendment was submitted in October 2007 to limit the population) received FOLFIRI plus cetuximab or FOLFIRI plus bevacizumab (48). There was no significant difference in the primary endpoint of ORR (65.3% with cetuximab *vs* 58% with bevacizumab, HR 1.18, *P*=0.18). The median PFS was similar in both the groups (10 months in the cetuximab group and 10.3 months in the bevacizumab group [HR 1.06, 95% CI 0.88–1.26; *P*=0.55]). Surprisingly, when evaluating *KRAS* exon 2 wt patients, OS was significantly better in patients treated with cetuximab (OS 33.1 *vs* 25.6 months favouring cetuximab over bevacizumab, *P*=0.011).

CALGB 80405 was a large randomised phase III trial in which patients with previously untreated mCRC received either FOLFIRI or FOLFOX at enrolment and were then randomised to either bevacizumab, cetuximab, or both (46). Initially, as for the FIRE-3, this trial included patients unselected for *RAS* status, with an amendment restricting eligibility to patients with *KRAS* wt tumours. The findings demonstrated similar results across all four groups, suggesting that either chemotherapy backbone in combination with either an anti-EGFR or anti-VEGF therapy is an acceptable therapy option in patients with *RAS* wt tumours.

mCRC is a clinically and molecularly heterogeneous disease, which is partially explained by the anatomic location of the tumour, given that left and right-sided tumours are derive from different embryonic structures (64). Furthermore, the left and right colon also have physiologically distinct functions with different contacts and exposure to nutrients, and thus different microbiomes can be found from the proximal to the distal colon (65). Regarding these differences, studies have retrospectively investigated the correlation between laterality and response to treatment, concluding that sidedness of colon cancer is a predictive biomarker of response to biological agents. From a meta-analysis published in 2017 covering all first line studies, a significant predictive benefit was demonstrated for chemotherapy plus EGFR antibody therapy in patients with left-sided tumours (HR 0.75 [95% CI 0.67-0.84] and 0.78 [95% CI 0.70-0.87] for OS and PFS, respectively) (66–68). However, there was a trend, albeit no significant benefit for patients treated with chemotherapy with or without bevacizumab with right-sided tumours (HRs 1.12 [95% CI 0.87-1.45] and 1.12 [95% CI 0.87-1.44] for OS and PFS, respectively). Recent data presented at ASCO 2022 from the PARADIGM trial, that randomised patients with *RAS* wt mCRC to receive panitumumab plus mFOLFOX or bevacizumab plus mFOLFOX, demonstrated a clear benefit of anti-EGFR therapy for patients with left-sided colon cancer (OS 37.9 *vs* 34.3 months; HR 0.82, *P*=0.031) (69). Thus, for patients with left-sided *RAS* wt disease, a cytotoxic doublet plus an anti-EGFR antibody should be the treatment of choice. For patients with right-sided *RAS* wt disease or *RAS* mutant, cytotoxic combination with bevacizumab is the preferred option.

The combination of both VEGF and anti-EGFR treatments is not recommended for first-line therapy of mCRC in light of the results of the PACCE and CAIRO2 trials (70, 71).

Maintenance treatment is a therapeutic strategy that envisages a period of high-intensity chemotherapy, after which agents that are mainly responsible for cumulative toxicity are stopped, leaving patients with a more simple and non-toxic combination of treatments until progression disease. This approach differs from treatment interruption, in which drug withdrawal is permitted with treatment-free intervals. Maintenance is active and should be part of the mCRC treatment strategy, as active maintenance with fluoropyrimidines and bevacizumab has demonstrated improvement of PFS (but not OS) (72, 73).

3.1..2 Advanced disease: Second line setting

Several anti-angiogenic agents have demonstrated efficacy in mCRC in the second line setting.

Aflibercept is a fully humanised recombinant fusion protein composed of a modified immunoglobulin domain of VEGFR-1 joined to another immunoglobulin domain of human VEGFR-2 and fused to a fragment crystallizable portion of a human immunoglobulin, thus providing complete blockade of angiogenesis by targeting VEGF-A, VEFGF-B, and PIGF (74). In 2012, in the absence of evidence of improvement of OS in the second line in mCRC after progression on a first line oxaliplatin-containing regimen, the VELOUR trial was initiated (52). This randomised phase III double-blind study randomised 1226 patients into two groups, aflibercept or placebo every 2 weeks plus FOLFIRI. Data demonstrated advantages in OS, PFS and RR of aflibercept combined with FOLFIRI *vs* chemotherapy alone. Prior treatment with bevacizumab was permitted. The results showed an OS benefit favouring the aflibercept group, with an OS of 13.5 *vs* 12.1 months (HR 0.817; P=0.0032), PFS of 6.9 *vs* 4.67 months (HR 0.758; P<0.0001), and an ORR of 19.8% *vs* 11.1% (P=0.0001) with aflibercept plus FOLFIRI compared with placebo plus FOLFIRI, respectively. The effects of aflibercept exhibited a consistent trend of improved OS and PFS in pre-specified subgroup analyses based on previous treatment with bevacizumab. This higher efficacy of aflibercept was associated with an expected increase in adverse effects, with grade 3 and 4 adverse events (AEs) reported in 85.3% and 62.5% of patients, respectively.

The TML18147 trial was a randomised phase III trial that assessed the efficacy of bevacizumab beyond progression in patients with mCRC who had received first line treatment with bevacizumab (54). In this study, patients received bevacizumab with chemotherapy or chemotherapy alone, and demonstrated an improvement in OS for patients in the bevacizumab plus chemotherapy group (11.1 *vs* 9.8 months, respectively; P=0.0062).

Ramucirumab is a human monoclonal antibody that targets VEGFR-2. The phase III RAISE study evaluated the efficacy and safety of ramucirumab in combination with second line FOLFIRI compared with FOLFIRI plus placebo in mCRC patients who had progressed during or after first line therapy with bevacizumab and FOLFOX, independent of *KRAS* status (50). In this trial, a total of 1,072 patients were randomised to FOLFIRI with or without ramucirumab, showing a significant improvement in both OS and PFS (13.3 *vs* 11.7 months and 5.7 *vs* 4.5 months, respectively).

The results of these phase III trials support the benefit of continuing VEGF inhibition following prior exposure to bevacizumab. No direct comparison has been done, however the effects across all studies are of a similar magnitude, therefore the selection of bevacizumab, aflibercept or ramucirumab should be based on evaluating the toxicity profile, the interval free of bevacizumab, patient's preference, reimbursement policy of each country and previous anti-EGFR in all patients with *RAS* wt mCRC.

3.1.3 Advanced disease: Refractory setting

Regorafenib is an oral inhibitor that blocks the activity of multiple protein kinases active in oncogenesis and the tumour microenvironment, with anti-angiogenic activity due to its dual-targeted VEGFR2 tyrosine kinase inhibition (75). The efficacy of regorafenib in the mCRC refractory setting was demonstrated in the CORRECT trial (76). This phase III trial explored the efficacy in terms of OS of regorafenib *vs* best supportive care in patients with mCRC who progressed on standard therapy. Previous anti-angiogenic treatment was permitted. Patients were randomised in a 2:1 ratio to receive either best supportive care plus oral regorafenib or placebo once daily. Median OS was 6.4 months in the regorafenib group *vs* 5.0 months in the placebo group (HR 0.77; P=0.0052).

