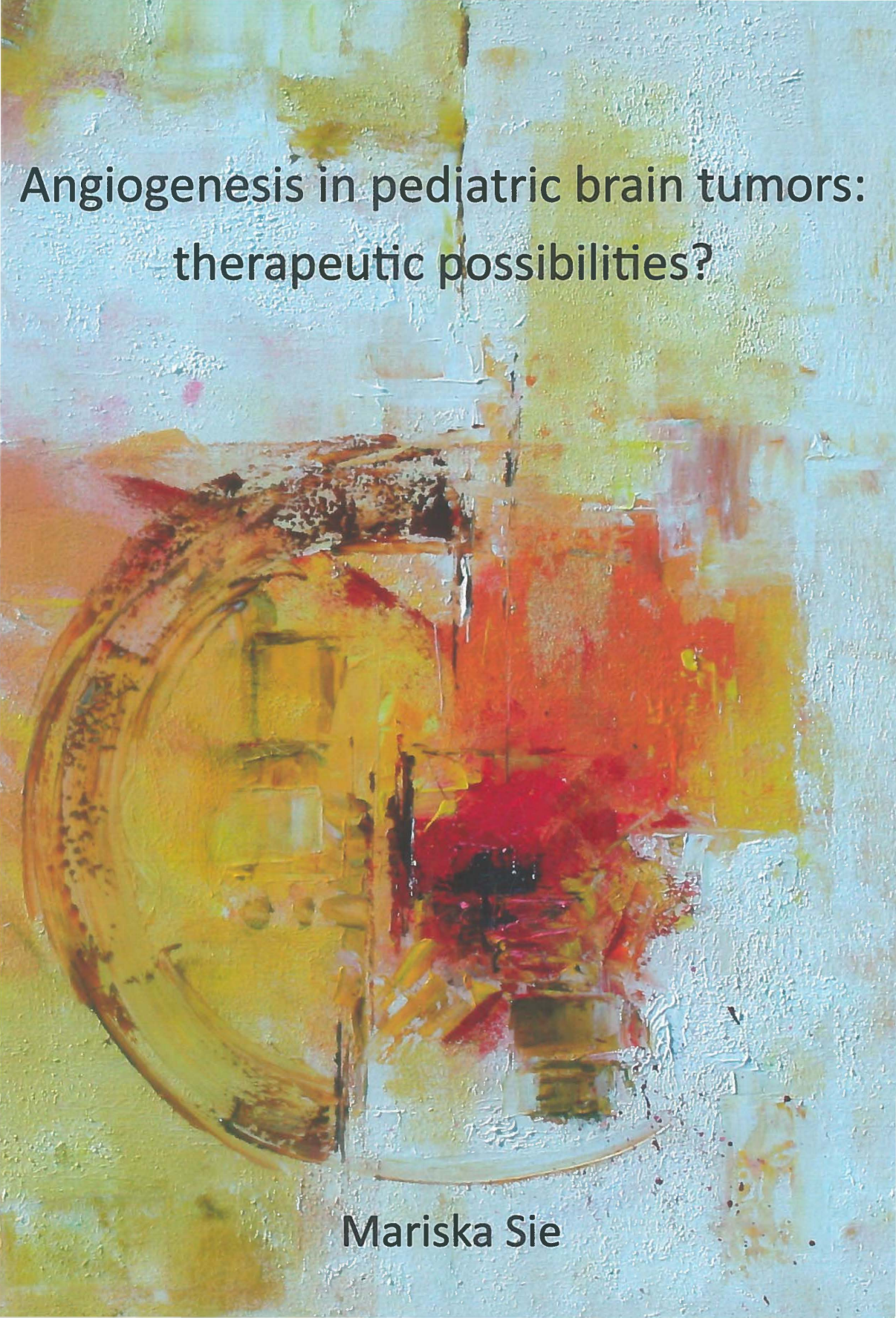
Angiogenesis in pediatric brain tumors

Sie, M.

Angiogenesis in pediatric brain tumors: therapeutic possibilities?



Mariska Sie

Angiogenesis in pediatric brain tumors: therapeutic possibilities?

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Stellingen

behorend bij het proefschrift

Angiogenesis in pediatric brain tumors: therapeutic possibilities?

1. De associatie tussen een hogere angiopoietine-1/angiopoietine-2 balans en langere overleving bij volwassen patiënten met een primair glioblastoom, suggereert mogelijkheden voor anti-angiogene therapie dat met name gericht is op vaatnormalisatie. (dit proefschrift)

2. Gezien het pilocytair astrocytoom en in mindere mate het ependymoom een cruciale overlap toont met het glioblastoom in tumorvasculatuur en het angiogene profiel kan anti-angiogene therapie mogelijk een belangrijke rol spelen in de behandeling van deze kinderhersentumoren. (dit proefschrift)

3. In laaggradige astrocytomen is het remmende effect van anti-VEGF op tumorgroei voornamelijk toe te schrijven aan de indirecte werking op het tumormilieu. (dit proefschrift)

4. Groeifactor gedreven tumor ontsnappingsmechanismen tijdens behandeling met receptor tyrosine kinase (RTK) remmers kunnen overwonnen worden door 'multi targeted' therapie in laaggradige astrocytomen en ependymomen. (dit proefschrift)

5. Preklinische *in vivo* en *ex vivo* modellen voor anti-angiogene therapie in kinderhersentumoren zullen geoptimaliseerd moeten worden om een rationele vertaling naar de kliniek te kunnen maken. (dit proefschrift)

6. The inquiry, knowledge and belief of truth is the sovereign good of human nature. *Francis Bacon*

7. De vorm van een labjournaal zegt niets over de inhoud.

8. Hoewel de interesse voor een medisch specialisme ontwikkeld kan worden op basis van theoretische kennis, laat de kliniek je de voorkeur pas echt overkomen.

9. Het is mogelijk in een ander land een gevoel van thuiskomen te ervaren zonder dat je er ooit eerder bent geweest.

10. Je kunt alleen maar winnen als je niet bang bent te verliezen.

11. Hangmatteren kun je leren.

Mariska Sie
11 september 2013

CMB.

university of groningen

RIJKSUNIVERSITEIT GRONINGEN

Angiogenesis in pediatric brain tumors: therapeutic possibilities?

Proefschrift

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Copromotor:	Dr. W.F.A. den Dunnen
Beoordelingscommissie:	Prof. dr. R.G. Grundy Prof. dr. J.M. Kros Prof. dr. G. Molema

Een race tegen de klok die je toch nooit zal winnen.
De trui af willen hebben voor de wol op is.
Langzaam vlieg ik weg,
naar het land van onbezorgdheid.

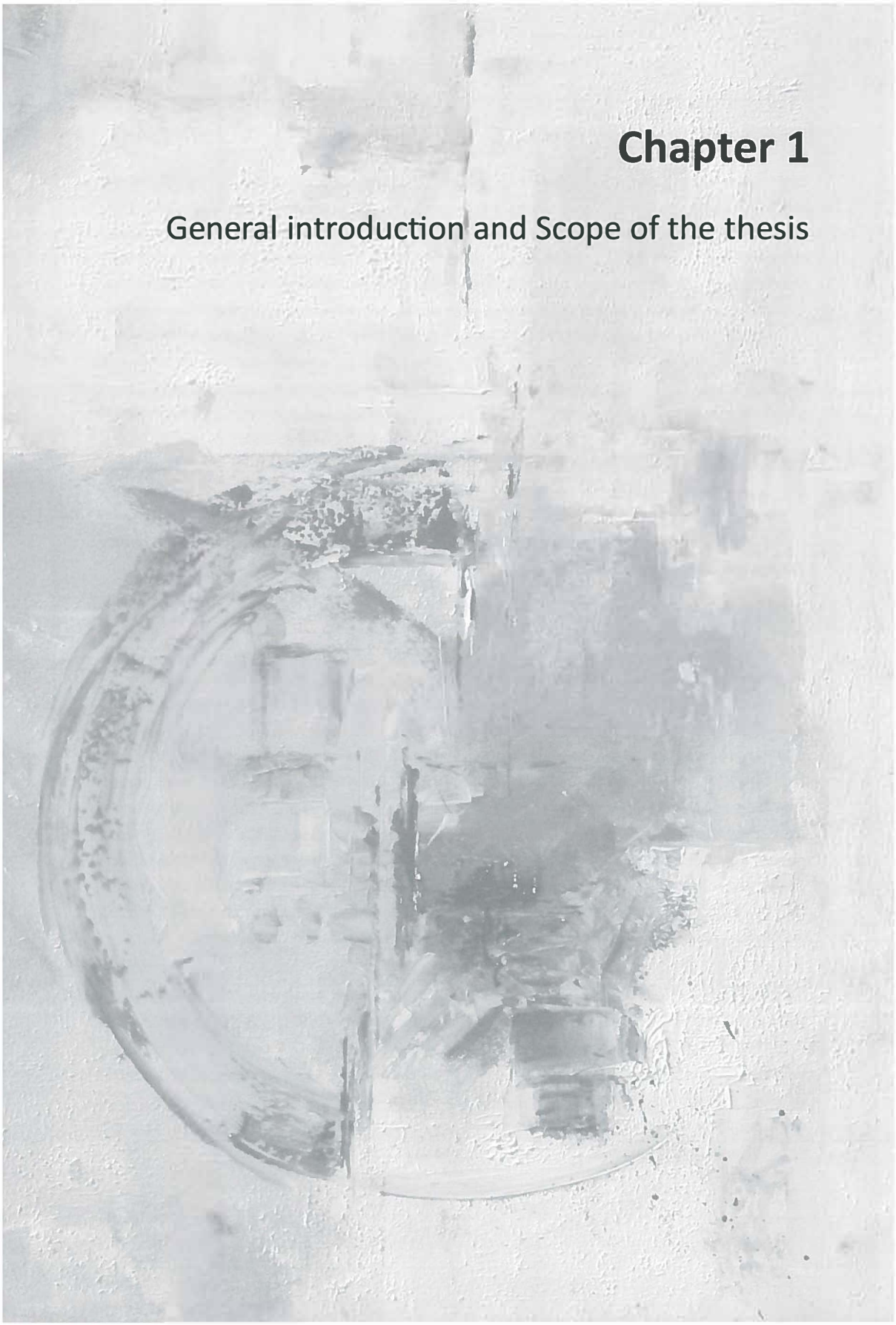
Floortje Peneder, 1993

Paranimfen:	Marloes J.M. Gooden
	Frank J.G. Scherpen

Contents

Chapter 1

General introduction and Scope of the thesis



Introduction

Brain tumors account for approximately 1.4% of all cancers in adults and 2.3% of all cancer-related deaths. Contrary, in children, brain tumors are the most frequent solid tumors, accounting for nearly 20% of all childhood cancers and are the leading cause of cancer morbidity and mortality among children.[1] Brain tumors present particular difficult challenges, especially in children, in that they interfere with normal brain function and development which are essential for healthy ageing. Due to its localization, a brain tumor is one of the most challenging tumor types to treat. Given the high morbidity and mortality numbers despite intensive therapeutic options including neurosurgical resection, intensive chemotherapy and craniospinal radiotherapy, alternative treatment strategies are warranted. The concept that tumor growth and progression are angiogenesis-dependent, has resulted in the development of various strategies inhibiting angiogenesis as a therapeutic implication.[2-6] However, in this research field, limited studies have been published about angiogenesis and tumor vasculature in pediatric brain tumors compared with adult solid tumors. In the present thesis we will focus particularly on the angiogenic profile in pediatric brain tumors. To study angiogenesis in various pediatric brain tumors it is compared to glioblastoma. Glioblastoma (WHO grade IV), the most frequent primary brain tumor in adults, is often seen as a model for studying angiogenesis.[7]

Glioblastoma

Glioblastomas, accounting for approximately 50% of all intrinsic brain tumors and 80% of malignant brain tumors,[8] are among the most vascularized tumors. Despite the short duration of symptoms of less than 3 months in more than 50% of the patients, the tumors are often surprisingly large at the time of presentation. Glioblastoma occurs most often in the subcortical white matter of the cerebral hemispheres with a particularly typical fronto-temporal location. Tumor infiltration often extends into the adjacent cortex and through the corpus callosum into the contralateral hemisphere.[7] Especially in children, glioblastoma of the basal ganglia, thalamus and brain stem (malignant brain stem glioma) can occur and is seen in 2-3% of the children diagnosed with a brain tumor (exact percentages are given in Figure 1). The overall survival is dramatically poor with median survival rates of 14 months reported in clinical trials,[9] which could even be overestimated as they showed bias towards recruitment of younger adult patients and patients with high preoperative Karnofsky performance scores, which are both predictors of a more favorable clinical outcome.

Pediatric brain tumors

Low grade astrocytomas, including pilocytic astrocytoma (WHO grade I) and diffuse astrocytoma (WHO grade II), are the most frequent brain tumors in children (Figure 1). Pilocytic astrocytomas are relatively circumscribed, slowly growing, often cystic tumors, but highly vascular, as is evidenced by their contrast enhancement on magnetic resonance imaging (MRI).[10] These tumors arise in most pediatric patients in the infratentorial region. The most common supratentorial site is the hypothalamus/optic pathways, followed by the thalamic/basal ganglia region.[7]

Diffuse astrocytomas (WHO grade II) are characterized by a high degree of cellular differentiation and slow growth, occurring throughout the central nervous system but preferentially located supratentorially. The intrinsic tendency of diffuse astrocytoma for malignant progression is rarely found in children.[7, 11] Overall low grade astrocytomas have a good prognosis with 5-year overall survival rates of 80-90%. The 5-year survival rate for high grade astrocytomas including anaplastic astrocytomas (WHO grade III) ranges from merely 20-40% and is even worse for glioblastoma (5-15%).[12] Interestingly, although pediatric high grade astrocytoma may resemble adult glioblastoma on histopathological criteria, there are significant differences both clinically and within the molecular biology of the tumors.

The astrocytic tumors, are followed in frequency of occurrence by medulloblastomas (WHO grade IV) which are malignant, invasive embryonal tumors arising at least in 75% of the childhood cases in the vermis, and project into the fourth ventricle. Involvement of cerebellar hemisferes increases with the age of the patient.[7, 13] Ependymomas (WHO grade I-III) represent the third most common pediatric brain tumors after astrocytomas and medulloblastomas. They most commonly develop in the fourth ventricle and in the spinal cord, followed by the lateral ventricles and the third ventricle, although supratentorial parenchymal ependymomas may occur outside the ventricular system.[14] Histopathological grading of ependymomas according to the WHO classification (II-III) seems to be hardly reproducible, and not correlated with clinical outcome, especially in young children.[15]

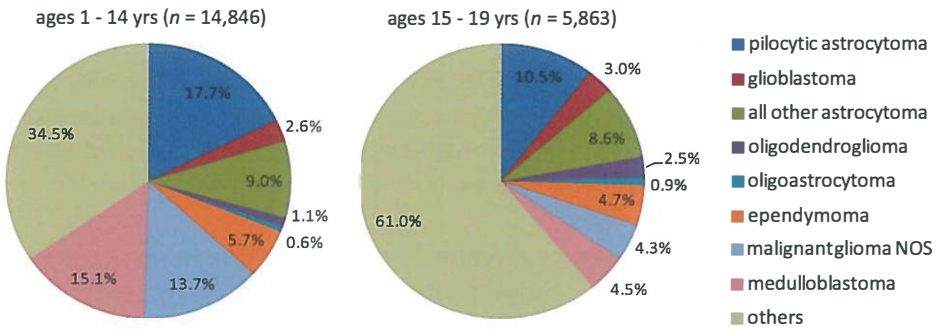


Figure 1. Distribution of childhood primary central nervous system tumors by histology.[8]

Angiogenesis and tumor vasculature

Angiogenesis, the formation of vessels from a pre-existing vasculature, is one of the main processes through which tumors acquire blood supply to establish a source of nutrients and oxygen and to eliminate cellular waste products. In addition, several other modes of vessel formation in tumors have been identified, including co-options of pre-existing vessels, the recruitment of endothelial progenitor cells, vessel splitting known as intussusceptions and vasculogenic mimicry which includes the formation of tube-like structures by tumor cells instead of endothelial cells.[16-19] Crucial factors in the process of angiogenesis are *e.g.* angiopoietins (ANGPTs), regulating angiogenesis and vessel (in)stability by binding to the endothelial cell-specific receptor tyrosine kinase Tie2, and vascular endothelial growth factor (VEGF), promoting angiogenesis and cell survival.[3, 19, 20]

Despite having an abundant number of vessels, tumors are mainly characterized by hypoxia, low pH and high interstitial fluid pressure due to an abnormal vasculature with immature and leaky vessels.[19] This hostile tumor microenvironment can impede the transport and/or distribution of chemotherapeutics to the tumor and thereby sustaining tumor survival and even tumor growth.