TAS-102 is an oral combination of a thymidine-based nucleic acid analogue, trifluridine and a thymidine phosphorylase inhibitor, tipiracil hydrochloride. TAS-102 has demonstrated efficacy in terms of OS compared to best supportive care in patients with refractory mCRC (5.3 months with placebo *vs* 7.1 months with TAS-102; HR 0.68, 95% CI 0.58 to 0.81; P<0.001) (77). The combination of bevacizumab and TAS-102 was explored in a phase II trial which randomised patients to receive standard-dose TAS-102 with or without bevacizumab (78). Combination therapy was associated with a modest, although statistically significant, improvement in median PFS (4.6 *vs* 2.6 months, HR 0.45, 95% CI 0.29-0.72) and OS (9.4 *vs* 6.7 months, HR 0.55, 95% CI 0.32-0.94) and a higher ORR (67% *vs* 51%).

3.2 Predictive biomarkers of anti-angiogenic drugs in colorectal cancer

Despite the importance of anti-angiogenic treatment for targeting this critical pathway of the disease, not all patients with mCRC benefit from this treatment, and in addition, a large proportion of them present severe AEs. There is an unmet

clinical need driving the search for biomarkers of response to anti-angiogenic therapy in mCRC. Nevertheless, established biomarkers to predict response to anti-angiogenic treatments in mCRC are yet to be identified, as the different biomarkers tested to date have failed to show clear clinical utility (79, 80).

Retrospective data suggest that hypertension could predict treatment efficacy of bevacizumab in patients with mCRC (81). Some studies are researching the role of imaging in the assessment of vascularity of mCRC by radiomics of MRI and CT scan, attempting to translate medical images into biological information about tumour angiogenic status (82). A post-hoc analysis of the VELOUR trial showed that patients with previous bevacizumab treatment showed higher levels of VEGF-A and PIGF, suggesting that it could be a mechanism of resistance, and a negative prognostic marker in these patients, without differences in OS or PFS regarding plasma levels of VEGF-A and PIGF (83). Other retrospective data support this prognostic role of plasma levels of VEGF-A, without implications in prediction of response to anti-angiogenic treatment (84–86). Furthermore, the pattern of histopathological growth may influence response to anti-angiogenic agents (87). Vessel co-option is implicated as a major mechanism of resistance to these therapies and could represent a simple yet valuable biomarker of response (88).

3.3 Novel therapeutic strategies targeting angiogenesis in colorectal cancer

As previous reviewed, bevacizumab, aflibercept, regorafenib and ramucirumab have significantly improved both PFS and OS of mCRC in different clinical settings, from first line to the refractory scenario. Novel antiangiogenic agents and innovate combinations have been developed in recent years.

Fruquintinib is a novel receptor TKI inhibiting VEGFR 1, 2 and 3. Safety of this novel molecule was evaluated in a phase Ib trial, enrolling Asian patients with refractory mCRC, showing a manageable toxicity profile with the dosage of 5 mg once daily for 3 weeks with a 4-week cycle, giving a disease control rate (DCR) of 83.3% and 16-week PFS of 65% (89). The phase III FRESCO trial was a multicentre Asian trial in which 416 patients were randomised using a 2:1 ratio to receive fruquintinib with best supportive care or placebo plus best supportive care (90). Patients who received previous VEGFR inhibitors were excluded. Significant improvements were seen in the active fruquintinib treatment arm compared with placebo for OS (9.3 *vs* 6.6 months; HR: 0.65; $P<0.001$), PFS (3.7 *vs* 1.8 months; $P<0.001$), ORR (4.7% *vs* 0.0%; $P=0.01$) and DCR (62.2% *vs* 12.3%; $P<0.001$). This benefit was independent of previous treatment with anti-angiogenic agents or molecular status. The global FRESCO-2 trial (NCT04322539) is ongoing to confirm the results of the phase III FRESCO trial conducted in China.

Microsatellite stable (MSS) mCRC patients do not respond to monotherapy immunotherapy as demonstrated in many clinical trials (91–94). This population represents 95% of all patients with mCRC. Different combinations of immunotherapy with cofactors are being tested to achieve a change of a cold immune microambient to a hot microambient. One promising combination explored is the association of anti-angiogenic treatment with immunotherapy, as the blockade of VEGF leads to vasculature normalisation, thus permitting tumour infiltration with effector immune cells and the maturation of DCs (95–97).

Lenvatinib is a multiple kinase inhibitor. It inhibits the three main VEGFRs, VEGFR1, 2 and 3, as well as FGFR1, 2, 3 and 4, PDGFR, c-Kit and the RET proto-oncogene. Combination of pembrolizumab and lenvatinib has demonstrated the activation of CD8+ T cells, reduction of TAMS, leading to tumour reduction in murine models (98). A phase II non-randomised trial explored the combination of pembrolizumab with lenvatinib in MSS mCRC, demonstrating an ORR of 22% and a median PFS of 2.3 months (99). An ongoing randomised phase III trial (NCT04776148) is comparing lenvatinib plus pembrolizumab to standard of care in refractory MSS mCRC patients.

4 Hepatocellular carcinoma

HCC is the third leading cause of cancer-related death worldwide and its incidence is increasing globally (5). Most patients will be diagnosed at or progress to advanced stages, where systemic therapies remain the only effective option (100). Anti-angiogenic therapies constitute the treatment backbone of advanced HCC and their combination with immunotherapies has provided unprecedented benefits to this population (100–102). However, this has not yet been translated into the intermediate and early disease settings, and is an area of active research (103).

4.1 Clinical overview of anti-angiogenic drugs in HCC

4.1.1 Advanced disease: First line setting

Sorafenib is the first TKI to demonstrate increased survival in HCC. It was approved in 2007 based on the results of the SHARP trial, an international, placebo-controlled phase III trial that randomised 602 patients with advanced HCC (BCLC-C or BCLC-B stage not amenable to transarterial chemo-embolisation [TACE]) with preserved liver function and performance status of 0-2, to sorafenib or placebo (Table 2).

TABLE 2 Overview of selected phase III trials evaluating anti-angiogenic agents in advanced HCC.

Trial	Disease setting	Treatment arms	N patients	OS		PFS		ORR (%)	DCR (%)	Grade 3-4 TRAEs (%)
				Median (mo)	HR (95% CI)	Median (mo)	HR (95% CI)			
IMbrave150	Advanced – 1st line	Atezolizumab + bevacizumab	336	19.2	0.66 (0.52-0.85)*	6.9	0.65 (0.53-0.81)*	30*	74*	43
		Sorafenib	165	13.4		4.3		11*	55*	46
SHARP	Advanced – 1st line	Sorafenib	299	10.7	0.69 (0.55-0.87)*	NA	NA	2	43*	45*
		Placebo	303	7.9				1	32*	32*
Asia-Pacific	Advanced – 1st line	Sorafenib	150	6.5	0.68 (0.5-0.93)*	NA	NA	3.3	35	NA
		Placebo	76	4.2				1.3	16	NA
REFLECT	Advanced – 1st line	Lenvatinib	478	13.6	0.92 (0.79-1.06)*	7.4	0.66 (0.57-0.77)*	24.1*	75.5	57
		Sorafenib	476	12.3		3.7		9.2*	60.5	49
COSMIC-312	Advanced – 1st line	Atezolizumab + cabozantinib	432	15.4	0.90 (0.69-1.18)	6.8	0.63 (0.44-0.91)*	11	78	53.8
		Sorafenib	217	15.5		4.2		3.7	65	31.9
		Cabozantinib	188		NA	5.8	0.71 (0.51-1.01)	6.4	84	55.2
LEAP-002	Advanced – 1st line	Lenvatinib + pembrolizumab	395	21.2	0.84 (0.71-0.99)	8.2 8.1	0.83 (0.71-0.98)	26.1		61.5
		Lenvatinib	399	19				17.5		56.7
Qin et al.	Advanced – 1st line	Rivoceranib + camrelizumab	272	22.1	0.62 (0.49-0.8)*	5.6 3.7	0.52 (0.41-0.65)*	78.3		80.9
		Sorafenib	271	15.2				5.9*	53.9	52.4
Qin et al.	Advanced – 1st line	Donafenib	334	12.1	0.83 (0.70-0.99)*	3.7	0.91 (0.76-1.08)	4.6	30.8	38
		Sorafenib	334	10.3		3.6		2.7	28.7	50
SUN1170	Advanced – 1st line	Sunitinib	530	7.9	1.3 (1.13-1.5)*	3.6	1.13 (0.99-1.3)	NA	NA	82.1
		Sorafenib	544	10.2		3				74.2
BRISK-FL	Advanced – 1st line	Brivanib	577	9.5	1.07 (0.94-1.23)	NA	NA	12	66	67
		Sorafenib	578	9.9				8.8	65	65
LIGHT	Advanced – 1st line	Linifanib	514	9.1	1.05 (0.9-1.22)	NA	NA	13	NA	85.3
		Sorafenib	521	9.8				7		75
SEARCH	Advanced – 1st line	Sorafenib + erlotinib	362	9.5	0.93 (0.78-1.11)	NA	NA	6.6	43.9*	64.9
		Sorafenib	358	8.5				3.9	52.5*	63.7
RESORCE	Advanced – 2nd line	Regorafenib	379	10.6	0.63 (0.5-0.79)*	3.1	0.46 (0.37-0.56)*	11	65	50
		Placebo	194	7.8		1.5		4	36	17
CELESTIAL	Advanced – 2nd line	Cabozantinib	470	10.2	0.76 (0.63-0.92)*	5.2	0.44 (0.36-0.52)	3.8	64	68
		Placebo	237	8		1.9		0.4	33	37
REACH-2	Advanced – 2nd line	Ramucirumab	197	8.5	0.71 (0.53-0.95)*	2.8	0.45 (0.34-0.60)*	59.9		NA
		Placebo	95	7.3	1.6	1		38.9		
Qin et al.	Advanced – 2nd line	Apatinib	267	8.7	0.79 (0.62-1)*	4.5	0.47 (0.37-0.60)*	11	61	77
		Placebo	133	6.8	1.9			2	29	19

DCR, disease control rate; HR, hazard ratio; mo, months; N, sample size; NA, not available; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; TRAEs, treatment-related adverse events. *Indicates statistically significant differences.

Sorafenib increased OS (median 10.7 *vs* 7.9 months, HR 0.69, 95% CI 0.55-0.87) and time to radiologic progression (median 5.5 *vs* 2.8 months, HR 0.58, 95% CI 0.45-0.74). The median duration of treatment was 5.3 months and the overall incidence of treatment-related AEs was 80% (104). These results were further supported by the Asia-Pacific trial, a randomised, confirmatory phase III trial with a similar design that was performed in China, South Korea and Taiwan, and randomised 271 patients to sorafenib or placebo (105). Sorafenib increased OS (median 6.5 *vs* 4.2 months, HR 0.68, 95% CI 0.5-0.93) although the median survival times were less than in the SHARP trial, owing to the inclusion of more

advanced patients, with a higher proportion of BCLC-C patients (95% *vs* 82%), extrahepatic spread (69% *vs* 53%) and worse performance status (ECOG PS1 69% *vs* 38%).

Since the approval of sorafenib, it became the standard comparator arm in all phase III trials, most of which led to disappointing results (106–109). Lenvatinib is the first TKI to have demonstrated non-inferiority in terms of OS compared with sorafenib (110). The REFLECT trial was a phase III, international, sorafenib-controlled study that randomised 954 advanced HCC patients without main portal vein thrombosis, less than 50% of liver occupation and absence of invasion of the bile duct, to lenvatinib or sorafenib. Lenvatinib showed non-inferiority in terms of OS (median 13.6 *vs* 12.3 months, HR 0.92, 95% CI 0.79-1.06) but did not achieve superiority. However, lenvatinib did show superior PFS (median 7.4 *vs* 3.7 months, HR 0.66, 95% CI 0.57-0.77) and ORR (24.1% *vs* 9.2%, odds ratio 3.13, 95% CI 2.15-4.56) according to investigator assessment using mRECIST (110, 111). The open-label design of the study may have influenced the unexpected differences in treatment duration and time to progression between the sorafenib and lenvatinib arms (112), although subsequent real-world studies have confirmed the efficacy of lenvatinib (113).

The combination of atezolizumab-bevacizumab has become the new standard of care first line treatment in advanced HCC (114, 115). The IMbrave150 trial was a phase III international, sorafenib-controlled trial that enrolled 501 patients randomised in a 2:1 ratio to atezolizumab-bevacizumab or sorafenib. The trial met its primary endpoint of OS, showing an increase of 5.8 months at final analysis (median 19.2 *vs* 13.4 months, respectively, HR 0.66, 95% CI 0.52-0.85) (116). Additionally, the combination improved PFS (median 6.9 *vs* 4.3 months, HR 0.65, 95% CI 0.53-0.81) and ORR (30% *vs* 11%, $P<0.001$) (116). Treatment-related grade 3-4 AEs were observed in 43% of the patients in the atezolizumab-bevacizumab arm and 46% of the patients in the sorafenib arm. Importantly, five fatal upper gastrointestinal bleeding events were observed in the experimental arm, which were attributed to bevacizumab (116). Two previous phase II trials testing bevacizumab monotherapy had shown an increased risk of upper gastrointestinal bleeding in 7-11% of patients (117, 118). To mitigate this risk, a mandatory esophagogastroduodenoscopy had to be performed in the 6 months prior to enrolment and any varices had to be treated per local standard of care (114). A similarly designed phase III trial was reported in China and evaluated the combination of sintilimab, a programmed cell death protein 1 (PD-1) inhibitor, with IBI-305, a bevacizumab biosimilar. The trial randomised 571 patients to sintilimab-bevacizumab biosimilar or sorafenib in a 2:1 ratio and showed an improvement in OS (median not reached *vs* 10.4 months, HR 0.57, 95% CI 0.43-0.75) and PFS (median 4.6 *vs* 2.8 months, HR 0.56, 95% CI 0.46-0.7) (119). Both of these trials have demonstrated the efficacy of combining anti-VEGFA antibodies with ICIs.