Anti-angiogenic therapy

'Traditionally', anti-angiogenic therapy attempt to inhibit new vessel formation and/or to destroy existing vessels to starve the tumor from its nutrients. Interestingly, various other theories have been described. For example, anticipating on the previously described vascular abnormalities in tumors, anti-angiogenic therapy has been suggested as a strategy to normalize tumor vasculature by inhibiting pro-angiogenic factors like VEGF which is highly expressed in tumors, leading to improved blood supply with subsequently increased delivery of chemotherapy and oxygen for higher efficacy of radiation.[21, 22] Furthermore angiogenic inhibitors targeting receptor tyrosine kinases, the so-called RTK inhibitors are not only effective through angiogenic pathways targeting the tumor indirectly, but also directly by inhibiting tumor cell growth.[23, 24] However, in response to anti-angiogenic therapy tumor resistance or rescue mechanisms could rise, including upregulation of alternative angiogenic growth factors.[25, 26]

Scope of the thesis

The research described in the present thesis aimed to analyze the angiogenic profile, tumor vasculature and growth-factor-driven rescue to receptor tyrosine kinase (RTK) inhibitors in pediatric brain tumors. By determining these aspects of tumor angiogenesis, more insight has been obtained into this mechanism, possibly resulting in the identification of a potential therapeutic window for anti-angiogenic therapy in pediatric brain tumors.

Up to now, numerous clinical studies of anti-angiogenic therapy in pediatric brain tumors have been described. **Chapter 2** reviewed these published studies and provided an overview of recently started clinical trials inhibiting angiogenesis in pediatric brain tumors. Moreover, response measuring and clinical monitoring of anti-angiogenic therapy will be evaluated. Based on pre-clinical results, clinical developments and future perspectives, this review discussed the question if anti-angiogenic therapy is an effective strategy in the treatment of children with brain tumors.

With this clinical point of view in mind, this thesis continued with analyses of more biological tumor characteristics in terms of the angiogenic profile and tumor vasculature in glioblastoma as prototype of angiogenesis in brain tumors. In **chapter 3**, the angiopoietin (ANGPT)1/ANGPT2 balance as an indicator for vessel stability was determined in the context of therapeutic outcome in 62 adult patients with primary glioblastoma. Moreover, microvessel density, turnover of both endothelial and tumor cells, and VEGF-A-D expression were evaluated in immunohistochemically stained tumor slides.

As glioblastomas are often seen as a model for studying angiogenesis, tumor tissue from the previous study was used as a comparable prototype to characterize the angiogenic profile and tumor vasculature in tumor tissue obtained from respectively 27 pediatric intracranial ependymoma (**chapter 4**) and 59 pilocytic astrocytoma patients (**chapter 5**). Besides the former aspects of studying tumor vessel biology in glioblastoma, both pediatric brain tumor studies enriched their methods with analyses of vessel maturity in terms of basement membrane and pericyte coverage. Furhermore, as in pilocytic astrocytoma two different methods were used to determine microvessel density, more insight was obtained into blood vessel architecture in these tumors.

Subsequently, as each VEGF isoform, arises through differential splicing of the VEGF-A gene, could contribute differently to the process of angiogenesis resulting in differences in blood vessel architecture, **chapter 6** aimed to further analyze various VEGF-A isoforms (VEGF-$A_{121}a$-$_{189}a$) in correlation with tumor vessel morphology in pediatric pilocytic astrocytoma and adult glioblastoma. Interestingly, it has been suggested that anti-angiogenic VEGF-A isoforms (VEGF-$A_{xxx}b$) contain binding domains for the vast majority of anti-VEGF-A antibodies, like bevacizumab, and therefore inhibiting the effect of these drugs in tumors expressing significant levels of VEGF-$A_{xxx}b$.[27, 28] Tumors with relatively higher pro- than anti-angiogenic isoform expression will be probably more sensitive to anti-angiogenic therapy. Because of this possible clinical relevance, this study secondly aimed to determine VEGF-$A_{xxx}a$: VEGF-$A_{xxx}b$ ratios in both pilocytic astrocytoma and glioblastoma.

In **chapter 7** and **chapter 8** anti-angiogenic therapies were analyzed in pediatric brain tumors with respectively anti-VEGF monoclonal antibodies and various receptor tyrosine kinase (RTK) inhibitors. By VEGF targeting the process of angiogenesis can be disrupted, effecting tumor growth indirectly. RTK inhibitors prevent receptor binding of growth factor ligands resulting not only in inhibition of angiogenic pathways, but also downstream survival signaling pathways, including the PI3K/Akt and MAPK/Erk pathways. As such, tumor cell growth can be attacked directly. However, inhibition of a specific RTK could trigger the tumor to upregulate RTK ligands, through autocrine tumor-cell production, paracrine contribution from tumor stroma or systemic production, eventually contributing to tumor resistance to the RTK inhibitor.[26]
So in chapter 7 the anti-VEGF monoclonal antibodies, bevacizumab (Avastin®, anti human VEGF) and B20-4.1.1 (anti human and mouse VEGF) were studied on cell viability and proliferation level in pediatric low grade astrocytoma cell lines. Moreover an intracranial pediatric low grade astrocytoma xenograft mouse model was developed to determine *in vivo* effects. Chapter 8 aimed to investigate growth-factor-driven rescue to RTK inhibitors (sorafenib, dasatinib, canertinib, crizotinib) in pediatric low grade astrocytoma and ependymoma cell lines. Furthermore as a strategy to overcome tumor resistance multi targeted therapy will be evaluated.

Finally, in **chapter 9** the results of the research described in this thesis are summarized and discussed in a broader context with future perspectives about the possible therapeutic window for anti-angiogenic therapy in pediatric brain tumors.

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Chapter 2

Anti-angiogenic therapy in pediatric brain tumors: an effective strategy?

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Abstract

Brain tumors are still the leading cause of cancer morbidity and mortality among children, despite different therapeutic options including neurosurgery, chemotherapy and radiation. As angiogenesis is highly crucial in brain tumor growth and progression, numerous clinical trials evaluating diverse anti-angiogenic agents have been described. In the present review, we aimed to answer the question if anti-angiogenic therapy is an effective strategy in the treatment of children with brain tumors. Although some encouraging results have been published of anti-angiogenic therapy targeting vascular endothelial growth factor (VEGF)/VEGF receptor signaling or epidermal growth factor receptor (EGFR), still more insight is warranted to be highly conclusive about the efficacy of anti-angiogenic therapy with currently potential upcoming anti-angiogenic agents in pediatric brain tumors. However, given the need for new therapeutic strategies, multi targeted therapy with anti-angiogenic agents anticipating on possible tumor escape mechanisms could be effective in the future treatment of pediatric brain tumors.

Introduction

Brain tumors account for nearly 20% of all childhood cancers and are characterized by a large diversity of morphologic entities. The most common brain tumor subtype occurring in children and young adults is glioma, representing more than 50% of all tumors.[1] Gliomas are classified into low-grade glioma (LGG), including the most frequent occurring pilocytic astrocytoma (WHO grade I) and diffuse astrocytoma (WHO grade II) and high-grade glioma (HGG), including anaplastic astrocytoma (WHO grade III) and glioblastoma (WHO grade IV). WHO grade III tumors of oligodendroglial or mixed oligoastrocytic origin are less commonly found in children.[2] Tumor vascularity is associated with a higher WHO grade, except for pilocytic astrocytoma which are as grade I astrocytoma highly vascular tumors.[2-4] Preferred sites of low-grade astrocytoma include the optic nerve, optic chiasm/ hypothalamus, thalamus and basal ganglia, cerebral hemispheres, cerebellum and brain stem.[2] Overall these tumors have a good prognosis with 5-year overall survival rates of 80-90%. However, the 5-year survival rate for anaplastic astrocytomas ranges from merely 20-40% and is even worse for glioblastoma (5-15%).[5] Diffuse intrinsic brain stem gliomas (DIPG) which are mainly grade III or IV astrocytomas have the most infaust prognosis.[6] The overall survival of these children remains approximately 9 months, and most patients die from the disease within 2 years.[7]

Medulloblastoma, one of the embryonal brain tumor types, is the second most frequent tumor subtype after glioma. Peak occurrence is at 4 years of age with one third of the cases are present in the first years of life.[2, 6] Management of these very young patients remains challenging since the immature brain is particularly susceptible to the toxicity of current treatment options. Ependymoma is the third most common pediatric brain tumor subtype with a peak incidence between birth and 4 years of age. Ependymomas are classified into myxopapillary ependymoma (WHO grade I), grade II ependymoma (cellular, papillary, clear cell, tancytic) and anaplastic ependymoma (WHO grade III),[2] although clinical studies have failed to show a correlation between grade and clinical outcome.[8, 9] 5-year overall survival was reported to be 57.1% in which infratentorial location was associated with better survival rates compared with supratentorially located tumors.[10]

Although the prognosis for pediatric patients with brain tumors has improved over the last few decades with diverse intensive therapeutic modalities as neurosurgery, chemotherapy and radiation, many brain tumors remain difficult to treat and are associated with a poor prognosis. The long term survival for children with DIPG has not even changed over the last decades. Overall brain tumors are still the leading cause of cancer morbidity and mortality among children. So to reduce this morbidity as well as mortality, alternative therapeutic strategies have been developed, extrapolating from adult studies, including anti-angiogenic therapy.

In 1971, Folkman firstly proposed that tumor growth is angiogenesis-dependent, and hence, blocking angiogenesis could be a strategy to arrest tumor growth.[11] This possibility stimulated an intensive search for pro- and anti-angiogenic molecules, resulting in the identification of crucial angiogenic factors including vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR) and platelet derived growth factor (PDGF). Later, various strategies inhibiting the process of angiogenesis were described, specific anti-angiogenic inhibitors were developed and tested in pre-clinical and clinical settings in adults and subsequently in children.

Nowadays, numerous clinical studies of anti-angiogenic therapy also in pediatric brain tumors have been described. In the present review an introduction in the process of angiogenesis and its mediators in pediatric brain tumors will be followed by a description of the general effects of and possible tumor resistance mechanisms to anti-angiogenic therapy. Next, we provide an overview of both published clinical pediatric brain tumor studies and recently started clinical trials inhibiting angiogenesis in different pediatric brain tumor subtypes. Moreover, we will evaluate clinical results and monitoring of anti-angiogenic therapy. Finally, the question if anti-angiogenic therapy is an effective strategy in the treatment of children with brain tumors and the future perspectives will be discussed.

Angiogenesis and its mediators in pediatric brain tumors

Vascularization of the brain begins during embryogenesis, continues into the post-natal period, and involves a tightly regulated process of vasculogenesis followed by angiogenesis.[12-14] Angiogenesis is defined as blood vessel generation from pre-existing blood vessels, whereas vasculogenesis refers to in situ differentiation of undifferentiated precursor cells (angioblasts) to endothelial cells that assemble into a vascular labyrinth.[15, 16] During adulthood most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and in the placenta during pregnancy, and in physiological repair processes such as wound healing.[17, 18] Angiogenesis is thought to be the result of a delicate balance between pro- and anti-angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietins (ANGPT), fibroblast growth factor (FGF), epidermal growth factor receptor (ErbB/HER) and platelet derived growth factor (PDGF).[16, 19] Mitogens, such as VEGF, are activated to enhance endothelial cell migration and proliferation.[20] Endothelial cells migrate along newly deposited extracellular matrix tracts and form vessel sprouts. This process requires extracellular matrix dissolution facilitated by the presence of proteases such as matrix metalloproteinase (MMP). Subsequently, the occurring tube and basement membrane formation triggers further processes which involve pericyte recruitment by endothelial cells, and the eventual formation of a mature blood vessel.[21, 22] Normal blood vessels are distributed at regular and closely spaced intervals having a highly organized blood flow. In the brain, astrocytes induce endothelial cell and pericyte differentiation, but it appears that pericytes migrate faster to capillary-like structures and cover the capillary-like structures more extensively than astrocytes, which provide the astrocyte end-feet that eventually invest the outer wall of the blood vessel.[23] The basement membrane exists between endothelial cells and astrocytes, and is functionally important in the brain in restricting extravasation of protein rich fluids and blood components into the parenchyma.[24] Interestingly, hypoxia is a key regulator of angiogenesis and affects the expression of crucial angiogenic factors.

Tumor angiogenesis has become widely accepted as the mechanism by which tumors induce the formation of new blood vessels from the pre-existing microvasculature. Both tumor growth and invasion are angiogenesis-dependent.[25] Angiogenesis has been extensively analyzed in glioblastoma, which is the most frequent intrinsic brain tumor in adults and is often seen as a model for studying angiogenesis. Much of the knowledge about angiogenesis and its mediators in brain tumors has been derived from this 'angiogenic prototype' and extrapolated to pediatric brain tumors. Relatively limited studies have been published about angiogenesis in pediatric brain tumors. In general, during tumorigenesis, tumor cells co-opt blood vessels of the surrounding brain until the limiting diffusion distance to a vessel has been reached and the tumor become hypoxic. In response to changes in the local milieu caused by tissue hypoxia, growth factor stimulation from both tumor and endothelial cells, metabolic and/or mechanical stress, genetic mutations and immune response, the so called 'angiogenic switch' (*i.e.* the balance between pro-angiogenic and anti-angiogenic molecules) is tipped towards a pro-angiogenic state.[22, 26, 27]

The imbalance between pro- and anti-angiogenic factors results in abnormal blood vessels and immature tumor vasculature. In contrast to normal blood vessels, tumor vasculature is highly disorganized: the immature vessels are tortuous and dilated, vessel diameter varies extensively, pericyte coverage is incomplete, the basement membrane is abnormally thick or thin, there is excessive branching and shunting. Consequently, tumor blood flow is chaotic and variable and leads to hypoxic and acidic regions in tumors.[26, 28] Moreover structural abnormalities of the endothelial lining form the basis for an increased microvascular permeability and loss of blood brain barrier function.[29] All these abnormalities in tumor vasculature could lower effectiveness of regular therapeutic strategies including chemotherapy and radiation. In pediatric brain tumors differences in vessel architecture have been found. In pediatric pilocytic astrocytoma and to a lesser extend in pediatric ependymoma overlapping tumor immaturity and instability was found compared with adult glioblastoma.[3, 30]

One of the most extensively investigated angiogenic molecules is vascular endothelial growth factor (VEGF), which has been described as the key mediator of angiogenesis. The VEGF family currently comprises seven members: VEGF-A to VEGF-F (also known as FIGF) and placental growth factor (PlGF or PGF) of which VEGF-A is the best characterized VEGF member.[20, 31] The VEGF-A gene consists of eight exons that give rise to various isoforms (VEGF-$A_{121-206}$) through differential splicing. All isoforms contain exon 1-5 and the terminal exon, exon 8, except for VEGF-A_{148}. The presence or absence of the heparin-binding domains encoded by exon 6 and 7 determines binding to the extracellular matrix and therefore these domains influence solubility. VEGF isoforms containing exon 6 (VEGF-A_{145}) or both exon 6 and 7 (VEGF-A_{183}, VEGF-A_{189}, and VEGF-A_{206}) are bound tightly to cell surface heparin-containing proteoglycans in the extracellular matrix,[20, 32-34] whereas those lacking exon 6 (VEGF-A_{165}) or both exons 6 and 7 (VEGF-A_{121}) are moderately till highly diffusible.[34, 35] The affinity of the VEGF isoforms to bind heparin has an overall effect on isoform spatial distribution; these variations determine whether blood vessel growth is organized and directed, or disordered.[34-36] Longer isoforms like VEGF-A_{189} are associated with more vessel maturity,[37] showing good levels of pericyte coverage, while VEGF-A_{121} shows major defects in mural cell recruitment resulting in immature blood vessels that are fragile and leaky.[38]

Besides these pro-angiogenic isoforms, it has been described that alternative 3' splice site selection in exon 8, could give different C-terminal sequences, resulting in the anti-angiogenic VEGF isoforms.[39-41] It is suggested that anti-angiogenic VEGF-A isoforms contain binding domains for the vast majority of anti-VEGF-A antibodies, and therefore inhibiting the effect of these drugs in tumors expressing significant levels of anti-angiogenic isoforms.[42-44] The overlapping pro- and anti-angiogenic VEGF-A isoform ratio between pediatric pilocytic astrocytoma and adult glioblastoma could suggest possibilities for anti-angiogenic therapy not only in glioblastoma, but also in pilocytic astrocytoma.[37] Notably, the anti-angiogenic character of these VEGF isoforms have been questioned and more recently the pitfalls of interpreting VEGF expression patterns were highlighted, emphasizing the complexity of the VEGF-A isoform biology and carefulness of the interpretation of the results.[45, 46]

VEGF-A expression can be induced by hypoxia and acidosis, but can also be regulated by multiple oncogenes and tumor-suppressor genes, hormones, cytokines and various signaling molecules, including nitric oxide and mitogen-activated protein kinases. Moreover, VEGF-A can be released from other cells and the extracellular matrix. VEGF-A mediates its effects by interacting with two high-affinity tyrosine kinase receptors (VEGFR-1 or Flt-1 and VEGFR-2 or KDR), in which VEGFR-2 is responsible for mediating microvascular permeability, endothelial cell migration, proliferation and anti-apoptotic effects, while VEGFR-1 may have an independent role in stimulating cell migration but may also dampen certain signaling pathways mediated by VEGFR-2 (Figure 1).[31, 47] However, there are possible indications for VEGFR-1 induced tumor angiogenesis.[48] In pediatric brain tumors, VEGF-A is highly expressed and is thought to be partly responsible for the loss of the blood brain barrier during tumor growth. Neuropilins can also serve as a receptor for some members of the VEGF family and interact directly with VEGFRs, modulating angiogenesis (Figure 1).[49]

Other critical angiogenic factors are the angiopoietins, Ang-1 (ANGPT1) and Ang-2 (ANGPT2) that bind to the endothelial cell–specific receptor tyrosine kinase Tie2 (TEK) and regulate angiogenesis (Figure 1). The working mechanism is still under debate. Initially, it was suggested that Ang-1 activates Tie2 to promote blood vessel maturation and stabilization. In contrast, Ang-2, which is highly expressed by tumor endothelial cells, is thought to inhibit Tie2 activity and destabilize blood vessels, thereby facilitating VEGF-dependent vessel growth. However, recent findings indicate that Ang-2 plays a protective rather than a destabilizing role in tumor endothelial cells by activating Tie2 instead of inhibiting.[50-52] Previous studies demonstrated different oligomeric or multimeric forms of the angiopoietins. It has been indicated that the higher oligomeric forms (at least tetramers) are needed for Ang-1-specific Tie2 binding and activation in endothelial cells.[53] The lower oligomerization state of Ang-2 seems to be crucial for the Ang-2-specific Tie2 redistribution, whereas multimeric structures of Ang-2 could induce responses similar to Ang-1.[54] These data should help to explain the versatile effects of Ang-2 in angiogenesis, acting as both agonist and antagonist of Tie2.