The results of the COSMIC-312 trial, a phase III trial that tested the combination of cabozantinib and atezolizumab (120), enrolled 877 patients who were randomly assigned to the combination, cabozantinib or sorafenib in a 2:1:1 ratio. The dual primary endpoints are OS and PFS. The interim analysis demonstrated an improvement in PFS in the modified intention-to-treat population comprising the first 372 randomised patients (median 6.8 *vs* 4.2 months, HR 0.63, 95% CI 0.44-0.91), however, no improvement in OS was observed in the intention-to-treat population (median 15.4 *vs* 15.5 months, HR 0.9, 95% CI 0.69-1.18). The combination of TKIs with immunotherapies has been recently explored in two additional trials (121, 122). The LEAP-002 trial is an international, phase III, randomized, double blind study that enrolled 794 patients with advanced HCC and were randomly assigned to the combination of lenvatinib-pembrolizumab (N=395) or lenvatinib alone (N=399). The trial did not reach the prespecified threshold for none of the dual primary endpoints (OS and PFS). However, the median OS for the combination arm was the longest survival reported to date in the first-line setting (21.2 months). Importantly, this data further supported the role of lenvatinib monotherapy in this setting, with a median OS of 19 months (121). A second trial reported at ESMO 2022 was the combination of camrelizumab, an anti-PD1 monoclonal antibody, and rivoceranib, a VEGFR2-TKI, in the first-line setting. This was an international, phase III, open-label study that compared the combination to sorafenib (122). The dual primary endpoints were PFS and OS. The trial met it's endpoints and showed a significant increase in OS (median 22.1 vs 15.2, HR 0.62 95% CI 0.49-0.8) and PFS (median 5.6 vs 3.7, HR 0.52 95% CI 0.41-0.65), as well as an increase in ORR (25.4 vs 5.9%). Despite these encouraging results, the combination will have to be tested in other populations as most of the included patients were Chinese. Furthermore, the open-label design of the study led to a high number of consent withdrawals in the control arm, which will have to be explored to understand its potential impact on the study results.

In China, the TKI donafenib has proved to be superior to sorafenib in the first line setting of advanced HCC (123).

4.1.2 Advanced disease: Second line setting

Three TKIs have demonstrated improved outcomes in the second line setting after progression on sorafenib, regorafenib (124), cabozantinib (125) and ramucirumab in patients with alpha-feto protein (AFP) levels ≥400 ng/mL (126). The RESORCE trial was an international, phase III, double-blind, placebo-controlled trial that randomised 573 patients to regorafenib or placebo and was stratified based on region, performance status, macrovascular invasion, extrahepatic spread and AFP levels (124). Importantly, only patients who had previously tolerated sorafenib, defined as patients who had received ≥400 mg/day for ≥20 of the last 28 days of treatment,

were included. Regorafenib significantly improved OS compared to placebo (median 10.6 *vs* 7.8 months, HR 0.63, 95% CI 0.5-0.79) and PFS assessed by mRECIST (median 3.1 *vs* 1.5 months, HR 0.56, 95% CI 0.37-0.56) (124). The rate of grade 3-4 treatment-related AEs was 50% in the regorafenib arm compared with 17% in the placebo arm.

The CELESTIAL trial had a similar design to the RESORCE trial although prior tolerance to sorafenib was not mandatory and patients could have progressed on up to two lines of systemic treatment. The trial randomised 707 patients in a 2:1 ratio to cabozantinib or placebo, stratified by region, macrovascular invasion, extrahepatic spread and disease etiology (125). It met its primary endpoint of OS (median 10.2 *vs* 8 months, HR 0.76, 95% CI 0.63-0.92) and showed a significant prolongation of PFS (5.2 *vs* 1.9 months, HR 0.44, 95% CI 0.36-0.52). Therefore, cabozantinib is the only TKI with evidence of efficacy in HCC following two prior lines of systemic treatment.

Ramucirumab was initially tested in the phase III REACH trial in the second line setting of advanced HCC following progression to sorafenib (127). Despite the trial being negative, an exploratory subgroup analysis showed significant benefit in patients with a baseline AFP level of ≥400 ng/mL. Hence, the REACH-2 study was designed as an international, phase III, double-blind, placebo-controlled trial and randomised 292 patients with an AFP level ≥400 ng/mL to ramucirumab or placebo (126). Ramucirumab increased OS (median 8.5 *vs* 7.3 months, HR 0.71, 95% CI 0.53-0.95) and PFS (2.8 *vs* 1.6 months, HR 0.45, 95% CI 0.34-0.6). A pooled analysis of all patients with baseline AFP levels ≥400 ng/mL (*N*=542) in both REACH and REACH-2 trials confirmed the survival benefit (median 8.1 *vs* 5 months, HR 0.69, 95% CI 0.57-0.84) (126).

In China, the VEGFR-2 inhibitor apatinib has improved survival in the second or third line settings of advanced HCC following treatment with sorafenib or FOLFOX (128).

4.2 Early and intermediate setting

The use of anti-angiogenic therapies in earlier settings of HCC have so far provided disappointing results. In the early setting, the only phase III trial to have tested TKIs as adjuvant therapy is the STORM study, an international, phase III, double-blind, placebo-controlled trial that enrolled 1114 patients with HCC suitable for local treatment (either ablation or resection) and a high or intermediate risk of recurrence (defined as tumours >2 cm or vascular invasion or satellites) to either adjuvant sorafenib for 4 years or placebo. No difference was observed in the primary endpoint of recurrence-free survival (median 33.3 *vs* 33.7 months, HR 0.94, 95% CI 0.78-1.13) or in OS (median not reached, HR 0.995, 95% CI 0.76-1.3) (129).

In the intermediate setting, three TKIs have been tested in combination with TACE in four phase III trials (130), namely, sorafenib (131, 132), brivanib (133) and orantinib (134). Unfortunately, none of these trials demonstrated an OS benefit compared with TACE alone. Whilst all four trials had a similar design, the primary endpoints were different: BRISK-TA (133) and ORIENTAL (134) trials assessed OS, while the trials testing sorafenib used time to progression (131) or PFS (132). Despite these discouraging results, the TACTICS trial was recently published, testing the combination of sorafenib initiated 2-3 weeks before TACE compared with TACE alone (135). This was a phase II, open-label, multicentre trial that enrolled 156 patients with a co-primary endpoint of OS and PFS. However, the definition of progression in this trial was unconventional and included untreatable tumour progression, transient deterioration to Child-Pugh C or appearance of vascular invasion/extrahepatic spread. The trial demonstrated a significant improvement in PFS (median 25.2 *vs* 13.5 months, HR 0.59, 95% CI 0.41-0.87) (135) but did not show any improvement in OS at the final analysis (median 36.2 *vs* 30.8 months, HR 0.86, 95% CI 0.61-1.22), casting doubts on the true value of this unconventional definition of progression (136).

Therefore, to date, no anti-angiogenic therapy is recommended in earlier settings of HCC.

4.3 Predictive biomarkers of anti-angiogenic drugs in HCC

The field of biomarker discovery in HCC is daunting and has so far led to disappointing results. The only available FDA-approved biomarker to guide treatment decision is AFP before initiating ramucirumab, based on the results of the REACH and REACH-2 trials (126, 127). No other biomarker has proven capable of predicting response to other anti-angiogenic therapies.