Yet another mediator in angiogenesis is fibroblast growth factor (FGF) of which 18 family members have been described (FGF1-FGF10 and FGF16-FGF23). FGF is a potent mitogen for cells of neuroectodermic and mesodermic orgin, including endothelial cells, and stimulates angiogenesis by binding to FGF structurally related receptor tyrosine kinase receptors (FGFR1-5) (Figure 1).[55-57] FGF in combination with VEGF acts synergistically on endothelial cell proliferation and migration.[58]

Furthermore, epidermal growth factor receptor (ErbB/HER) and platelet derived growth factor (PDGF-A-D) may be involved in angiogenesis (Figure 1). The human ErbB family comprises four related receptors (EGFR/ErbB1/HER-1, ErbB2/HER-2/c-neu, ErbB3/HER-3 and ErbB4/HER-4) which are transmembrane glycoproteins containing an extracellular ligand binding domain and an intracellular receptor tyrosine kinase domain.[59] Dysregulation of ErbB/HER pathways by overexpression or constitutive activation can promote tumor processes including angiogenesis,[59] although the importance of EGFR promoting angiogenesis in different pediatric brain tumors is debatable.[60-64] PDGF is a mitogen for multiple cells of mesenchymal and neuroectodermal origin that acts through the PDGF receptors (PDGFRα and β). PDGF-B can upregulate VEGF and exerts autocrine effects on endothelial and perivascular cells.[65] PDGFRα is overexpressed in pediatric high-grade brain stem glioma,[66] and the expression is significantly associated with malignant histology in pediatric glioma, but it does not represent an independent prognostic factor.[67]

Effects of targeting angiogenesis

The inhibition of angiogenesis may affect tumor growth through several mechanisms. As the angiogenic profile is not quite similar in all pediatric brain tumors subtypes, different mechanisms will have a more or less important role depending on tumor type. Moreover, in some tumors angiogenesis-targeted therapy may act through parallel mechanisms. One such proposed mechanism is that of anti-angiogenesis where it simply inhibits the formation of new blood vessels. Inhibition of new vessel growth may occur with induction of endothelial cell apoptosis and blockade of incorporation of bone marrow-derived endothelial progenitor cells. However, there are no adequately powered clinical trials that showed really a decrease in tumor vessel count or microvessel density after anti-angiogenic therapy.[68]

Anti-angiogenic therapy may also act by its direct effects on blood vessel function through vascular constriction or vascular normalization. Vascular constriction after anti-angiogenic therapy is probably due to decreases in production of the vasodilators nitric oxide and prostacyclin. It has been hypothesized that these vasodilatators are chronically increased in tumor vessels owing to high levels of VEGF. Vasoconstriction decreases perfusion and could possibly lead to hypoxia and limited tumor growth.[68] Unfortunately this vasoconstriction is not limited to the tumor vasculature, as one of the most common adverse effects of angiogenesis-targeted therapy is hypertension, possibly resulting in posterior reversible encephalopathy syndrome (PRES), although the role of hypertension and changes in cerebral perfusion in the pathogenesis of PRES remains a matter of debate.[69]

According to the vascular normalization theory, anti-angiogenic therapy could correct the balance between pro- and anti-angiogenic factors, thereby normalizing the abnormal tumor vasculature. Theoretically, this normalization of the tumor vascular network can lead to improved blood flow with subsequently increased delivery of chemotherapy and oxygen for higher efficacy of radiation.[70] However, although some studies described an increase in delivery of chemotherapy to tumors with anti-angiogenic therapy,[71, 72] others didn't show any change in oxygenation/improved blood flow over time.[73]

Furthermore, anti-angiogenic therapy could have effect on tumor cells suppressing similar and functional receptors as endothelial cells, like VEGFRs and neuropilins, to impair tumor growth directly. This mechanism could be more or less important in different pediatric brain tumors, as for example high-grade gliomas express VEGFR-2 on their tumor cells, and the expression of VEGFR-2 in pilocytic astrocytoma is particularly limited to the endothelial cells.[74] Another mechanism of anti-angiogenic therapy is by counteracting the effects of angiogenic factors induced by hypoxia. In tumors, stress conditions like hypoxia are present, resulting in high levels of angiogenic factors, in turn, contributing to tumor growth. However, expression of these angiogenic factors is also upregulated in reaction on genotoxic stress induced by chemotherapy and radiation. The proposed mechanism of action is neutralizing the adaptive stress responses of tumor cells to genotoxic stress by adding anti-angiogenic therapy to chemotherapy and radiation.[68, 75]

(Overcome) resistance to anti-angiogenic therapy

Despite the benefits of anti-angiogenic treatments, tumor progression seems inevitable due to several mechanisms of acquired or adaptive resistance to anti-angiogenic therapy. Moreover, pre-existing non-responsiveness has been described as a second mode of tumor resistance. This last resistance mechanism is possibly attributable to intrinsic properties of tumor cells and angiogenic factor secretions prior to their exposure to anti-angiogenic therapy. Various strategies to overcome both types of resistance mechanisms have been hypothesized and are still under debate.

Resistance to anti-angiogenic therapy

One of the described acquired tumor resistance mechanisms includes activation and/or upregulation of alternative pro-angiogenic signaling pathways within the tumor than the goal targeted with the specific anti-angiogenic drug, such as FGF and ephrins during anti-VEGF therapy. Furthermore, anti-angiogenic therapy causing vessel regression and hypoxia could lead to recruitment of vascular progenitor cells and pro-angiogenic monocytes from the bone marrow that have the capacity to fuel tumors by eliciting new blood vessels. Another resistance mechanism suggests increased pericyte coverage of the tumor vasculature, serving to support the tumor's integrity and attenuate the necessity for angiogenic-mediated survival signaling. Alternatively, tumors can adopt an invasive phenotype, in which they co-opt existing cerebral vasculature. Some evidence supports a critical role for angiopoietins in this process.[75-78]

In sharp contrast with the vascular normalization theory purposed by Jain et al,[16, 70] is the hypothesis that VEGF/VEGFR-targeted therapy could lead to its own resistance mechanism, which is still being discussed.[79-81] It was noted in preclinical models that VEGF-inhibition decreased permeability of the blood brain barrier, resulting in an impaired effect of chemotherapy, possibly due to restoration of the blood brain barrier and obstruction of chemo-distribution to tumor cells. However, despite visual normalization of the blood brain barrier by anti-VEGF other studies showed no limitation in intratumoral concentrations of chemotherapy.[82-84] Up to now, these studies have only been performed in preclinical adult high-grade glioma models.

Other possible contributing resistance mechanisms included changes in the dominant VEGF isoforms (VEGF-A_{121}, VEGF-A_{165} or VEGF-A_{189}) or in neuropilin 1 as a VEGFR co-receptor, and loss of endothelial cell VEGF dependence from downregulation of VEGFR-2.[85] Additional mechanisms for receptor tyrosine kinase inhibitors, could be under-dosing owing to increasingly rapid clearance of the inhibitor[86] and inhibitor inactivation by uptake and lysosomal sequestration in tumor cells.[87] Besides the acquired resistance mechanisms in which tumors develop the capability of regrowth or progression in the face of continued anti-angiogenic therapy, pre-existing or intrinsic resistance mechanisms have been determined. It has been suggested that some tumors have an inherent upregulation of multiple pro-angiogenic pathways or can mediate a pre-existing inflammatory state where the presence of a high proportion of pre-existing inflammatory cells confers a resistant phenotype to anti-angiogenic therapy.[76] Moreover tumor-surrogate blood vessel subtypes exhibit differential susceptibility to anti-angiogenic therapy, as recently formed, immature and unstable blood vessels are more sensitive than older and mature vessels.[88]

Overcome resistance to anti-angiogenic therapy

To overcome resistance mechanisms different strategies have been described, including the addition of low-dose (metronomic) chemotherapy or radiation to anti-angiogenic treatment. However, the underlying mechanism of the added benefit is currently under debate. Anti-angiogenic therapy could lead to vascular normalization, resulting in increased delivery and efficacy of chemotherapy and possibly improved tumor oxygenation and radiosensitivity.[70] However, others suggest that hypoxia inducible factor (HIF)1α blockade by chemotherapy could offset the hypoxic effects of vascular pruning when paired with an anti-angiogenic agent. Alternatively, radiotherapy sensitizes endothelial cells to anti-angiogenic therapy, suggesting that anti-angiogenic therapy should cause greater slowing of tumor growth when preceded by radiation than when followed by radiation.[78] The fact that abnormal/immature tumor vasculature is more vulnerable to anti-angiogenic therapy, therapeutic destabilization of tumor vessels should possibly be a potential approach for overcoming resistance to anti-angiogenic therapy. The inhibition of Notch signaling by blocking delta-like protein 4 (DLL4) stimulates the growth of abundant vascular structures but slows tumor growth in preclinical models because the vessels that are derived from endothelial hypersprouting are poorly functional and intratumoral hypoxia is increased.[89] The abnormal vasculature is more responsive to inhibition of VEGF signaling, and tumor growth is slowed even in some models that are usually unresponsive to VEGF inhibition. Theoretically, as single targeted angiogenic therapy could lead to upregulation of alternative angiogenic factors, multi targeted therapy should also overcome resistance to anti-angiogenic therapy.[90] Other methods to overcome resistance is inhibition of immune cell recruitment or processes that favor tumor progression, like the VEGF-inducible factors HIF1α and insulin-like growth factor I (IGF1) or hypoxia increased c-Met (hepatocyt growth factor receptor, HGFR) which is associated with tumor invasiveness.[78]

Clinical strategies in pediatric brain tumors

The first anti-angiogenic compounds described in pediatric brain tumors were interferon (INF) and thalidomide (Table 1). INFs are a family of glycoproteins with anti-proliferative, anti-viral, immune-modulating, and anti-angiogenic effects. INF-α and INF-β both function through inhibition of bFGF (Figure 1),[91] while INF-γ has anti-angiogenic effect by enhanced secretion of anti-angiogenic chemokines.[92] Standard chemotherapeutic agents, when modified by the frequency and dose administration, can also target angiogenesis. This approach is referred to as anti-angiogenic chemotherapy, low-dose chemotherapy, or metronomic chemotherapy.[93] Thalidomide acts as an inhibitor of VEGF and bFGF-mediated angiogenesis (Figure 1). Later, lenalidomide was introduced, a potent structural and functional thalidomide analog that demonstrates anti-angiogenic, pro-apoptotic, and anti-inflammatory activities in addition to its immunomodulatory effects. Anti-angiogenic agents targeting VEGF/VEGFR signaling and the ErbB family were evaluated next (Table 1, Figure 1). Currently, new agents like crizotinib inhibiting c-Met, ALK and RON (Figure 1) and dasatinib, an oral Src family, bcr-abl, PDGFRα, -β and c-kit TK inhibitor are under investigation (Table 2). In general, mainly pediatric patients with recurrent high-grade astrocytoma were included. Both the retrospective character of part of the studies and the absence of controlled case series makes it difficult to be conclusive about the efficacy of the clinical strategies in pediatric brain tumors.

Interferon

In 1991 the first phase I/II study was published in which children with recurrent or progressive brain tumors were treated with INF-β (Table 1).[94] Although promising results were shown, results in the following studies including combination treatment with INF and chemotherapy and/or radiation in newly diagnosed brain tumors were somewhat disappointing. INF-α showed no improvement in the 2-year survival in pediatric patients with diffuse intrinsic pontine glioma compared with a historic control population.[95] Moreover, INF-β in combination with radiation in brain stem glioma and INF-γ combined with low dose cyclophosphamide in high-grade glioma were not sufficient beneficially effective.[96, 97] Even possible responses on a combination treatment with INF-β, chemotherapy and radiation in brain stem glioma were not convincing.[98, 99] Overall, given the lack of efficacy and the potential side effects including hepatotoxicity, hematologic toxicity and neurotoxicity, it appears unlikely that interferons will have a future role in anti-angiogenic treatment in pediatric brain tumors.

Metronomic chemotherapy

Metronomic chemotherapy refers to the use of chemotherapy agents in low, frequent (often daily) rhythmic doses with minimal breaks that target endothelial rather than tumor cell proliferation. A number of agents have been used in metronomic schedules against pediatric brain tumors: etoposide, temozolomide, cyclophosphamide or topotecan are given alone or in combination in daily schedules.[100-104] Low dose administration is less toxic compared with conventional or high dose chemotherapy. However, the optimal dosing for metronomic therapy has yet to be determined and may differ for each drug and tumor type.

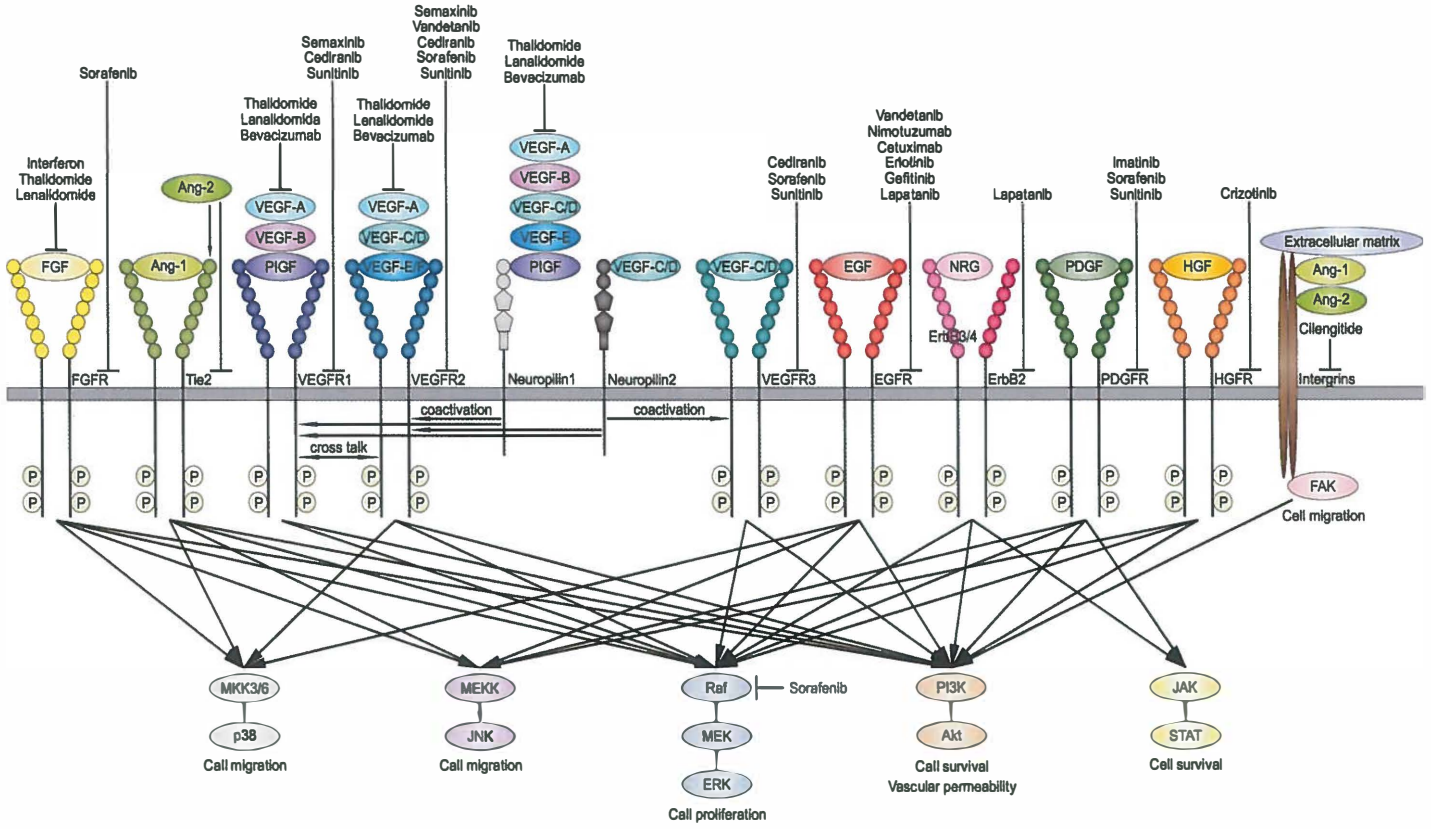

Figure 1. Schematic illustration showing numerous angiogenic mediators activating downstream pathways contributing to the process of tumor angiogenesis. Anti-angiogenic agents are included which are analyzed in published pediatric brain tumor clinical studies or in still ongoing clinical trials.

Up to now, beneficial findings of metronomic chemotherapy in pediatric brain tumors are limited to medulloblastoma which have been described in case report settings.[101, 103] Peyrl et al combined metronomic chemotherapy with the newer anti-angiogenic agent bevacizumab which seems also particularly beneficial in medulloblastoma.[104] At the moment, no clinical trial is ongoing studying metronomic chemotherapy in pediatric brain tumors.

Thalidomide and lenalidomide

The efficacy of thalidomide in pediatric brain tumors is doubtful. Although, a prospective study suggested a better outcome in children with diffuse intrinsic pontine gliomas treated with a combination of thalidomide, temozolomide and radiation, the authors compared the results with literature in which only radiotherapy was given.[105] So the therapeutic benefit could not be exclusively attributed to thalidomide. Also as tumor types varying from low to high-grade glioma were included, it is hard to be conclusive about the efficacy of thalidomide.[106] Thalidomide combined with radiation in newly diagnosed brain stem gliomas even failed to demonstrate any benefit with increased toxicity compared with historical use of radiation alone.[107] Given these moderate results, using thalidomide for future clinical strategies in pediatric brain tumors is not advisable. In contrast, lenalidomide seems to have effect in pediatric brain tumors. However, whether possible antitumor activity and toxicity were dose related has to be further analyzed.[108] Currently, two clinical trials analyzing lenalidomide are recruiting pediatric patients (Table 2).

Anti-VEGF monoclonal antibody and VEGFR tyrosine kinase inhibitors

Bevacizumab is a humanized immunoglobulin G1 monoclonal antibody that binds to and inhibits the activity of VEGF (Figure 1). Results of the efficacy of bevacizumab in clinical studies are various from objective radiographic response and clinical improvement in low-grade glioma[109] and prolonged progression free survival in medulloblastoma[104, 110] till only minimal efficacy in high-grade glioma[111-113] and ependymoma[114] (Table 1). Cessation of initial therapy resulted in tumor progression. However, more interestingly, despite previous exposure to bevacizumab, these patients showed clinical and/or radiographic improvement or stability with re-treatment.[109]
As the majority of the patients was no part of prospective trials, the issue of selection bias raises. Moreover all published studies included recurrent brain tumors that most likely have multiple oncogenic mutations, making them resistant to hypoxia and less dependent on angiogenesis for survival. Since anti-angiogenic therapy is likely to work better in the setting of minimal tumor burden and earlier in the disease course of such patients, it might be worthwhile using bevacizumab at the time of initial diagnosis. In line with this theory and the possible trend of therapeutic benefit by anti-VEGF, bevacizumab is still under investigation and will probably have a future role in the treatment of pediatric brain tumors. The possible synergistic effect of bevacizumab and chemotherapy in pediatric brain tumors is still unclear. Remarkable is that bevacizumab was well tolerated in pediatric patients, sometimes even better than in adults. However, VEGF-targeted medications and dexamethasone are both associated with changes in blood pressure, possibly resulting in PRES, so close monitoring and management of hypertension is required.[109, 115]

Besides bevacizumab as monoclonal VEGF antibody, small molecule tyrosine kinase inhibitors of the VEGF receptors, including semaxanib, vandetanib, cediranib, sorafenib and sunitinib were described in published studies (Table 1) or are still under research in ongoing studies (Table 2) (Figure 1).[116, 117] However, the clinical development of semaxanib has been halted in part due to the limited single-agent activity, as well as the significant toxicity related to the semaxanib dependent co-administration in the cremophor vehicle.[116] In contrast, vandetanib, showed encouraging results[117] and is continually being studied also in combination with other small molecule inhibitors like dasatinib (Table 2).

Anti-EGFR monoclonal antibodies and ErbB tyrosine kinase inhibitors

Two EGFR monoclonal antibodies including nimotuzumab and cetuximab, and three different ErbB tyrosine kinase inhibitors were evaluated in pediatric brain tumors (Table 1, Figure 1). Although modest activity was observed of nimotuzumab in resistant or relapsed high-grade glioma in particular DIPG, nimotuzumab combined with radiotherapy was not more efficacious than chemotherapy.[118] The prospective study of Saurez et al was minor contributory to insights into nimotuzumab´s efficacy due to a high heterogeneity of included tumors and variety of treatment regimens.[119] Up to now, little is known about their efficacy as these agents were yet mainly analyzed in phase I studies. The only phase II study in which one of the EGFR tyrosine kinase inhibitors was tested, analyzed patients with newly diagnosed brain stem glioma and supratentorial malignant gliomas treated with gefitinib combined with radiotherapy (Table 1). Unfortunately, progression free survival and overall survival data were consistent with the generally disappointing therapeutic results observed for these tumors.[120]

In general, the agents targeting the ErbB family showed to be well tolerated in children with brain tumors, also in combination with chemotherapy or radiotherapy.[121-124] The recommended dose was even higher in children compared with adults, mainly due to pharmacokinetic rather than pharmacodynamic particularities.[123, 124] However, potentially life-threatening intratumoral hemorrhage remains a substantial risk,[125] comparable with VEGF-targeted therapy, so close monitoring is required during ErbB-targeted treatment. Because there is yet not much known about the efficacy of these agents in pediatric brain tumors and encouraging results about the crucial role of ErbB family members in the process of tumor angiogenesis have been described, currently numerous studies investigate ErbB targeting agents, given this therapeutic strategy a future perspective in the treatment of pediatric brain tumors. On the basis that lapatinib, an EGFR and ErbB2 tyrosine kinase inhibitor, may be synergistic with bevacizumab, a phase II trial of this combined strategy is currently being conducted in children with recurrent ependymoma (Table 2).

Table 1. Clinical studies of anti-angiogenic therapy in pediatric brain tumors

Drug	Target in angiogenesis	Combination therapy	Tumor type (no. patients)	Phase	Publication
Interferon-β (i.v.)	bFGF	-	Brain tumor (29)	I/II	Allen 1991[94]
		ACNU, RT	BSG (16)		Wakabayashi 1992[98]
		RT	BSG (32)	I/II	Packer 1996[97]
		Low dose CPM	HGG (40)		Wolff 2006[96]
Interferon-γ (i.v.)		MCNU, RT	BSG (15)	retro	Ohno 2009[99]
Interferon-α (i.v.)		-	DIPG (32)	II	Warren 2012[95]
Thalidomide (oral)	TNF-α, VEGF, bFGF, IL-8	RT	BSG, glioblastoma (13)	II	Turner 2007[107]
		Carboplatin, vincristine, fluvastatin, RT	BSG (9)	II	López-Aguilar 2008[142]
		TZM, RT	DIPG (17)	pros	Kim 2010[105]
Lenalidomide (CC5013) (oral)	TNF-α, VEGF, bFGF, IL-6	-	Brain tumor (51)	I	Warren 2011[108]
Bevacizumab (Avastin®) (i.v.)	VEGF	Metronomic anti-angiogenic therapy, RT	Brain tumor (30)	retro	Reismüller 2010[115]
		Irinotecan	HGG (12)	retro	Narayana 2010[112]
		Irinotecan	HGG, DIPG (31)	II	Gururangan 2010[111]
		Irinotecan, TZM, CCNU	HGG (8)	retro	Parekh 2011[113]
		Irinotecan, TZM	Medulloblastoma (2)	CR	Aguilera 2011[110]
		Thalidomide, celecoxib, fenofibrate, etoposide, cyclophosphamide	Embryonal brain tumor (16)		Peyrl 2012[104]
		Irinotecan	Ependymoma (15)	II	Gururangan 2012[114]
		Irinotecan	LGG (14)	retro	Hwang 2013[109]
Semaxinib (SU5416) (i.v.)	VEGFR-1, -2	-	Brain tumor (excl BSG) (33)	I	Kieran 2009[116]
Vandetanib (oral)	VEGFR-2, EGFR	RT	DIPG (21)	I	Broniscer 2010[117]
Nimotuzumab (i.v.)	EGFR	CT, RT	Brain tumor (22)	pros	Saurez 2009[119]
		-	HGG (47)	II	Bode 2012[118]
		RT	DIPG (42)	III	Bode 2012[118]
Cetuximab (i.v.)	EGFR	Irinotecan	Brain tumor (26)	I	Trippett 2009[143]
Erlotinib (Tarceva®) (oral)	EGFR	TZM	Brain tumor (20)	I	Jakacki 2008[121]
		RT	HGG (23)	I	Broniscer 2009[122]
		RT	Malignant brain tumor (51)	I	Geoerger 2011[123]
Gefitinib (oral)	EGFR	RT	BSG, HGG (23)	I	Geyer 2010[125]
		RT	DIPG (43)	II	Pollack 2011[144]
Lapatinib (oral)	EGFR, ErbB2	Steroids	Malignant brain tumor (59)	I	Fouladi 2010[145]
Imatinib (oral) (Gleevec®)	PDGFRα, bcr-abl, c-kit	-	PA (1)	CR	McLaughlin 2003[146]
		RT	Glioma (84)	I	Pollack 2007[127]
		- / CT	HCG (6)		Peyrl 2009[126]
		-	Brain tumor (19)	II	Baruchel 2009[128]
Cilengitide (i.v.)	αvβ3, αvβ5 integrins	-	Brain tumor (31)	I	MacDonald 2008[129]

bFGF: basic fibroblast growth factor; RT: radiotherapy; BSG: brain stem glioma; CPM: cyclophosphamide; HGG: high-grade glioma; retro: retrospective; DIPG: diffuse intrinsic pontine glioma; TNF-α: tumor necrosis factor α; VEGF: vascular endothelial growth factor; IL: interleukin; TZM: temozolomide; pros: prospective; LGG: low-grade glioma; CR: case report; VEGFR: vascular endothelial growth factor receptor; EGFR: epidermal growth factor receptor; CT: chemotherapy; PDGFRα: platelet derived growth factor receptor α; PA: pilocytic astrocytoma; HCG: hypothalamic-chiasmatic glioma.

Table 2. Ongoing studies of anti-angiogenic therapy in pediatric brain tumors

Drug	Target in angiogenesis	Combination therapy	Tumor type	Phase	Estimated completion year
Thalidomide (oral)	TNF-α, VEGF, bFGF, IL-8	TZM	Brain tumor	II	Kieran (2010)
		Carboplatin, RT	BSG	II	Goldman (2007)
Lenalidomide (CC5013) (oral)	TNF-α, VEGF, bFGF, IL-6	RT	Brain tumor	I	Warren (2012)
		RT	DIPG, HGG	I	Warren (2016)
Bevacizumab (Avastin®) (i.v.)	VEGF	*Lapatinib*	Ependymoma	II	Rytting (2013)
		Irinotecan, TZM	Brain tumor	I	Stapleton (2013)
		Valproic acid, RT	HGG	II	Su (2014)
		Irinotecan hydrochloride, TZM	Medulloblastoma, PNET	II	Levy (2013)
		RT, *Erlotinib*/TZM	DIPG	II	Kieran (2013)
		Vorinostat/TZM	HGG	II/III	Fouladi (2019)
		Thalidomide, celecoxib, fenofibrate acid, etoposide, cyclophosphamide	Medulloblastoma	II	Peyrl (2018)
Vandetanib (oral)	VEGFR-2, EGFR	RT	DIPG	I	Broniscer (2011)
		RT, *Dasatinib*	DIPG	I	Broniscer (2013)
Cediranib (AZD2171) (oral)	VEGFR-1, -2, -3	-	Brain tumor	I	Kieran (2012)
Sorafenib (oral)	VEGFR-2, -3, PDGFRβ, Raf, c-kit, BRAF, FGFR-1	-	LGG	II	Karajannis (2014)
Sunitinib (SU11248) (oral)	VEGFR-1, -2, -3, PDGFRα, β, RET, c-kit, CSF-1R	-	Malignant glioma, ependymoma	II	Wetmore (-)
Nimotuzumab (i.v.)	EGFR	RT	DIPG	II	Epelman (2012)
		-	DIPG	II	Bouffet (2010)
Cetuximab (i.v.)	EGFR	External beam RT, Irinotecan	DIPG, HGG	II	Dunkel (2013)
Erlotinib (Tarceva®) (oral)	EGFR	(Versus Etoposide)	Ependymoma	II	APGD (2013)
		-	Ependymoma	II	APGD (2013)
		RT	Glioma	I/II	Broniscer (2015)
		RT	Malignant brain tumor, BSG, LGG	I	Hargrave (-)
		Rapamycin	Embryonal brain tumor, choroid plexus carcinoma, ependymoma, HGG	I	Packer (2017)
		CT, (Erlotinib versus Etoposide)		-	Gajjar (2019)
Cilengitide (i.v.)	αvβ3, αvβ5 integrins	-	HGG	II	MacDonald (2011)
		RT	DIPG	I	Leblond (2015)
		TZM	Glioma	II	Kramm (2015)
Crizotinib (oral)	c-Met, ALK, RON	*Dasatinib*	DIPG, HGG	I	Broniscer (2015)

TNF-α: tumor necrosis factor α; VEGF: vascular endothelial growth factor; bFGF: basic fibroblast growth factor; IL: interleukin; TZM: temozolomide; RT: radiotherapy; BSG: brain stem glioma; DIPG: diffuse intrinsic pontine glioma; HGG: high-grade glioma; VEGFR: vascular endothelial growth factor receptor; EGFR: epidermal growth factor receptor; PDGFR: platelet derived growth factor receptor; FGFR: fibroblast growth factor receptor; LGG: low-grade glioma; CSF-1R: colony stimulating factor 1 receptor; CT: chemotherapy; ALK: anaplastic lymphoma kinase; RON: recepteur d'origine nantais.

Remaining drugs

Imatinib mesylate disrupts PDGF/PDGFR autocrine and paracrine loops and inhibits bcr-abl and c-kit (Figure 1). The results of imatinib in pediatric brain tumors were disappointing, except for possible activity of imatinib in progressive hypothalamic-chiasmatic astrocytoma.[126] However, in a phase I trial it was suggested that imatinib may increase the risk of intratumoral hemorrhage,[127] and a phase II study failed to observe objective tumor response despite immunohistochemical target confirmation in pediatric brain tumor samples (Table 1).[128] One potential explanation for the lack of response was that, despite the possibility for partial blood brain barrier disruption, less than 5% of the corresponding imatinib plasma levels were found in the cerebrospinal fluid, perhaps due to poor penetration of the blood brain barrier. Rationally, as imatinib showed overall no convincing therapeutic beneficial effects in pediatric brain tumors, up to now, no clinical trials evaluating imatinib in pediatric brain tumors are ongoing.

Another, more promising anti-angiogenic agent is cilengitide, an antagonist of αvβ3 and αvβ5 integrins which are particularly important in angiogenesis through their ability to bind to arginine-glycine-aspartic acid amino acid sequences found on matrix proteins, allowing endothelial cells to attach to the extracellular matrix (Figure 1). Cilengitide inhibits tumor growth in a dose-dependent manner in *in vivo* medulloblastoma and glioblastoma models. MacDonald et al described the first phase I clinical trial in which cilengitide in children with refractory brain tumors was well tolerated (Table 1).[129] As the demonstrated responses were encouraging for preliminary evidence of activity of this agent against pediatric brain tumors, currently three cilengitide studies are ongoing (Table 2).