An exploratory analysis of 10 plasma markers (Ang2, EGF, bFGF, VEGF, sVEGFR-2, sVEGFR-3, HGF, s-c-KIT, IGF-2 and Ras) of patients enrolled in the SHARP trial found that despite that Ang2 and VEGF independently predicted survival in the entire cohort, none of the biomarkers assessed could predict response to sorafenib (137). In the sorafenib arm, high s-c-KIT and low HGF showed a trend towards enhanced survival (*P*-values of interaction 0.081 and 0.073, respectively) (137). Given the inherent difficulties of acquiring tissue specimens from advanced HCC patients, the same authors performed a thorough transcriptomic assessment of patients enrolled in the STORM trial, who were surgically resected and received sorafenib in the adjuvant setting (138). Tumour specimens from 188 patients were analysed by gene expression profiling, targeted exome sequencing, immunohistochemistry and fluorescence *in situ* hybridisation for VEGFA. None of the tested biomarkers, gene signatures or mutations predicted survival. A 146-gene signature was generated that could predict improved recurrence-free survival with sorafenib, although this has not been translated into the clinical setting

due to lack of validation (138). Additionally, genomic variations of the *SCL15A2* gene, involved in drug transport, have been proposed as an additional biomarker of response to sorafenib (139). In line with this, a retrospective study found that the expression of OCT1 (another major player involved in sorafenib uptake) in the plasma membrane was associated with improved outcomes following sorafenib treatment (140). These studies highlight the importance of SLC transporters in sorafenib uptake and underline their possible impact on patient survival.

To identify potential biomarkers of response to regorafenib, an analysis of plasma from patients enrolled in the RESORCE trial was performed (141). The authors analysed 294 plasma proteins and 750 miRNAs. Additionally, next-generation sequencing of tumour tissue from 7 responders and 10 non-responders and expression of 770 genes involved in oncogenic and inflammatory pathways in 46 tumour tissues was performed (141). Decreased baseline plasma concentrations of five proteins (angiopoietin 1, cystatin B, the latency-associated peptide of transforming growth factor beta 1, oxidised low-density lipoprotein receptor 1 and C-C motif chemokine ligand 3) was associated with improved survival with regorafenib. Additionally, nine miRNAs were also associated with improved survival with regorafenib (MIR30A, MIR122, MIR125B, MIR200A, MIR374B, MIR15B, MIR107, MIR320, and MIR645) (141).

In a similar plasma analysis including VEGF, ANG2, FGF19, FGF21 and FGF23 of 407 patients included in the REFLECT study, a higher baseline level of FGF21 was predictive for longer OS with lenvatinib compared with sorafenib (*P*-value of interaction 0.0397) (142). Similarly, a plasma analysis of 674 patients included in the CELESTIAL trial did not identify any biomarkers predictive of response to cabozantinib (143). High levels of MET, HGF, GAS6, IL-8 and ANG2 and low levels of IGF-1 were associated with shorter survival in the placebo arm and this association was also observed for MET, IL-8, and ANG2 in the cabozantinib group (143).

More recently, an integrated molecular analysis was performed, comprising RNA sequencing, DNA sequencing and simple and multiplex immunohistochemistry of 358 patients included in the phase Ib GO30140 (144) and the phase III IMbrave150 trial (114, 116). This showed that pre-existing immunity, including the expression of a T effector transcriptomic signature and CD8+ T cell infiltration, predicted response to the combination of atezolizumab-bevacizumab, but not to sorafenib (145). Importantly, improved outcomes for the combination *vs* atezolizumab monotherapy was associated with high VEGFR-2 expression. Conversely, reduced benefit from the combination was associated with a low Treg/effector T cell ratio (145). These data highlight the synergistic effects of the combination of atezolizumab-bevacizumab and suggest several predictive biomarkers that will need validation in future trials.

4.4 Novel therapeutic strategies targeting angiogenesis in HCC

The breakthrough marked by the IMbrave150 trial with the atezolizumab-bevacizumab combination, has fuelled the development of multiple combinations that are being tested across all settings of this disease (102). Preclinical data strongly support combining anti-angiogenic drugs with immunotherapies and local treatments in the intermediate setting (101, 103). Local ablation or TACE increases the release of antigens, proinflammatory cytokines and proangiogenic factors (such as VEGF-A and HIF1) which promote an immune response that can be further sustained by increasing the activation of cytotoxic cells through immune checkpoint inhibition and decreasing the infiltration of immunosuppressive cells such as MDSCs and Tregs through the inhibition of angiogenesis (103). This constitutes the rationale for the design of trials combining TACE with durvalumab/bevacizumab (NCT03778957), atezolizumab/bevacizumab (NCT04712643) or pembrolizumab/lenvatinib (NCT04246177) (Table 3). More intriguingly, the outstanding survival outcomes observed with the atezolizumab and bevacizumab combination in the advanced setting, with a median OS of 19.2 months - which is similar to the expected survival of intermediate-stage HCC treated with TACE (100) - has sparked the development of two trials comparing standard TACE directly with systemic treatment (atezolizumab/bevacizumab [NCT04803994] or regorafenib/nivolumab [NCT04777851]) (Table 3).

Applying these combinations in earlier settings, when cure is still possible, is being eagerly pursued in phase III clinical trials. Three trials are currently exploring atezolizumab-bevacizumab (NCT04102098), camrelizumab/apatinib (NCT04639180) and sintilimab/bevacizumab (NCT04682210) in the postsurgical setting to decrease the risk of recurrence (Table 3). However, T-cell priming after the tumour was removed as this can be less efficient due to the close-to-non-existent tumour antigen burden (101). In addition, the high response rate achieved with new combinations could facilitate downstaging and improve tumour resectability when applied in the pre-surgical setting. Accordingly, a phase Ib study that enrolled 15 patients with unresectable HCC who received 8 weeks of neoadjuvant cabozantinib and nivolumab found that 13 patients ultimately underwent resection, 12 of whom had no residual tumour and 5 had major or complete pathological response. Importantly, none of the patients presented disease progression according to RECIST 1.1 (146).

Novel treatments and combinations are being explored in the advanced setting of HCC (Table 3). Lenvatinib-pembrolizumab constitutes one of the most promising combinations based on the phase Ib KEYNOTE-524 trial that enrolled 100 advanced HCC patients who had received no prior

TABLE 3 Overview of selected trials testing novel antiangiogenic agents and combinations in HCC.