Finally, new tyrosine kinase inhibitors are upcoming, like crizotinib inhibiting c-Met combined with dasatinib targeting Src in diffuse intrinsic glioma and high-grade glioma (Table 2). c-Met showed to be associated with tumor invasiveness as tumor escape mechanism during anti-angiogenic therapies. The importance of Src in pediatric brain tumors was previously determined with kinome profiling.[130] So, this combination treatment of crizotinib and dasatinib could be a promising strategy.

Response measuring and clinical monitoring

The evaluation of anti-angiogenic therapy in pediatric brain tumors relies on the overall survival as an ultimate gold standard or, more commonly in patients with recurrent disease, on clinical (neurologic) response, quality of life, progression free survival and the radiographic response rate. Symptom-based end points are subjective and are often influenced by factors not directly related to tumor burden. The tools to assess objectively and reproducible quality of life in children are limited with a high interpatient variability. Unfortunately, up to now, no blood, urine or cerebrospinal fluid biomarkers have been identified that could reliably reflect response or predict response to anti-angiogenic agents in pediatric brain tumors. Moreover, even regular lumbar punctures are too invasive for routinely clinical use. To actually measure and monitor objective response to anti-angiogenic agents, radiologic assessment of tumor response will be more crucial during anti-angiogenic therapy.

However, in the radiologic measurement of objective tumor response to anti-angiogenic therapy difficulties have been raised, including the phenomenon termed 'pseudo-response'.[131-133] Pediatric brain tumors that display contrast enhancement on T1 weighted magnetic resonance imaging (MRI) do so as a consequence of contrast extravasation in abnormally leaky blood vessels.[133] Anti-angiogenic therapy can modify vascular permeability and interstitial pressure; restore the integrity of the blood brain barrier, thereby reducing contrast leakage from the vasculature and thus the extent and distribution of gadolinium extravasation. In these possible 'pseudo-response' cases it is questionable to which extent a reduction in contrast enhancement reflects true antitumor activity or may purely reflect vascular changes. If this effect reflects only vascular permeability, interruption of anti-angiogenic therapy may lead to a rebound resulting in an overestimation of tumor growth on MRI.[131-134] Moreover, low-grade astrocytomas (WHO grade II) for example are less contrast enhancing tumors in imaging and ependymoma may have varying degrees of contrast enhancement,[2, 131] making it even more difficult to distinguish between tumor response or tumor progression. Furthermore increased T1 enhancement can also be induced by a variety of non-tumoral processes such as corticosteroid doses, seizure activity, post-surgical changes, ischemia, subacute radiation and radiation necrosis.[135-138]

To detect 'pseudo-response' and non-enhancing tumor progression, T2 and fluid-attenuated inversion recovery (FLAIR) MRI sequences have been recommended besides T1 weighted MRI. This combination of MRI sequences could also determine possible tumor escape mechanism during anti-angiogenic therapy in which a non-enhancing portion of tumor increased, suggestive of infiltrative tumor progression despite the continuing radiologic response of the enhancing lesions.[132] However, measurement of T2-FLAIR signal is challenging and distinguishing tumor from radiation-related changes, edema, and even seizures remains difficult.

The use of volumetric assessment would possibly allow more accurate determination of the contrast-enhancing and nonenhancing volumes and overcome limitations of two-dimensional measurements of lesions surrounding a surgical cavity.[139] It has also been suggested that advanced MRI techniques such as perfusion, permeability, and diffusion imaging, magnetic resonance spectroscopy, and [18F]-fluorothymidine and amino acid positron emission tomography (PET) may allow differentiation of non-enhancing tumor from other causes of increased FLAIR signal or predict tumor response.[133] However these techniques require rigorous validation before they can be used in pediatric clinical trial setting.

Recently, the institution of the Response Assessment in Pediatric Neuro-Oncology (RAPNO) has been established to develop a consensus on the radiological assessment for clinical trials involving children with brain tumors, and better define response so that it reflects drug activity. Initially, response criteria for three tumor subtypes will be developed and subsequently validated in clinical trials: low-grade glioma, high-grade glioma and diffuse intrinsic pontine glioma, each with its own inherent radiographic features. Inclusion of advanced MRI techniques will be determined per tumor type. Eventually, a more personalized imaging approach based on tumor characteristics, treatment modality and clinical factors may be necessary to adequately define radiographic response in pediatric brain tumors.[140]

Conclusions and future perspectives

The research field of tumor angiogenesis and anti-angiogenic therapy has undergone many crucial developments. More insight has been generated into the mechanism of angiogenesis and possible tumor escape mechanisms to anti-angiogenic treatment. New anti-angiogenic agents have been developed and replaced the relative older ones with low efficacy and significant toxicity levels. However, a substantial part of the generated knowledge has been derived from extensive research in adult glioblastoma, often seen as a model for studying angiogenesis. These insights have been extrapolated to pediatric brain tumors. It is questionable if the relatively limited studies in pediatric brain tumors and the extrapolation from adult glioblastoma are sufficient to make a rational translation into clinical trials in which pediatric patients are exposed to anti-angiogenic agents. Moreover, it has to be noted that inclusion of specific tumor subtypes including diffuse intrinsic pontine glioma in pediatric clinical trials is not only based on their expected susceptibility to anti-angiogenic therapy but also because of its fatal character despite intensive conventional therapies. Given the fact that many clinical trials in pediatric brain tumors are in the phase I setting and new agents are upcoming, we are still at the beginning of the development and optimization of anti-angiogenic therapy as an innovative clinical strategy. Up to now, some studies showed encouraging data of anti-angiogenic therapy in pediatric brain tumors, however results were partly generated in uncontrolled cases and not unambiguously presenting an anti-angiogenic strategy that significantly increases overall survival. So further research is definitely warranted in this field, especially focused on pediatric brain tumors.

Future therapeutic strategies will possibly combine anti-angiogenic agents with conventional treatment strategies like chemotherapy, as the possible synergistic effect is still unknown in pediatric brain tumors. However, multi targeted therapy with various potential anti-angiogenic agents could be of even more interest, anticipating on possible tumor escape mechanisms. These strategies could be especially suitable for children with an unresectable brain tumor, taking into account that introducing this strategy at initial diagnosis could be highly effective. Although potential anti-angiogenic agents are generally well tolerated in pediatric patients, strict clinical monitoring remains required which will be accompanied by radiologic challenges. In conclusion, because of the need for new therapeutic strategies in pediatric brain tumors as leading cause of cancer morbidity and mortality among children, multi targeted therapy with anti-angiogenic agents could have a future therapeutic role. There are also arguments for a personalized targeted therapy based on tumor marker analyses of recently derived pediatric brain tumor tissue.[141] A step forward would be the discovery of biomarkers or tumor marker patterns, which will predict accurately the benefit of which drug.

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Chapter 3

The angiopoietin 1/angiopoietin 2 balance as a prognostic marker in primary glioblastoma multiforme

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Abstract

Object: In the present study, the authors analyzed the ANGPT1/ANGPT2 balance in the context of therapeutic outcome in 62 patients with primary glioblastomas multiforme (GBMs).

Methods: The tumor tissue used was obtained in adult patients who underwent neurosurgical debulking. Microvessel density was assessed by morphometric analysis. Double immunostaining for Ki 67/CD34 and cleaved caspase-3/CD34 was used to investigate the proliferation and apoptotic fraction of both endothelial and tumor cells. The expression of VEGFs (A-D) was evaluated on immunohistochemistry. To measure tumor vascular stabilization, the ANGPT1/ANGPT2 mRNA balance was determined using real-time reverse transcriptase polymerase chain reaction.

Results: Within the hypoxic perinecrotic tumor area, the apoptotic fraction of endothelial cells was positively correlated with VEGFA expression ($p < 0.001$). Higher levels of VEGFA correlated with greater proliferation of endothelial cells in the intermediate tumor area ($p = 0.031$). Vascular endothelial growth factor D was significantly more highly expressed within the perinecrotic tumor area compared with the intermediate tumor area ($p < 0.001$). Multivariate analysis showed a significant association between the ANGPT1/ANGPT2 balance and the survival time of patients with GBMs ($p = 0.035$).

Conclusions: The results of the present study suggest that the ANGPT1/ANGPT2 balance has prognostic value in patients with primary GBMs. The authors' findings support the need for further studies of the feasibility of antiangiogenic therapy in primary GBMs, with a special focus on the normalization of tumor vasculature.

Introduction

Glioblastoma multiforme is the most frequent and malignant brain tumor in adults, making up 12–15% of all intracranial neoplasms.[1-3] Despite combined modality treatment with neurosurgery, radiotherapy, and chemotherapy, the median patient survival is 14.6 months,[4] underscoring the aggressive growth properties of GBM. Glioblastoma multiforme is characterized by endothelial cell proliferation and prominent vascularization[2, 5] resulting from a combination of vessel cooption and tumor angiogenesis.[6, 7]

Because angiogenesis is a key event in tumor growth and progression,[6, 7] understanding its molecular control has been the focus of many studies. Vascular endothelial growth factor (VEGF) and the angiopoietins (ANGPTs) are critically involved in angiogenesis.[5, 8-10] There are 7 members of the VEGF family, of which VEGFA, VEGFB, VEGFC, and VEGFD are of interest in the study of human tumors.[10] Vascular endothelial growth factor A, induced by hypoxia, increases microvascular permeability, induces endothelial cell migration and division, promotes endothelial cell survival, prevents senescence, and induces angiogenesis.[9] Little is known about the roles of VEGFB, VEGFC, and VEGFD in tumor angiogenesis. Vascular endothelial growth factor B promotes *in vivo* angiogenesis.[11] Vascular endothelial growth factor C and D are essential to the development of the lymphatic system and can also induce angiogenesis and increase vascular permeability.[12]

Angiopoietin 1 helps to maintain and stabilize mature vessels by acting as a maturation factor that promotes the recruitment of pericytes and smooth muscle cells to the developing vessel.[13] The functions of ANGPT1 and VEGF may be complementary, with VEGF providing most mitogenic activity and ANGPT1 responsible for ensuring vascular stability.[14] Angiopoietin 2 is the more dynamic protein of these, is expressed at sites of vascular remodeling, and acts as a competitive antagonist of ANGPT1. In the absence of VEGF, ANGPT2 destabilizes vessels via TEK dephosphorylation, ultimately leading to vessel regression. In the presence of VEGF, however, ANGPT2 facilitates angiogenesis.[15, 16] The authors of some studies have demonstrated that VEGF and angiopoietins are both markedly upregulated in the vast majority of human tumors, including gliomas.[5, 17] A shift in the balance between ANGPT1 and ANGPT2 in favor of ANGPT2 appears to correlate with tumor angiogenesis.[5]

In various tumors the production of proangiogenic factors, such as ANGPT2, leads to abnormal vessel structure and signs of vascular immaturity (such as that reflected by a reduction of pericyte coverage).[15, 18] By correcting the balance in these angiogenic factors, the immature vasculature in tumors can be normalized, leading to increased tumor perfusion, increased partial oxygen pressure, and a reduction in the interstitial pressure in the tumor. As a consequence, the efficacy of radiotherapy and chemotherapy may be increased.[15, 18]

Although authors of previous studies have explored the expression of VEGF, ANGPT1, and ANGPT2 in primary GBMs as separate features, in the present study we aimed to directly quantify the ANGPT1/ANGPT2 balance within these tumors. We analyzed MVD, proliferation and apoptotic fraction of endothelial and tumor cells, VEGF expression, and angiopoietin balance in the context of therapeutic outcome in patients with primary GBMs.

Methods

Patient population and baseline characteristics

From January 1998 to January 2003, 132 patients received a diagnosis of primary GBM (WHO grade IV astrocytoma) at our institution. Patients who underwent neurosurgical debulking were eligible for this study. Exclusion criteria included stereotactic biopsy (in 57 patients), secondary GBM (in 10), and mixed tumors containing zones with sarcomatous or oligodendroglial differentiation (in 3 patients). Finally, tumor tissues from 62 patients were included in this study.

The baseline patient characteristics included age at diagnosis, sex, preoperative tumor volume, period between start of symptoms and surgical debulking, degree of debulking as indicated by the surgical report, postoperative radiotherapy (up to a total of 60 Gy), and survival time. Age at diagnosis was defined as the patient's age on the date that the imaging study (MR imaging or CT) used to diagnose the tumor was obtained. Preoperative tumorvolume was calculated as described by Kuijlen et al.[19] The period between the start of symptoms and operation was defined as the time between the first manifestation of clinical symptoms, as registered by the referring neurologist, and the day of surgical debulking. The survival time was calculated as the period from the date of surgery until death.

Immunohistochemical staining

Deparaffinized GBM tissue was used to evaluate the proliferation and apoptotic fraction of both endothelial and tumor cells (4-µm-thick tissue slices), and the expression of VEGFA, -B, -C, and -D (5-µm-thick slices). This evaluation was performed based on double staining for Ki 67/CD34 and cleaved caspase-3/CD34, and single staining for VEGFA, -B, -C, and -D. For Ki 67/CD34 and cleaved caspase-3/CD34 staining, antigen retrieval was performed using 10 mM Tris/1 mM EDTA (pH 9) and 1 mM EDTA (pH 9), respectively, in a microwave at 700 W. Antigen retrieval for VEGFA staining was performed with 0.1 mM Tris/HCl (pH 9). For VEGFB, VEGFC, and VEGFD staining, 0.1 mM Tris/0.1 mM EDTA (pH 9) and subsequent microwave treatment at 400 W was used. Endogenous peroxidase and biotin were blocked using routine techniques.

The slides for Ki 67/CD34 staining were incubated with the first primary antibody, Ki 67 (Clone MIB-1; Dako) at room temperature for 1 hour, followed by application of the secondary antibody peroxidase-conjugated rabbit anti-mouse serum (Dako), and the tertiary antibody peroxidase-conjugated goat anti-rabbit serum (Dako), for 30 minutes each. For cleaved caspase-3/CD34 staining, sections were incubated with the primary antibody for cleaved caspase-3 (Asp175; Cell Signaling Technology) at room temperature for 1 hour. This was also followed by application of the same secondary and tertiary antibodies as used for the Ki 67/CD34 assay for 30 minutes each. The first primary antibodies were diluted 1/100 in 1% BSA/PBS. The secondary and tertiary antibodies were diluted 1/100 in 1% BSA/PBS with 1% AB serum. Color development was performed with 3,3'-diaminobenzidine (Sigma) for 10 minutes.

For Ki 67/CD34 staining, a blocking step with 0.1 M glycine/HCl (pH 2) was performed next for 45 minutes. Thereafter, the slides for both Ki 67/CD34 and cleaved caspase-3/CD34 were incubated with the second primary antibody CD34 (prediluted Clone Qbend10; Immunotech) at room temperature for 1 hour, followed by the application of secondary antibody alkaline phosphatase-conjugated goat anti-mouse (1/50 in 1% BSA/PBS with 1% AB serum; Dako) for 30 minutes, and subsequent staining with Fast Red (Sigma) for 20–30 minutes.

The slides for VEGF were incubated with the primary antibody VEGFA (sc-152), VEGFB (sc-13083), VEGFC (sc-9047) and VEGFD (sc-13085; Santa Cruz Biotechnology) for 60 minutes. Primary antibodies were diluted 1/50 in 1% BSA/PBS. Biotinylated peroxidase-conjugated goat anti-rabbit serum (1/300 in 1% BSA/PBS with 1% AB serum; Dako) was applied as the secondary antibody for 30 minutes, followed by the tertiary antibody peroxidaseconjugated StrepABComplex (Ko377, 1/100 in 1% BSA/PBS with 1% AB serum; Dako) for 30 minutes. The slides were subsequently stained with 3'3-diaminobenzidine (Sigma) for 10 minutes, followed by nuclear counterstaining with hematoxylin.

Histological evaluation

The slides were scanned for hot spots of blood vessels in both the perinecrotic and intermediate tumor area. The perinecrotic tumor area is a relatively hypoxic area directly adjacent to necrosis, showing increased expression of HIF-1α.[20] The intermediate tumor area lies between the perinecrotic area and the tumor periphery, which contains the invasion front into the normal brain tissue. The intermediate tumor area showed no HIF-1α expression and less VEGFA expression compared with the perinecrotic tumor area.

The MVD for both the perinecrotic and intermediate tumor area was assessed using the Chalkley point overlap morphometric technique.[21] This method entails the use of an ocular grid with 25 random points. After the tumor was scanned to identify 10 hot spots per slide (5 in the perinecrotic and 5 in the intermediate area), the ocular grid was turned to maximize the overlap between points on the grid and the CD34-stained vessels. The number of overlapping points was counted for every hot spot. The MVD was obtained by calculating the mean of 5 hot spots per tumor area.