Trial	Treatment	Phase	Setting	Enrolment target	Primary endpoint
NCT04102098 IMbrave050	Atezolizumab + bevacizumab	III	Early-adjuvant	668	RFS
NCT04639180	Camrelizumab + apatinib	III	Early-adjuvant	674	RFS
NCT04682210	Sintilimab + bevacizumab	III	Early-adjuvant	246	RFS
NCT03778957 EMERALD-1	TACE + durvalumab + bevacizumab	III	Intermediate	724	PFS
NCT04712643	TACE + atezolizumab + bevacizumab	III	Intermediate	342	OS TACE-PFS
NCT04246177 LEAP-012	TACE + pembrolizumab + lenvatinib	III	Intermediate	950	OS PFS
NCT05220020	TACE + lenvatinib	III	Intermediate	299	2y OS
NCT04803994 ABC-HCC	Atezolizumab + bevacizumab	III	Intermediate	434	TFTS
NCT04777851 RENOTACE	Regorafenib + nivolumab	III	Intermediate	496	PFS
NCT05320692	TACE + camrelizumab + apatinib	III	Intermediate	360	PFS
NCT05301842 EMERALD-3	TACE + durvalumab + tremelimumab +/- lenvatinib	III	Intermediate	525	PFS
NCT04194775	CS1003 + lenvatinib	III	Advanced – 1st line	525	OS PFS
NCT04465734	HLX10 + HLX04	III	Advanced – 1st line	477	OS PFS
NCT04344158	AK105 + anlotinib	III	Advanced – 1st line	648	OS
NCT04560894	SCT-I10A + SCT510	III	Advanced – 1st line	621	OS PFS
NCT04723004	Toripalimab + bevacizumab	III	Advanced – 1st line	280	OS PFS
NCT04541173	Y90 TARE + atezolizumab + bevacizumab	II	Advanced – 1st line	128	PFS
NCT05377034 STRATUM	SBRT + atezolizumab + bevacizumab	II	Advanced – 1st line	176	ORR
NCT04976634	Lenvatinib + pembrolizumab + bezulfitan	II	Advanced – 1st line	400	DLT Safety ORR
NCT04524871 Morpheus-Liver	Diverse drugs and combinations	I/II	Advanced – 1st line	280	ORR
NCT04310709 RENOBATE	Regorafenib + nivolumab	I/II	Advanced – 1st line	42	ORR
NCT04770896 IMbrave251	Atezolizumab + sorafenib/lenvatinib	III	Advanced – 2nd line	554	OS
NCT04170556 GOING	Regorafenib + nivolumab	I/IIa	Advanced – 2nd line	78	Safety
NCT04718909 REGSIN	Regorafenib + sintilimab	II	Advanced – 2nd line	180	PFS
NCT04212221	MGD013 + brivanib	I/II	Advanced – 2nd line	300	DLT Safety ORR
NCT03475953 REGOMUNE	Avelumab + regorafenib	I/II	Advanced – 2nd line	482	ORR

DLT, dose-limiting toxicity; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; TACE, transarterial chemoembolization; TFTS, time to failure of treatment strategy; y, year.

systemic treatment (147). The combination achieved an ORR of 46% according to mRECIST, with a disease control rate of 88%, a median OS of 22 months and median PFS of 9.3 months (147). This combination is currently being evaluated in a phase III trial (NCT03713593). To optimize the sequencing of TKIs and ICIs, the GOING trial (NCT04170556) is evaluating the priming effect of regorafenib monotherapy administered for 8 weeks prior to incorporating nivolumab into the regimen. Finally, the

IMbrave251 trial (NCT04770896) is evaluating the combination of atezolizumab with sorafenib or lenvatinib in the second line following progression on atezolizumab-bevacizumab. These and other trials shown in Table 3 are likely to change the treatment landscape of HCC in the near future.

5 Gastroesophageal cancer

Gastroesophageal cancers are a group of aggressive and highly lethal neoplasms. Gastric cancer represents the fifth most common cancer and the fourth most common cause of cancer-related death, while oesophageal cancer ranks seventh in terms of incidence and sixth in terms of mortality (5). Despite substantial advances over the last decade, prognosis remains poor, with an overall 5-year OS rate of 29% and 20% for gastric and oesophageal cancer, respectively (5). The pre-malignant form of oesophageal adenocarcinoma, known as Barrett's oesophagus, expresses high levels of VEGFR2 (148). In gastric cancer, VEGF expression in tumour tissue or blood are correlated with prognosis, stage and risk of metastasis (149). Human epidermal growth factor receptor 2 (HER2) therapy and anti-angiogenic agents are the only two biological targeted therapies that have improved OS in patients with gastric or gastroesophageal junction adenocarcinoma.

There are currently no anti-angiogenic therapies approved for the treatment of oesophageal cancer. Small anti-angiogenic molecules such as sunitinib (in combination with paclitaxel or FOLFIRI) or sorafenib (in combination with docetaxel and cisplatin), erlotinib (with bevacizumab and neoadjuvant chemoradiation), apatinib (as maintenance treatment after chemo-radiation in localised oesophageal squamous cell carcinoma) or anlotinib (as monotherapy in the refractory setting) have shown limited or no efficacy in small phase II trials (150–154). Combinations of TKIs with immunotherapy are being evaluated (155). Bevacizumab was evaluated in combination with chemotherapy in two phase II trials, and was safe but with limited benefits (156, 157).

In gastric cancer, bevacizumab was evaluated in the AVAGAST phase III trial, comparing standard chemotherapy with or without bevacizumab, failing to show improvement in OS (158, 159). The phase III REGARD trial randomised patients with advanced gastric cancer to receive ramucirumab or placebo as second line treatment, with an OS of 5.2 *vs* 3.8 months, respectively (HR 0.776; *P*=0.047) (160). Following these results, the FDA approved ramucirumab for advanced gastric and gastroesophageal junction adenocarcinomas in 2014. The RAINBOW phase III trial compared weekly paclitaxel in combination with ramucirumab or placebo in patients refractory to a fluoropyrimidine plus platinum combination. Median OS was 9.6 months in the ramucirumab plus paclitaxel arm *vs* 7.4 months in the placebo plus paclitaxel group (HR 0.807; *P*=0.017), and paclitaxel plus ramucirumab became a recommended standard second line treatment for gastric cancer (161). RAINFALL was a global phase III trial that compared cisplatin plus capecitabine or 5-FU in combination with ramucirumab or placebo in the first line setting of patients with gastric or gastroesophageal junction adenocarcinoma (162). This trial demonstrated an improvement in PFS (5.7 *vs* 5.4 months; HR 0.75, *P*=0.011) but not in OS (11.2 *vs* 10.7 months; HR 0.96). Multiple trials are testing the combination of immunotherapy with ramucirumab, with promising signals of efficacy (163–165).

Apatinib is a small TKI that was tested in a Chinese phase III trial that compared apatinib with placebo in patients with refractory advanced gastric cancer, showing a statistically significant difference in OS (6.5 *vs* 4.7 months; *P*=0.0156) that led to its approval by the Chinese regulatory agency (166). The ANGEL trial (NCT03042611) is ongoing to confirm these results in the global population.

Other molecules such as aflibercept (in combination with FOLFOX), sorafenib (in combination with capecitabine plus cisplatin in the first line setting), sunitinib (in combination with FOLFIRI in second or third line) pazopanib (in combination with 5-FU plus leucovorin plus oxaliplatin) or fruquintinib (in combination with paclitaxel in second line treatment in China) have been tested in phase II trials, showing only marginal benefit in PFS (151, 167–171).

6 Neuroendocrine cancer

Neuroendocrine tumours (NETs) are a heterogeneous family of neoplasms that can arise from almost everywhere throughout the body, as they originate from the diffuse neuroendocrine system. NETs are rare (less than 7 new cases/100,000 inhabitants/year), however their incidence has increased over the last few decades (172). Well-differentiated NETs are characterised by rich vascularisation, with this phenomenon known as the "neuroendocrine paradox", as the vascularisation is inversely related to the grade of aggressiveness of the tumour. NETs show high expression of PDGFR and c-Kit, demonstrating the importance of the angiogenesis pathway of these tumours (173). Sunitinib and surufatinib have shown activity in phase III trials *vs* placebo.