To determine the proliferation fraction of the endothelial and tumor cells in both the perinecrotic and intermediate tumor area, 5 hpfs in Ki 67/CD34-doublestained slides were evaluated at an objective magnification of 40. A minimum of 100 CD34+ endothelial cells were counted per slide, and the fraction of cells that stained for both CD34 and Ki 67 was recorded as the proliferation fraction of endothelial cells. Similarly, a minimum of 800 tumor cells was counted and the fraction of Ki 67+ tumor cells was determined. The same procedure was used with the cleaved caspase-3/CD34 slides to assess the apoptotic fraction of both endothelial and tumor cells.

Expression of VEGFA, -B, -C, and -D in both the perinecrotic and intermediate tumor areas was evaluated using a semiquantitative staining intensity score: no staining was scored as 0, low staining as 1, moderate as 2, and high intensity staining as 3. Normal brain parenchyma was used as a control for the staining procedure. All slides were evaluated by a neuropathologist who was blinded to patient data.

Real-time reverse transcriptase PCR and RNA isolation

Paraffin-embedded GBM tissue obtained in 62 patients was deparaffinized. Isolation of RNA was performed using the protocol described by Specht et al.,[22] and RNA isolation was quantified using Nanodrop ND-1000 UV Spectrophotometer (NanoDrop Technologies). After this, the TURBO DNA-Free Kit (Ambion) was used to remove contaminating DNA from RNA preparations, and subsequently to remove the DNAse and divalent cations from the sample; 1.5 µl of 10 × TURBO DNAse Buffer and 1-µl TURBO DNAse (2 U/µl; Ambion) were added and the tube was incubated at 37°C for 30 minutes. Two microliters of DNAse Inactivator Reagent (Ambion) was added next, and the tube was incubated at room temperature for 2 minutes with occasional mixing. The tube was centrifuged at 13,000 × gravity at room temperature for 1 minute to make the DNAse Inactivation Reagent into a pellet. After centrifuging, the supernatant containing the RNA was carefully transferred into a clean tube and the RNA content was again quantified using Nanodrop.

Synthesis of cDNA was performed as described previously.[19] Real-time PCR was performed in triplicate with the TaqMan Universal PCR Master Mix (Applied Biosystems), using an ABI7900HT real-time sequence detection system in 384-well reaction plates. The following primers were used: ANGPT1 (Hs 00181613.m1), ANGPT2 (Hs 00169867.m1), and GAPDH (Hs 99999905.ml). The balance between ANGPT1 and ANGPT2 was calculated using the formula: $2^{\Delta Ct}$, in which ΔCt is the mean Ct value of ANGPT2 minus the mean Ct value of ANGPT1.

Statistical analysis

Statistical comparisons were made with the Student t-test if the data had a Gaussian distribution, and with the Mann-Whitney U-test if the data were not normally distributed. The nonparametric Spearman correlation test was used to compute correlations. The survival time was analyzed by the Kaplan-Meier method with use of log-rank statistics. Univariate and multivariate analyses were performed using the Cox proportional hazards model (SPSS 15.0 software). For all statistical analyses, a 2-tailed probability value < 0.05 was considered significant.

Results

Patient characteristics

All 62 patients had a Karnofsky Performance Scale scores ≥ 70 before debulking surgery. Radiotherapy was abrogated in 15 patients (24%) because of rapid postoperative deterioration (Karnofsky Performance Scale scores < 70); the remaining 47 patients (76%) received postoperative radiotherapy. No patient in this study received any form of chemotherapy because chemotherapy was not part of the standard treatment for GBMs in the Netherlands during the study period.

The mean patient age at diagnosis in the study group was 59 years (Table 1). Two-thirds (68%) of patients fell into the expected peak incidence age group of 45–70 years,[2] with a male preponderance of 69%. The median survival time was 253 days in the study group. As expected,[1] the median survival period in patients who did not receive postoperative radiotherapy was less than in those who did: 50 days versus 303 days.

No differences in the other patient characteristics were found between the groups with and without radiotherapy. Our study group was thus representative of the population of patients with primary GBMs.

Table 1. Summary of characteristics of 62 patients with GBMs*

Characteristic	Total (62 patients)	RT group (47 patients)	No RT group (15 patients)	P value
Mean age at diagnosis in yrs (95% CI)	59 (57–62)	59 (56–62)	60 (54–67)	ns
Male sex (%)	43 (69)	33 (70)	10 (67)	ns
Female sex (%)	19 (31)	14 (30)	5 (33)	ns
Median time to op in days (range)#	43 (7–211)	51 (7–211)	31 (14–122)	ns
Extent of resection, no. of patients (%)				
GTR	35 (57)	27 (57)	8 (53)	ns
Partial	17 (27)	13 (28)	4 (27)	ns
Not recorded	10 (16)	7 (15)	3 (20)	ns
Mean preop tumor vol in cm^3 (95% CI)	43 (37–49)	42 (36–48)	45 (29–61)	ns
Median survival in days (range)	253 (17–792)	303 (61–792)	50 (17–91)	< 0.001

* GTR: gross-total resection; NS: not significant; RT: radiotherapy.
# Represents time period between symptom onset and surgery.

Distribution of MVD and proliferation/apoptotic fraction of endothelial and tumor cells

In all primary GBM tissue samples, vascular hot spots were found in both the perinecrotic and intermediate tumor areas (Figure 1). The intermediate tumor area showed a significantly higher MVD and proliferation fraction of tumor cells than the perinecrotic area (Table 2). The MVD of the intermediate tumor area did not correlate with the proliferation fraction of endothelial cells. Within the intermediate area, the proliferation fraction of tumor cells was negatively correlated with the MVD (Spearman $r = -0.325$, $p = 0.010$). The perinecrotic tumor area, a relatively hypoxic zone, showed a significantly higher apoptotic fraction of both endothelial and tumor cells. The morphometric data did not significantly correlate with the survival time of GBM patients.

Table 2. Morphometry within the perinecrotic and intermediate tumor area in 62 patients with primary GBMs*

Morphometry	Perinecrotic tumor area	Intermediate tumor area	P value
Mean MVD (95% CI)	4.6 (4.3–5.0)	5.4 (5.0–5.8)	0.007
Median PFEC (range)	6.8 (0.9–28.8)	9.5 (0.0–25.0)	ns
Median AFEC (range)	14.9 (0.2–67.6)	2.6 (0.0–12.5)	< 0.001
Median PFTC (range)	13.1 (3.3–48.8)	21.1 (3.9–72.6)	< 0.001
Median AFTC (range)	3.0 (0.2–30.6)	0.6 (0.0–7.8)	< 0.001

* AFEC: apoptotic fraction of endothelial cells; AFTC: apoptotic fraction of tumor cells; PFEC: proliferation fraction of endothelial cells; PFTC: proliferation fraction of tumor cells.

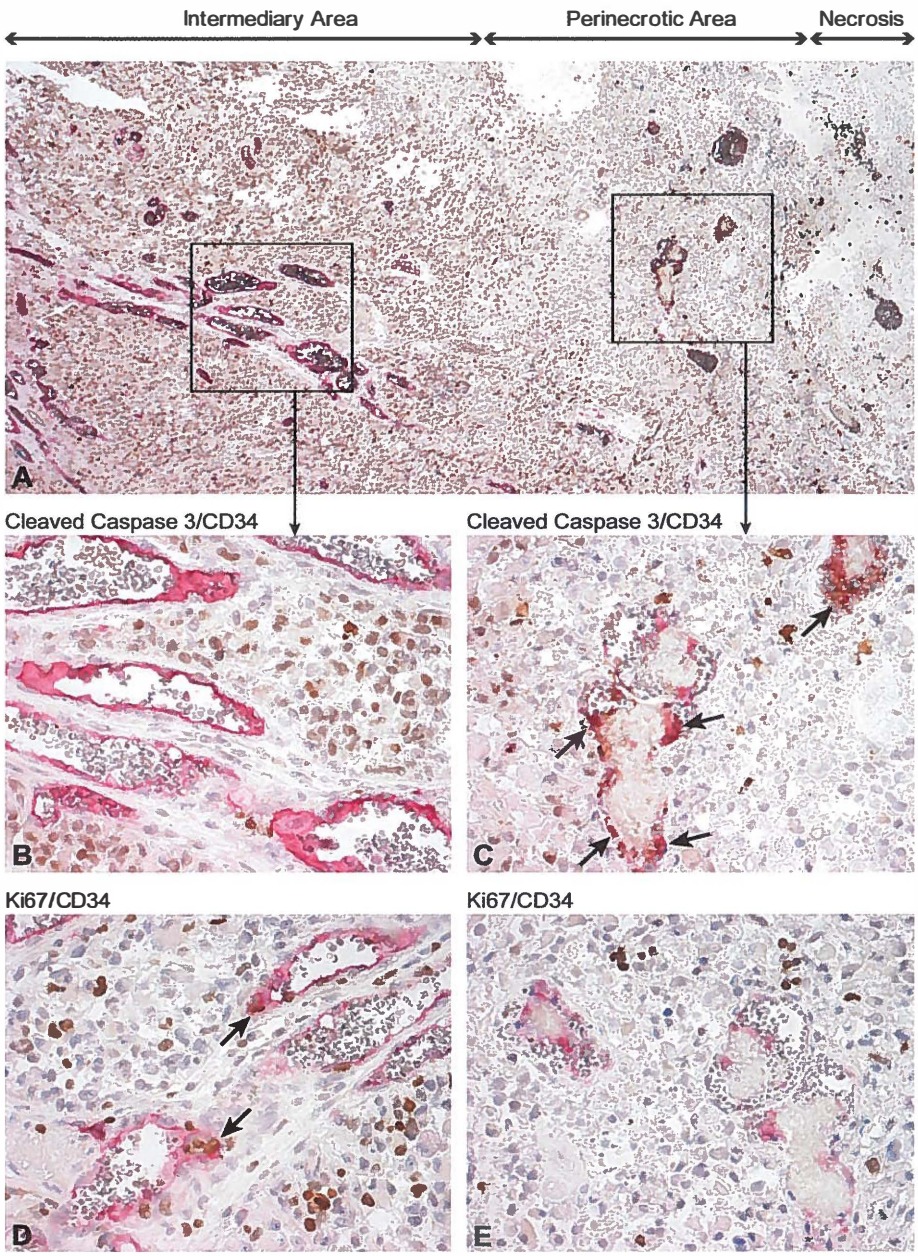

Figure 1. Photomicrographs of double-stained slides of a primary GBM. An overview of the different tumor areas with vascular hot spots in both the perinecrotic (*right*) and intermediate (*left*) tumor area is shown in **A**. Panels **B** and **C** respectively show details of vessels in the intermediate and perinecrotic tumor area, double-stained with cleaved caspase-3/CD34. Apoptotic endothelial cells are mostly observed in the perinecrotic tumor area (*arrows* in C). Panels **D** and **E** show details of vessels in the intermediate and perinecrotic tumor area, respectively, and are double-stained with Ki 67/CD34. Proliferating endothelial cells are mostly found in the intermediate tumor area (*arrows* in D). Original magnification × 100 (A), and × 400 (B-E).

Vascular endothelial growth factor expression

A significantly higher expression of VEGFA and VEGFD was found within the perinecrotic tumor area than in the intermediate tumor area (Figure 2). These data are consistent with the hypothesis that the production of VEGF is driven, at least in part, by hypoxia.[6,7] Although this is known for VEGFA, it has not been described previously for VEGFD. For VEGFB and VEGFC no significant differences were found in expression between the perinecrotic and intermediate tumor areas.

Within the perinecrotic tumor area, a positive correlation was found between the apoptotic fraction of endothelial cells and the expression of VEGFA (Spearman $r = 0.485$, $p < 0.001$). In addition, a higher level of VEGFA expression was correlated with a higher proliferation fraction of endothelial cells within the intermediate tumor area (Spearman $r = 0.277$, $p = 0.031$). However, in the intermediate area, the expression of VEGFA was not significantly correlated with the MVD (Spearman $r = 0.251$, $p = 0.051$). Furthermore, no significant correlation was found between the expression of VEGF and the survival time of patients with GBMs.

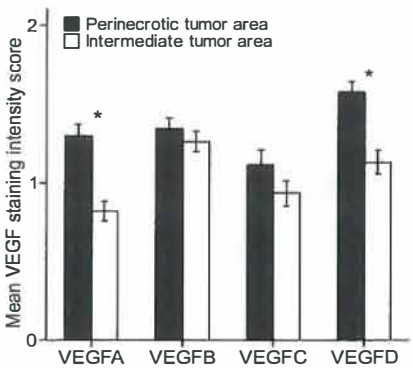

Figure 2. Bar graph showing the mean (± standard error) VEGFA, VEGFB, VEGFC, and VEGFD staining intensity score within the perinecrotic and intermediate tumor area in 61 patients with primary GBMs. The mean staining intensity score of VEGFA and VEGFD was 1.295 and 1.574 within the perinecrotic tumor area compared with 0.820 and 1.131 within the intermediate tumor area (*$p < 0.001$).

Angiopoietin 1/angiopoietin 2 balance

Expression of ANGPT1 and ANGPT2 could be determined in all primary GBM tissue samples, and the balance between them was calculated. The median ANGPT1/ANGPT2 balance was 0.268 (range 0.034–1.675) in the 62-patient study group and 0.289 (range 0.034–1.675) in the subgroup of 47 patients who underwent postoperative radiotherapy. The angiopoietin balance was positively correlated with the survival time of 62 patients with GBMs (Spearman $r = 0.322$, $p = 0.011$). Because patients who underwent radiotherapy had a significantly longer survival time than patients who did not, a subgroup analysis was indicated. The ANGPT1/ANGPT2 balance also significantly correlated with the survival time in patients who received radiotherapy (Figure 3).

Using the median ANGPT1/ANGPT2 balance of 0.289 as the cutoff in the radiotherapy subgroup, the median survival time in patients with an ANGPT1/ANGPT2 balance ≤ 0.289 was 253 days compared with 345 days in patients with an ANGPT1/ANGPT2 balance > 0.289 (Figure 4). Moreover, univariate analysis showed a significant association between the ANGPT1/ANGPT2 balance and the survival time in both the total study group and the subgroup that received postoperative radiotherapy (Table 3). Even corrected for known prognostic parameters in a multivariate analysis, the angiopoietin balance was still associated with the survival time of GBM patients.

Table 3. Univariate and multivariate analyses of data obtained in patients with primary GBMs*

	Univariate analysis			Multivariate analysis		
Variable	HR	95% CI	p value	HR	95% CI	P value
Entire study group (62 patients)						
Age at diagnosis	1.02	1.00–1.05	0.104	1.04	1.01–1.07	0.006
Sex	1.11	0.64–1.92	0.711	0.58	0.29–1.18	0.133
Preop tumor volume	1.01	0.99–1.02	0.298	1.01	0.99–1.02	0.519
Degree of debulking (GTR vs PR)	0.73	0.40–1.33	0.299	0.55	0.27–1.14	0.106
Radiotherapy	70.96	15.33–328.47	<0.001	73.32	13.85–388.13	<0.001
ANGPT1/ANGPT2 balance (≤0.268 vs >0.268)	1.86	1.11–3.10	0.018	1.99	1.05–3.76	0.035
RT group (47 patients)						
Age at diagnosis	1.03	1.00–1.06	0.080	1.06	1.02–1.10	0.002
Sex	1.12	0.59–2.12	0.732	0.57	0.25–1.29	0.177
Preop tumor volume	1.01	0.99–1.03	0.343	1.02	1.00–1.04	0.133
Degree of debulking (GTR vs PR)	0.66	0.33–1.33	0.245	0.53	0.24–1.17	0.116
ANGPT1/ANGPT2 balance (≤0.268 vs >0.268)	2.02	1.12–3.64	0.020	2.49	1.22–5.06	0.012

* HR: hazard ratio; RT: radiotherapy; GTR: gross-total resection; PR: partial resection

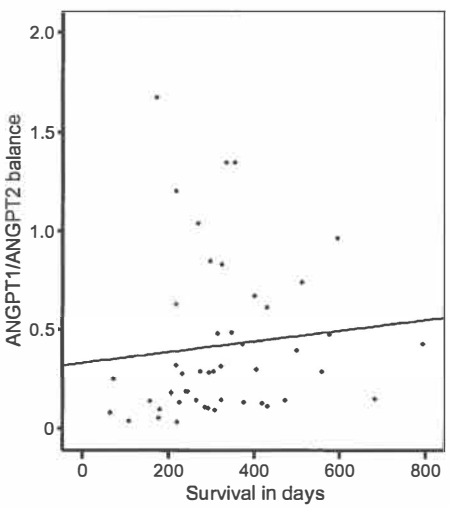

Figure 3. Scatterplot showing a positive correlation between the ANGPT1/ANGPT2 balance and the survival time in 47 patients with primary GBM and postoperative radiotherapy (Spearman r = 0.317, p = 0.030).

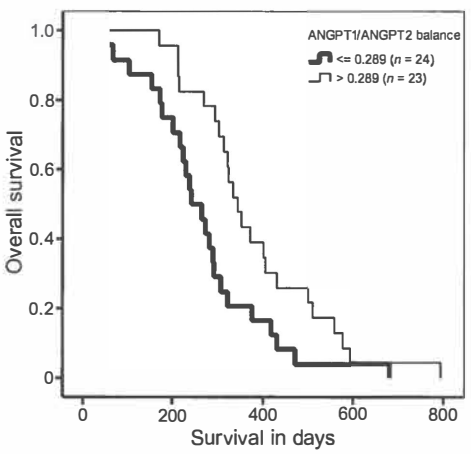

Figure 4. Kaplan-Meier curves of the overall survival of patients with primary GBM and postoperative radiotherapy (47 patients). Using the median ANGPT1/ANGPT2 balance of 0.289 as the cutoff, the median survival time in patients with an ANGPT1/ANGPT2 balance ≤ 0.289 was 253 days, and 345 days in patients with a higher ANGPT1/ANGPT2 balance (p = 0.018, log-rank test).

Discussion

We evaluated MVD, proliferation and apoptotic fraction of endothelial and tumor cells, VEGF expression, and ANGPT1/ANGPT2 balance in the context of therapeutic outcome in 62 patients with primary GBMs. Here, the ANGPT1/ANGPT2 balance was identified as a prognostic marker in patients with GBMs.

Of all the members of the VEGF protein family, VEGFA has been studied the most extensively. Our findings of increased staining of VEGFA within the perinecrotic area are consistent with the literature. This distribution within the tumor is explained by considering hypoxia as the primary trigger for the production of VEGFA. In the study by Holash et al.,[23] who investigated early vascularization of gliomas in a rat C6 model, hypoxia was the most specific signal that induced transcription of the VEGFA gene and stabilization of its mRNA. Although VEGFB, -C, and -D have been reported to be expressed in gliomas,[24-27] a distribution similar to VEGFA was not seen. Currie et al.[28] found an association between VEGFD, HIF-1α, and the HIF target gene DEC1 in primary human breast carcinomas. Although these findings support a role for hypoxiainduced VEGFD production, expression of VEGFD was not seen adjacent to areas of necrosis. In the present study, we show for the first time an increased staining intensity of VEGFD in the perinecrotic area.

Microvessel density is frequently used as a measure of angiogenic activity, so our finding that MVD does not correlate with either endothelial cell proliferation or VEGFA staining intensity seems counterintuitive. However, it has been proposed that MVD may depend more on the metabolic and mitotic activity of the tumor cells.[29] This concept is nicely illustrated by the negative correlation of MVD with tumor cell proliferation in the intermediate area, in absence of a correlation with endothelial cell proliferation and VEGF staining intensity. Few studies have analyzed the proliferation fraction of endothelial cells as a measure of angiogenic activity. Unlike MVD, however, the proliferation fraction of endothelial cells did correlate with VEGFA staining in the intermediate area. Analysis of the proliferation fraction of endothelial cells may very well be superior to MVD as a measure of angiogenic activity in tumors.[29, 30]

The angiopoietins are thought to actively control the initiation of tumor angiogenesis.[31] Angiopoietin 1 and 2 ligate TEK, an endothelial cell transmembrane receptor; TEK is phosphorylated by ligation with ANGPT1, whereas ANGPT2 acts as a competitive antagonist. The exact roles of both angiopoietins are uncertain, and contradictory reports on pro- and antiangiogenic activity of both proteins can be found in the literature.[32] Overall, it is clear that the presence of ANGPT1 leads to increased integration of the endothelial cells into their surroundings (adequate pericyte coverage), which is important for endothelial cell survival. Angiopoietin 2, however, destabilizes the vasculature and prepares the tumor endothelial lial cells to become responsive to proangiogenic factors such as VEGF. Consequently, in the presence of VEGF, sprouting angiogenesis can occur.

In line with this concept, overexpression of ANGPT2 in experimental tumors was found to lead to larger and heavier lesions.[33, 34] In preclinical models in astrocytomas, ANGPT2 has been identified as an early marker of tumor angiogenesis,[35, 36] and overexpression of ANGPT2 in intracranial tumors leads to enhanced infiltrative growth and increased angiogenesis in the areas of invasion.[37]

The angiopoietin/TEK system has been studied previously in human astrocytomas.[35, 36, 38, 39] Angiopoietin 1 mRNA is present in GBM tumor cells. Although the extent of expression varies locally, it can be seen throughout the tumor.[35, 36, 39] Most studies have shown ANGPT2 expression to be restricted to the tumor endothelial cells. Angiopoietin 2 expression appears to be induced by the presence of tumor cells. This is illustrated by ANGPT2 expression by endothelial cells surrounded by infiltrating tumor cells at the invasive edge of the tumor. The role of ANGPT2 as an early marker of tumor angiogenesis as described in preclinical studies in a glioma model has been confirmed in human glioma samples.[36]

Because ANGPT1 and ANGPT2 ligate the same receptor in competitive antagonism, the angiopoietin/TEK system is considered to function as a balance. Although the 2 angiopoietins are largely produced by different cells, the calculation of a direct ratio of the mRNA levels of both may reflect the general angiogenic activity of the tumor. We found that this ANGPT1/ANGPT2 balance was positively correlated with survival. Although this does not constitute a direct relationship between angiopoietin balance and survival, it does add to the evidence in support of the importance of the angiopoietin/TEK system in glioma growth and angiogenesis.

The concept of vessel normalization was introduced relatively recently into the field of angiogenesis.[40] Since the rise of angiogenesis research a few decades ago, attempts to use antiangiogenesis in the treatment of cancer have mainly focused on targeting the tumor vasculature for destruction and thereby starving the tumor or halting its growth. Vessel normalization, however, aims at reversing the changes that have occurred in the tumor vasculature during the process of vessel cooption or new vessel formation, thereby increasing the tumor's vulnerability to conventional therapies, such as radiation. The angiopoietins are strongly associated with the occurrence of these morphological and functional changes in the tumor vasculature and are thought to actively control the initiation of tumor angiogenesis.[31] Therefore, our finding of a prognostic significance of the angiopoietin balance in a group of patients who underwent radiation therapy supports further attempts to study the feasibility of vessel normalization as part of the treatment of patients with GBMs.

Conclusions

To our knowledge, this is the first study that examines the ANGPT1/ANGPT2 balance instead of studying ANGPT1 and ANGPT2 as separate features in human brain tumors. In our multivariate analysis, we found a significant association between the ANGPT1/ANGPT2 balance and survival in primary GBM patients. Our findings support the initiation of further studies into the feasibility of antiangiogenic therapy in primary GBMs, especially when focused on the normalization of tumor vasculature.

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Chapter 4

Tumor vessel biology in pediatric intracranial ependymoma

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Abstract

Object: This study aimed to characterize the pediatric intracranial ependymoma vasculature in terms of angiogenic activity and maturation status so as to provide indications for the applicability of vessel-targeted therapy in cases of pediatric intracranial ependymoma.

Methods: Tumor samples obtained in patients with ependymomas were immunohistochemically (double) stained for Ki 67/CD34, caspase 3a/CD34, vascular endothelial growth factor (VEGF)-A, -B, -C, -D, collagen Type IV, and smooth muscle actin to determine microvessel density, tumor and endothelial cell proliferation and apoptotic fraction, the relative expression of VEGF family members, and the coverage of the tumor endothelial cells by basal membrane and pericytes. Messenger RNA expression of angiopoietin-1 and -2 was analyzed by real-time reverse transcriptase polymerase chain reaction. These data were compared with those obtained in a glioblastoma series.

Results: Despite a low endothelial cell turnover, the microvessel density of ependymomas was similar to that of glioblastomas. In ependymomas the expression of VEGF-A was within the range of the variable expression in glioblastomas. The staining intensities of VEGF-B, -C, and -D in ependymomas were significantly lower ($p < 0.001$). The expression of angiopoietin-1 was higher in ependymomas than in glioblastomas ($p = 0.03$), whereas angiopoietin-2 expression was similar. The coverage of tumor endothelial cells with basal membrane and pericytes was more complete in ependymomas ($p = 0.009$ and $p = 0.022$, respectively).

Conclusions: The ependymoma vasculature is relatively mature and has little angiogenic activity compared with malignant gliomas. Therefore, the window for vessel normalization as a therapeutic aim might be considered small. However, the status of the tumor vasculature may not be a reliable predictor of treatment effect. Therefore, possible benefits of antiangiogenic treatment cannot be excluded beforehand in patients with ependymomas.

Introduction

Angiogenesis has been increasingly recognized as an important process in the outgrowth of solid tumors. Parameters related to tumor angiogenesis such as MVD and VEGF expression have been shown to be predictive of the prognosis in a host of tumors[1-4] and correlate with histological tumor grade in gliomas.[5,6] Moreover, clinical trials of therapies aimed at the neovasculature have produced promising results.[7] Glioblastomas have received special attention within the field of angiogenesis research because of their robust vasculature.[8,9] The different aspects of the angiogenic process—that is, the initiation of angiogenesis, endothelial cell proliferation, and migration, extracellular matrix breakdown, and tube formation—have been extensively studied in glioblastomas. The angiogenic potential of glioblastomas has initiated investigations of vessel-targeted therapy and has motivated study groups worldwide to start trials on the feasibility of using antiangiogenic drugs in the treatment of glioblastomas.

Less is known about the neovasculature of other brain tumors. In pediatric ependymomas, in which the neovasculature is less prominent, only a handful of studies have analyzed aspects of the angiogenic process.[10-14] The prognosis of pediatric ependymomas is limited. Despite developments of new surgical techniques, the introduction of conformal radiotherapy, and new chemotherapeutic strategies (for example, neoadjuvant chemotherapy), 5-year survival is approximately 57%.[15] Because late recurrences are no exception, 10-year survival is even worse. Therefore, new therapeutic strategies are needed.

In the present study we aimed to characterize the neovasculature in a series of pediatric intracranial ependymomas. We quantified MVD, VEGF expression, and tumor and endothelial cell proliferation and apoptosis. Furthermore, the balance between the expression of Ang-1 and Ang-2 was analyzed, as this may be indicative of the angiogenic potential of tumors. These data were interpreted in the context of what is known about the neovasculature of glioblastomas that has been reported.[16] As such, this study may provide a basis for further research into the possibilities of adding drugs with antiangiogenic activity to the treatment schedules of pediatric ependymoma.

Methods

Clinical data

Patients operated on between 1988 and 2004 were selected selected from the medical database of the University Medical Center Groningen and the national pathological database PALGA. Patients were included if 1) they were diagnosed with an intracranial ependymoma, 2) they were under 20 years of age, 3) there was access to clinical follow-up information, and 4) pre- and postoperative MR images were available. Cases involving spinal ependymomas were excluded. Tumor slides were reviewed to confirm the diagnosis, and, when available, tumor tissue samples were used for immunohistochemical analysis and real-time RT-PCR analysis. Table 1 summarizes the characteristics, treatment, and follow-up of the selected patients.

Data and analysis on glioblastoma vasculature were drawn from tissue samples of patients who underwent debulking surgery between 1998 and 2003 for a primary glioblastoma in the University Medical Center Groningen. These data have been previously reported.[16]

Table 1. Patients characteristics, treatment, and follow-up*

		Yrs of age		Location	
Parameters§	Total group	<3	>3	Infratentorial	Supratentorial
Patient					
No.	27	10	17	21	6
Age	4.2 (1.2–15.6)	2.0 (1.2–2.7)	6.4 (3.1–15.6)	3.7 (1.2–15.6)	4.6 (2.6–6.4)
Sex (M/F)	14/13	5/5	9/8	11/10	3/3
WHO Grade II/III	23/4	9/1	14/3	17/4	6/0
Treatment					
Biopsy	1	1	0	1	0
Resection	26	9	17	20	6
Partial	12	3	9	9	3
Gross-total	14	6	8	11	3
Radiotherapy	18	2Ŧ	16	13	5
Chemotherapy	6	4	2	4	2
Follow-up					
Follow-up (yrs)	9.5 (2.6–18.9)	8.8 (2.9–17.8)	9.5 (2.6–18.9)	7.8 (2.8–17.8)	12.6 (2.6–18.9)
Recurrence#	18 (27)	8 (10)	10 (17)	15 (21)	3 (6)
Time to recurrence (yrs)	1.3 (0–4.5)	1.3 (0–3.1)	1.2 (0–4.5)	1.3 (0–4.5)	1.1 (0–2.7)
5-yr PFS (%)	27.3	11.1	38.5	17.6	60
5-yr OS (%)	54.5	55.6	53.8	52.9	60
10-yr (OS)#	5 (13)	1 (5)	4 (8)	3 (9)	2 (4)

* OS: overall survival; PFS: progression-free survival.

§ All parameters are presented as median (range) unless stated otherwise.

Ŧ Patients received radiotherapy after reaching the age of 3 years.

Recurrences and 10-yr OS are presented in absolute numbers (total).

Immunohistochemistry

Tumor cell and endothelial cell proliferation and apoptosis, as well as the presence of VEGF protein, were evaluated by double-staining procedures for Ki 67/CD34 and caspase 3a/CD34, and by staining procedures for VEGF-A, -B, -C, and -D as described by Sie et al.[16]

The basal membrane and pericyte coverage of tumor vessels were evaluated by immunohistochemical staining for CD34, collagen Type IV, and SMA. Five-micrometer thick formalin-fixed, paraffin-embedded sections were cut and deparaffinized. For CD34 staining, antigen retrieval was performed with 0.1 M Tris/HCl (pH 9.5) in a microwave at 700 W. For collagen Type IV staining, antigen retrieval was performed using protein K 0.01% for 30 minutes. Endogenous peroxidase and biotin were blocked, and sections were incubated with the first primary antibody in PBS supplemented with 1% BSA for 1 hour at room temperature. This was followed by incubation with the secondary antibody in PBS supplemented with 1% BSA and 1% serum for 30 minutes. Endogenous peroxidase and biotin were blocked, and sections were incubated with the first primary antibody in PBS supplemented with 1% BSA for 1 hour at room temperature.

This was followed by incubation with the secondary antibody in PBS supplemented with 1% BSA and 1% serum for 30 minutes. Subsequently, sections were incubated with the third antibody for 30 minutes at room temperature in PBS supplemented with 1% BSA and 1% serum. Sections were then stained with 3,3'-diaminobenzidine (Sigma) for 10 minutes. Finally, nuclear counterstaining was performed with hematoxylin.

The primary antibodies used were SMA (m0851, DAKO), collagen Type IV (10760, Mp Biomedicals), and CD34 (Qbend10, Immunotech). The secondary antibody was rabbit anti-mouse (Rampo, DAKO, 1:100 dilution) for SMA, goat anti-rabbit (Garpo, DAKO, 1:100 dilution) for collagen Type IV, and alkaline phosphatase-conjugated goat anti-mouse (GAMAF, DAKO, 1:50 dilution) for CD34. The third antibody was goat anti-rabbit (Garpo, DAKO, 1:100 dilution) for SMA and rabbit anti-goat (Ragpo, DAKO, 1:100 dilution) for collagen Type IV and CD34.

Microvessel density, and tumor cell and endothelial cell proliferation and apoptosis

Microvessel density was quantified using the Chalkley point overlap morphometric technique.[17] First, sections were scanned for vascular hot spots (magnification × 100). The overlap of points on the Chalkley grid (Graticules, Ltd.) with endothelial cells or vessel lumen was counted in 5 hot spots per tissue section (magnification × 200). The MVD was obtained by calculating the mean of these 5 hot spots.

The Ki 67/CD34 stained sections were evaluated for number and proliferation fraction of tumor cells and endothelial cells in 5 random high power fields (magnification × 400). The same sequence was repeated with the caspase 3a/CD34 sections. From these the fractions of apoptotic tumors cells and apoptotic endothelial cells were calculated. In the glioblastoma data, which were used for comparison, a distinction was made between the perinecrotic areas and intermediate areas because of the heterogeneity within these tumors.[5] The more homogeneous nature of the ependymomas precluded such a distinction. The data of both areas in glioblastomas were used to compare the tumor vessel characteristics of ependymomas and glioblastomas.

Evaluation of VEGF, collagen type IV, and SMA staining

Vascular endothelial growth factor staining and analysis were performed on the 14 tissue samples large enough to facilitate a representative assessment (roughly > 5 mm in diameter).

Vascular endothelial growth factor immunohistochemical staining was analyzed semiquantitatively. The intensity of the staining was scored using a subjective scoring system: 0 = no staining, 1 = slight staining, 2 = moderate staining, 3 = maximal staining.

The extent of coverage of the tumor vessels by a basal membrane and pericytes was analyzed by quantifying the vessels as recognized by immunohistochemical staining for either CD34, collagen IV, or SMA in 5 random areas per tumor. The number of vessels was recorded for every area (magnification × 200). Vessel counts of collagen Type IV and SMA were subsequently divided by the CD34 vessel count to produce the percentage of tumor vessels that is covered by a basal membrane or pericytes, respectively. The analysis was performed on the samples of 16 ependymomas. For comparison, 10 glioblastomas were analyzed.