Sunitinib was tested in a phase III placebo-controlled trial of patients with advanced, well-differentiated pancreatic NETs (pNETs), in which patients could have received prior treatment (174). The study was discontinued early as a difference between placebo and sunitinib arm was observed benefiting patients in the active control arm. Median PFS was 11.4 months in the sunitinib group compared with 5.5 months in the placebo group (HR: 0.42; *P*<0.001). Posterior actualised data showed an improvement of median OS (38.6 *vs* 29.1 months in sunitinib *vs* placebo, respectively) (175). Surufatinib has shown benefit in a phase III placebo-controlled trial in advanced

pancreatic NETs in a Chinese population (median PFS 10.9 months for surufatinib *vs* 3.7 months for placebo; HR 0.49; *P*=0.0011) (176). This agent has also reported benefit over placebo for extra-pancreatic advanced NETs in a Chinese population (176). Different phase II trials have tested multikinase inhibitors such as pazopanib, lenvatinib and cabozantinib, showing clinical activity in patients with NETs (177–181). Two ongoing phase III trials are evaluating axitinib (NCT01744249) and cabozantinib (NCT03375320).

7 Pancreatic and biliary tract cancer

Pancreatic cancer is a highly lethal disease with a rising incidence of 0.5-1% per year and is expected to become the second leading cause of cancer death by 2030 in the United States (182). Multiagent chemotherapy is recommended across all stages of the disease, either perioperatively in resectable/borderline resectable disease or to improve survival outcomes in advanced stages (183, 184). To date, only gemcitabine combined with albumin-bound paclitaxel (185) and FOLFIRINOX (fluorouracil, irinotecan, oxaliplatin, leucovorin) (186) regimens have demonstrated superiority over gemcitabine monotherapy in the first line metastatic setting, while FOLFOX (fluorouracil, oxaliplatin, leucovorin) (187, 188) and fluorouracil combined with liposomal irinotecan (189) have improved outcomes in the second line setting following a gemcitabine-based regimen. Most trials testing anti-angiogenic agents were performed before these combinations were approved and were combined with gemcitabine monotherapy in the first line setting. Bevacizumab was tested in two independent trials (Table 4). First, the CALGB80303 phase III trial randomised 602 advanced pancreatic cancer patients to gemcitabine monotherapy or combined with bevacizumab and showed no improvement in OS (median 5.8 *vs* 5.9 months, HR 1.05, 95% CI 0.88-1.24) (190). A second study tested the combination of bevacizumab with gemcitabine and erlotinib compared with gemcitabine and erlotinib alone and found a significant improvement in PFS (median 4.6 *vs* 3.6 months, HR 0.73, 95% CI 0.61-0.86) but no difference in OS (median 7.1 *vs* 6 months, HR 0.89, 95% CI 0.74-1.07) (191). Aflibercept was explored in a similarly designed phase III trial in combination with gemcitabine but was stopped early due to futility (192) and in a phase III trial with elpamotide, a peptide targeting VEGFR-2, the primary endpoint of OS was not reached when combined with gemcitabine (193). Additionally, several randomised phase II and III investigations have explored the use of different TKIs combined with gemcitabine including axitinib (194), sorafenib (195) and sunitinib (196), also failing to show any significant survival improvement over gemcitabine alone. More recently, the HCRN GI14-198 phase II trial tested ramucirumab in combination with a multiagent chemotherapy and randomised 86 patients diagnosed with treatment-naïve advanced pancreatic cancer to modified FOLFIRINOX combined with ramucirumab or placebo (197). The trial failed to improve outcomes in terms of PFS, ORR and OS (197). The reasons behind the failure of these trials are largely unknown, although novel therapies modulating the desmoplastic microenvironment and tumour stroma may help to enhance the clinical activity of anti-angiogenic therapies in this disease.

Biliary tract cancer refers to a spectrum of malignancies including cholangiocarcinoma and gallbladder adenocarcinoma (198). Their incidence is increasing globally, with a 5-year OS rate bordering 10%, and they represent ~2% of all cancer-related deaths worldwide annually (199). In advanced stages, the combination of cisplatin and gemcitabine has remained the established first line chemotherapy regimen for the past 12 years (200, 201), although this is likely to change given the improved survival observed with the addition of durvalumab in the TOPAZ-1 trial (202). The use of anti-angiogenic drugs has only been explored in phase II investigations and none of these combinations has reached later stages of development (Table 4). The largest of these trials was a randomised, phase II, three-arm trial exploring the combination of ramucirumab, merestinib or placebo with cisplatin-gemcitabine. The trial failed to meet its primary endpoint of PFS (median 6.5 *vs* 7 *vs* 6.6 months, ramucirumab *vs* placebo HR 1.12, 95% CI 0.9-1.4) (203). An exploratory analysis of mutations and gene expression signatures identified no predictive biomarkers (203). The ABC-03 trial was a randomised phase II trial that tested the combination of cediranib, an oral VEGFR-1, -2 and -3 inhibitor, with cisplatin and gemcitabine and showed no improvement in outcomes compared with placebo (204). In an exploratory biomarker analysis, circulating PDGFbb levels predicted benefit from cediranib (P-value of interaction 0.002) (204). Other trials that compared suboptimal chemotherapy regimens combined with sorafenib (205) or vandetanib (206) also failed to improve survival. New strategies, including the potential synergy of anti-angiogenic therapies when combined with immunotherapies and chemotherapy, are leading to novel combinations that could change the treatment landscape in the near future.

8 Discussion and future prospects

Anti-angiogenic therapies have been extensively evaluated across many gastrointestinal and hepatobiliary tumours. In some malignancies, such as HCC or CRC, these therapies provide unquestionable survival benefits either alone or combined with immunotherapy or chemotherapy, respectively. In others, such as oesophageal, biliary tract or pancreatic cancers, anti-angiogenics do not improve outcomes when combined with currently approved therapies. The development of novel anti-angiogenic strategies has stalled in recent years, partly due to the concurrent development of novel and highly effective drugs, especially immune-based therapies (207). However, whilst new anti-angiogenic drugs are not expected to enter the clinical setting in the near future, the use of approved anti-angiogenic therapies is likely to increase exponentially, owing to their highly synergistic effect with immunotherapies and other

TABLE 4 Overview of selected phase II-III trials testing antiangiogenic agents in pancreatic and biliary tract cancer.

Trial	Disease setting	Treatment arms	N patients	OS		PFS		ORR (%)	DCR (%)	Grade 3-4 TRAEs (%)
				Median (mo)	HR (95% CI)	Median (mo)	HR (95% CI)			
CALGB 80803	Pancreatic – 1st line	Gemcitabine + bevacizumab	302	5.8	1.04 (0.88-1.24)	3.8	0.86 (0.74-1.01)	13	54	NA
		Gemcitabine + placebo	300	5.9		2.9		10	44	NA
Van Cutsem et al.	Pancreatic – 1st line	Gemcitabine + erlotinib + Bevacizumab	306	7.1	0.89 (0.74-1.07)	4.6	0.73 (0.61-0.86)*	14	62	74
		Gemcitabine + Erlotinib + Placebo	301	6		3.6		9	59	70
Kindler et al.	Pancreatic – 1st line	Gemcitabine + axitinib	316	8.5	1.01 (0.79-1.31)	4.4	1.01 (0.78-1.30)	5	35	NA
		Gemcitabine + placebo	316	8.3		4.4		2	35	NA
BAYPAN	Pancreatic – 1st line	Gemcitabine + sorafenib	52	9.2	1.27 (0.84-1.93)	5.7	1.04 (0.70-1.55)	19	65	88
		Gemcitabine + placebo	52	8		3.8		23	71	79
Rougier et al.	Pancreatic – 1st line	Gemcitabine + aflibercept	271	6.7	1.17 (0.92-1.47)	3.7	1.02 (0.83-1.25)	NA	NA	77
		Gemcitabine + placebo	275	7.8		3.7		NA	NA	67
Reni et al.	Pancreatic – 1st line	Gemcitabine + sunitinib	28	10.6	0.71 (0.4-1.26)	3.2	0.51 (0.29-0.89)	0	52	NA
		Gemcitabine + placebo	28	9.2		2		0	21	NA
Bergmann et al.	Pancreatic – 1st line	Gemcitabine + sunitinib	52	7	1.06 (0.69-1.63)	2.7	1.06 (0.71-1.58)	7	75	NA
		Gemcitabine + placebo	54	8.5		3.1		6	67	NA
PEGASUS-PC	Pancreatic – 1st line	Gemcitabine + elpamotide	100	8.4	0.87 (0.49-1.56)	3.7	NA	NA	60	NA
		Gemcitabine + placebo	53	8.5		3.8		60	NA	NA
HCRN GI14-198	Pancreatic – 1st line	FOLFIRINOX + ramucirumab	42	10.3	NA	5.6	NA	18	NA	NA
		FOLFIRINOX + placebo	40	9.7		6.7		23	NA	NA
ABC-03	Biliary tract – 1st line	Cisplatin + gemcitabine + cediranib	62	14.1	0.86 (0.58-1.27)	8	0.93 (0.65-1.35)	44*	78	NA
		Cisplatin + gemcitabine + placebo	62	11.9		7.4		19*	65	NA
Valle et al.	Biliary tract – 1st line	Cisplatin + gemcitabine + ramucirumab	106	10.5	1.33 (0.96-1.86)	6.5	1.12 (0.9-1.4)	31*	81	85
		Cisplatin + gemcitabine + placebo	101	13		6.6		33*	78	76
		Cisplatin + gemcitabine + merestinib	102	14	0.95 (0.67-1.34)	7	0.92 (0.73-1.15)	20*	83	79
Van Gogh	Biliary tract – 1st line	Vandetanib	56	7.5	NA	3.4	1.3 (0.86-1.96)	3*	25	NA
		Gemcitabine + vandetanib	57	9.3		3.7	1.3 (0.75-1.7)	19*	30	NA
		Gemcitabine + placebo	52	10.1	NA	4.9		14*	40	NA
Moehler et al.	Biliary tract – 1st line	Gemcitabine + sorafenib	49	8.4	1.20 (0.75-1.93)	3	1.28 (0.81-2.02)	14	86	NA
		Gemcitabine + placebo	48	11.2	4.9	1.28 (0.81-2.02)		10	90	NA

CI, confidence interval; DCR, disease control rate; HR, hazard ratio; mo, months; N, sample size; NA, not available; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; TRAEs, treatment-related adverse events. * Indicates statistically significant differences.

drug families (28). New combinations and applications in earlier disease settings is already being intensively explored in many diseases and will reshape the treatment scenario (101). Furthermore, recent studies have unveiled the heterogeneity of tumour endothelial cells and may support the use of patient-tailored antiangiogenic drug combinations to overcome this heterogeneity (40–42). Importantly, novel insights on resistance mechanisms support the combination of antiangiogenic drugs with distinct partners, such as inhibitors of the TGFß pathway, autophagy or CXCR4 (39). An intriguing area of research is the determination of the ideal dose of anti-angiogenic agents, especially when combined with other therapies. Until now, this has been

based on the maximum tolerated dose in most cases, reflecting the belief that the higher dose of a drug will lead to a higher efficacy. Although this may be true for cytotoxic agents, this principle may not apply to anti-angiogenic drugs, where normalisation of blood vessels may be more important than vessel depletion to improve their synergistic effects (22).

Finally, the development of robust and validated biomarkers represents a clear unmet need in this field. It is unlikely that a unique biomarker common to distinct anti-angiogenic therapies will be identified owing to their diverse and heterogenous mechanisms of action, as well as inter and intra-tumoural heterogeneity. Furthermore, the utility of these potential biomarkers may be dependent on the combination partner, further emphasising the need to develop unique biomarkers for specific diseases and specific combinations. Despite these challenges, the importance of biomarker discovery in this field remains paramount. Radiomics, the study of vasculature in preclinical *in vivo* models and the analysis of new circulating angiogenic factors could shed some light on this unmet clinical need. Clinicians need additional tools to select the optimal therapy in each individual patient, given the increasingly complex treatment scenarios resulting from the continual approval of novel agents and combinations. Effective biomarkers would enable a better selection of patients and avoid unnecessary toxicities in those patients who are not expected to derive benefit from these agents. Therefore, adapting modern clinical trials to integrate biomarker-based objectives and pre-planned exploratory post-hoc analysis of baseline and on-treatment patient samples is fundamental and will become standard practice in the design of future trials.

Author contributions

NS and FC have contributed equally to this work and share first authorship. All authors contributed to the article and approved the submitted version.

Acknowledgments

The authors thank Sarah MacKenzie, PhD, for providing medical writing support.

Conflict of interest

Author EE declares personal financial interests for consulting/advisory role and/or honoraria, travel grants and research grants from Amgen, Bayer, Hoffman-La Roche, Merck Serono, Sanofi, Pierre Fabre, MSD, Organon, Novartis and Servier. EE also declares institutional financial interests in the form of financial support for clinical trials or contracted research for Amgen Inc, Array Biopharma Inc, AstraZeneca Pharmaceuticals LP, BeiGene, Boehringer Ingelheim, Bristol Myers Squibb, Celgene, Debiopharm International SA, F. Hoffmann-La Roche Ltd, Genentech Inc, HalioDX SAS, Hutchison MediPharma International, Janssen-Cilag SA, MedImmune, Menarini, Merck Health KGAA, Merck Sharp & Dohme, Merus NV, Mirati, Novartis Farmacéutica SA, Pfizer, Pharma Mar, Sanofi Aventis Recherche & Développement, Servier, Taiho Pharma USA Inc. Author JT reports personal financial interest in form of scientific consultancy role for Array Biopharma, AstraZeneca, Bayer, Boehringer Ingelheim, Chugai, Daiichi Sankyo, F. Hoffmann-La Roche Ltd, Genentech Inc, HalioDX SAS, Hutchison MediPharma International, Ikena Oncology, Inspirna Inc, IQVIA, Lilly, Menarini, Merck Serono, Merus, MSD, Mirati, Neophore, Novartis, Ona Therapeutics, Orion Biotechnology, Peptomyc, Pfizer, Pierre Fabre, Samsung Bioepis, Sanofi, Scorpion Therapeutics, Scandion Oncology, Seattle Genetics, Servier, Sotio Biotech, Taiho, Tessa Therapeutics and TheraMyc. And also educational collaboration with Imedex, Medscape Education, MJH Life Sciences, PeerView Institute for Medical Education and Physicians Education Resource (PER).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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