Isolation of RNA and real-time RT-PCR for Ang-1 and Ang-2

Paraffin-embedded tissue samples were deparaffinized. Isolation of RNA was performed using the RT-PCR protocol described by Specht et al.[18] The RNA yield was quantified using Nanodrop (Nanodrop Technologies). To remove contaminating DNA from RNA preparations, the Ambion's TURBO DNA-free Kit was used according to the manufacturer's protocol and again RNA content was quantified using Nanodrop.

Synthesis of cDNA and real-time PCR was performed as previously described.[22] The RT-PCR analysis of Ang-1 and Ang-2 was performed on tumors of which material of sufficient quality was available (8 samples). The RNA content per sample relative to glyceraldehyde 3-phosphate dehydrogenase was calculated using the formula $2^{-\Delta Ct}$, in which ΔCt is the Ct value of the RNA molecule under study minus the Ct value of glyceraldehyde 3-phosphate dehydrogenase. The balance between Ang-1 and Ang-2 per tumor (the angiopoietin balance) was calculated using the formula $2^{\Delta Ct}$, in which ΔCt is the Ct value of Ang-2 minus the Ct value of Ang-1. As such, a higher angiopoietin balance coincides with a higher relative expression of Ang-1.

Statistical analysis

Statistical analysis was performed using the Microsoft Excel XP and SPSS package 12.01. To evaluate differences between groups a Student t-test was used if data had a Gaussian distribution and a Mann-Whitney U-test for 2 independent variables was used if data were not normally distributed. Probability values < 0.05 were considered significant.

Results

Microvessel density

Mean MVD (± SD) of the ependymomas was 5.7 ± 1.7 (20 specimens), with a wide range of 3.3–7.9 (Table 2). Microvessel density did not significantly vary with age, sex, tumor grade, or tumor location. Although it is remarkable that all supratentorial tumors had an MVD exceeding 5, there was no significant difference between the infra- and supratentorial tumors.

The MVD of the pediatric ependymomas compared well with that of the intermediate area in the glioblastoma series and exceeded that of the relatively hypoxic, perinecrotic area of glioblastoma (Table 2).

Tumor and endothelial cell proliferation and apoptosis

There were no significant differences in tumor cell proliferation or apoptosis between the different tumor locations (infra- or supratentorial), age groups (under or over 3 years of age), or tumor WHO grades (II and III). The endothelial cell apoptotic and proliferating fractions did not differ among the age groups and WHO grades. The fraction of proliferating endothelial cells was significantly higher in infratentorial tumors than in supratentorial tumors ($p = 0.01$).

To put the turnover of tumor and endothelial cells in pediatric ependymoma in context, the proliferation and apoptosis data were compared with those in the glioblastoma series (Table 2).

Tumor cell proliferation and apoptosis were significantly higher in glioblastomas irrespective of the tumor area (Table 2, Figures 1). Endothelial cell proliferation in the intermediate area of the glioblastoma exceeded that of the ependymoma with borderline significance. The endothelial cell apoptosis of glioblastomas was significantly higher only in the perinecrotic area compared with ependymomas (Table 2).

Semiquantitative analysis of VEGF staining

All samples were positive for VEGF-A, albeit to a variable degree (Table 2). Staining for VEGF-B, -C, and -D was less intense and less uniform (with 13 of 14, 4 of 14, and 9 of 14 being positive, respectively). Vascular endothelial growth factor staining did not vary significantly with age group, location, or grade.

Vascular endothelial growth factor-A staining intensity in the ependymoma samples was similar to staining intensity of the perinecrotic area in our glioblastoma series (Table 2), whereas the staining intensity of the intermediate area in glioblastomas was clearly lower. Staining intensities of VEGF-B, -C, and -D in glioblastomas exceeded that of ependymomas irrespective of tumor area (Table 2, Figure 1). These differences in staining intensities were highly significant.

Angiopoietin-1/Angiopoietin-2 ratio

A relative high expression of the Ang-2 gene compared with Ang-1 is thought to promote tumor vessel instability. This instability determines the susceptibility of the tumor vessels to the proangiogenic stimulus of VEGF. We analyzed expression of the angiopoietin gene and calculated the ratio of Ang-1 and Ang-2. Whereas the expression of Ang-2 in ependymoma was similar to that in glioblastoma, the expression of Ang-1 in ependymoma exceeded that in glioblastoma significantly (Table 2). The calculated angiopoietin balance, with seemingly higher ratios in ependymoma compared with glioblastoma mainly due to differences in Ang-1 expression, did not reach significance (Table 2). Together, these data may be indicative of a vascular status in ependymoma that is less sensitive to the induction of angiogenic sprouting through VEGF compared with glioblastoma.

Basal membrane and pericyte coverage of tumor vessels

Vascular basal membrane and pericyte coverage were analyzed in 26 tumors (16 ependymoma and 10 glioblastoma). In comparison with glioblastoma, the coverage of tumor vessels by a basal membrane or pericytes was more extensive in ependymoma (Figure 2). Nevertheless, individual ependymomas did overlap with the glioblastoma group (Table 2). These results indicate that in general the vasculature of ependymomas has a higher maturation status, although individual ependymomas may contain immature vessels similar to glioblastomas.

Table 2. Tumor vessel parameters of ependymomas and glioblastoma*

Parameter	Ependymomas†	Glioblastomas‡	P value
MVD (Chalkley count)	5.74 (4.95–6.52)	P: 4.63 (4.26–5.00)	0.024
		I: 5.37 (4.98–5.76)	0.408
Tumor cell			
Proliferation	4.22 (0–49.9)	P: 13.10 (3.3–48.8)	<0.001
		I: 21.05 (0.2–30.6)	<0.001
Apoptosis	0.25 (0–6.9)	P: 3.00 (0.2–30.6)	<0.001
		I: 0.60 (0–7.8)	0.007
Endothelial cell			
Proliferation	3.45 (0–25.2)	P: 6.75 (0.9–28.8)	0.135
		I: 9.45 (0–25.0)	0.052
Apoptosis	2.78 (0–9.6)	P: 14.85 (0.2–67.6)	<0.001
		I: 2.6 (0–12.5)	0.282
VEGF			
A	1.25 (1.00–1.5)	P: 1.30 (1.14–1.45)	0.938
		I: 0.82 (0.69–0.95)	0.002
B	0.64 (0.47–0.82)	P: 1.34 (1.2–1.48)	<0.001
		I: 1.26 (1.13–1.39)	<0.001
C	0.14 (0.01–0.28)	P: 1.11 (0.93–1.30)	<0.001
		I: 0.93 (0.77–1.10)	<0.001
D	0.46 (0.23–0.70)	P: 1.57 (0.44–1.71)	<0.001
		I: 1.13 (0.98–1.28)	<0.001
Ang-1§	0.03 (0.001–0.36)	0.009 (0.001–0.24)	0.030
Ang-2§	0.04 (0.03–0.14)	0.03 (0.006–0.74)	0.097
Ang-1/Ang-2§	0.99 (0.03–3.04)	0.27 (0.03–1.67)	0.089
Basal membrane¶	0.72 (0.22–1.14)	0.31 (0.14–0.81)	0.009
Pericytes¶	0.20 (0.03–0.81)	0.11 (0.02–0.32)	0.022

* MVD, VEGF-A, -B, -C, and -D are presented as mean ± SD; the other parameters are presented as median (range).

† Ependymoma: MVD, tumor cell and endothelial cell apoptosis (20 samples); tumor cell and endothelial cell proliferation (19); VEGF-A, -B, -C, and -D (14); Ang-1 and -2 (7); basal membrane and pericytes (16).

‡ The relative hypoxic perinecrotic (P) tumor area or the intermediate (I) tumor area; there were 62 samples for all parameters except basal membrane and pericytes (10 samples).

§ One ependymoma sample was disregarded as a result of outlier exclusion.

¶ Basal membrane and pericyte coverage of tumor vessels presented as the median number of tumor vessels staining for collagen Type IV or SMA, respectively, divided by the number of tumor vessels staining for CD34.

Discussion

Targeting the neovasculature of tumors is a promising addition to the current conventional treatments of cancer. A significant part of what is known about tumor vasculature and the process of angiogenesis has been analyzed in glioblastoma or glioblastoma models.[8, 9] In line with this, several clinical trials of antiangiogenic agents in patients with glioblastoma are ongoing. Less is known about the vasculature of other brain tumors. Pediatric ependymoma is a relatively rare tumor, and only a handful of studies have analyzed VEGF expression or MVD of this tumor.[10-14]

We studied several aspects of the ependymoma vasculature and compared the findings with data obtained in a glioblastoma series published previously. We found that ependymoma vasculature in general has a higher maturation status than glioblastoma vasculature, but that nevertheless a subset of ependymomas may contain immature vessels similar to glioblastomas.

The proliferation and apoptosis indices of tumor cells in glioblastomas were higher than in the ependymomas regardless of the tumor area. The tumor cell proliferation fraction of our ependymoma series was well within the range of 1–20.5 reported for ependymomas in the literature.[19, 20] The fraction of apoptotic tumor cells is in agreement with the apoptotic index of 0.9 reported for ependymomas by Korshunov et al.[11] The differences in proliferation and apoptosis indices of endothelial cells between ependymoma and glioblastoma were less uniform. Whereas the Chalkley counts of both the ependymoma and the glioblastoma series were similar, proliferation and apoptosis indices of endothelial cells were not. Compared with ependymomas, proliferation of endothelial cells in glioblastomas was higher in the intermediate area, whereas apoptosis was higher in the perinecrotic area. These regional differences reflect the heterogeneity within glioblastomas and may be indicative of the influence of hypoxia on the angiogenic process. The hypoxic conditions within the tumor, which are known to contribute to the production of VEGF,[21] may very well be of less importance in ependymoma, which has a more homogeneous architecture. Nonetheless, VEGF-A staining were of equal intensity in the 2 series. Although these results cannot be readily explained, alternative pathways of VEGF-A induction[21] may account for these findings. In contrast to our findings, 2 studies reported that the VEGF expression in ependymomas either coincides with the presence of necrosis[4] or is present mainly in areas of necrosis.[14] These findings may actually suggest a hypoxic drive similar to that seen in glioblastoma. Vascular endothelial growth factor-B, -C, and -D stained with much less intensity in the ependymoma series than in the glioblastoma series. In a previous study, we showed that the pattern of expression of VEGF-D suggests a role for hypoxia in the induction of the production of this protein, similar to that of VEGF-A.[16] The relative lack of VEGF-D shown here may therefore be illustrative of the absence of hypoxia as a driving force behind the production of VEGF in ependymoma.

The effect of VEGF-A on the initiation of angiogenesis depends in part on the angiopoietin/Tie-2 system.[22] Tie-2, a membrane-bound endothelial cell restricted receptor, is ligated by Ang-1 leading to phosphorylation of the Tie-2 receptor and vessel stabilization and coverage with mural cells, constituting so-called mature vessels. In contrast, Ang-2 is a competitive antagonist of Ang-1 that induces vessel destabilization. These changes may, in the absence of sufficient survival factors such as VEGF, lead to endothelial cell apoptosis. On the other hand, if VEGF is available, proliferation and migration of endothelial cells can ensue. Angiopoietin-1 is expressed in tissues under physiological circumstances. Angiopoietin-2 expression is increased in pathological sites such as wound healing and tumors. In gliomas the onset of the expression of Ang-2 has been identified to be instrumental in the initiation of angiogenesis. It is contended that it is the balance between Ang-1 and -2 that determines the susceptibility of the vasculature to the proangiogenic stimulus of VEGF.[23] In that case the Ang-1 and -2 balance and the presence or absence of VEGF can be used as a measure of angiogenic potential.

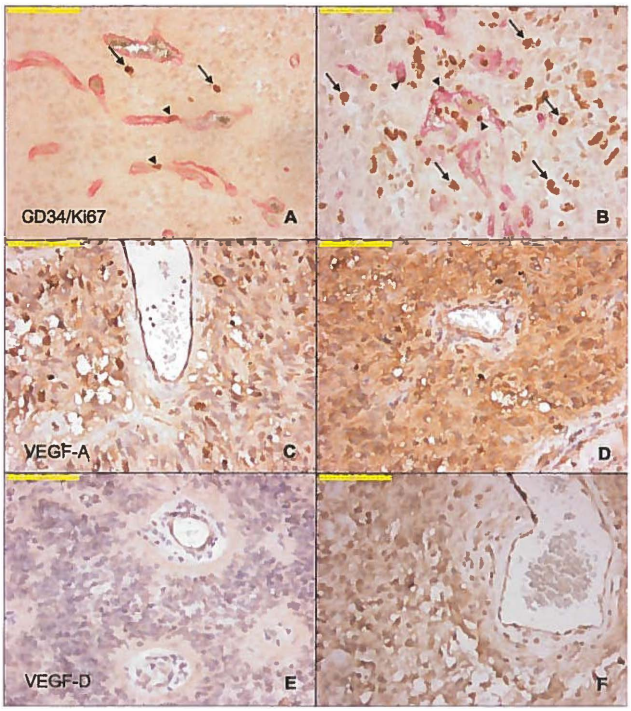

Figure 1. Immunohistochemical staining of ependymoma (**A**, **C**, and **E**) and glioblastoma (**B**, **D**, and **F**). Although the CD34/Ki 67 double staining of ependymoma (A) and glioblastoma (B) show similar MVDs, the number of proliferating tumor cells (*arrows*) and endothelial cells (*arrowheads*) is clearly higher in glioblastomas. Both ependymoma (C) and glioblastoma (D) show diffuse expression of VEGF-A, with intense staining of endothelial cells as well as tumor cells. Immunohistochemical staining for VEGF-D shows little to no staining in ependymoma (E), whereas staining in glioblastoma (F) is diffusely positive. Yellow bars represent 100 μm.

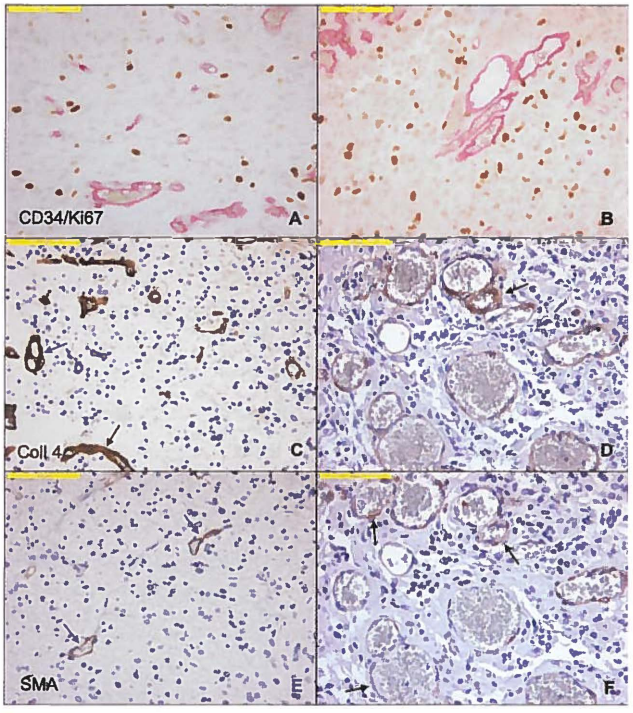

Figure 2. Immunohistochemical staining of ependymoma (**A**, **C**, and **E**) and glioblastoma (**B**, **D**, and **F**). Whereas the collagen Type IV (Coll 4) deposition of tumor vessels varies considerably in glioblastomas (D) from one vessel to the other (*arrow* indicates the only vessel in the photograph that is completely surrounded by a layer of collagen Type IV), collagen Type IV staining in ependymoma (C) is rather homogeneous and forms a thick layer around the vessels. Large and long vessels with multiple luminal areas (*arrows*) may appear as 2 or more separate structures on CD34 staining, which may account for the fact that collagen Type IV coverage of endothelial cells was less than 100% in ependymoma. In glioblastoma (F) SMA staining shows incomplete, heterogeneous, and scant coverage of tumor vessels with pericytes (*arrows*). In ependymoma (E), a subset of the microvessels shows SMA positivity. These vessels are more often than not completely surrounded by pericytes (*arrows*). Yellow bars represent 100 μm.

In theory, one would expect to find highly angiogenic tumors such as glioblastomas to express high amounts of Ang-2 relative to Ang-1 compared with ependymomas. This was confirmed by our results. It is surprising, however, that this is brought about by a relatively higher expression of Ang-1 in the ependymoma series, whereas the expression of Ang-2 was similar.

Microvascular immaturity is associated with a relatively low pericyte coverage and aberrant basal membrane makeup.[24] As such, our data on pericyte and basal membrane coverage are in line with the findings of the angiopoietin balance. In ependymoma, the pericyte coverage of microvessels is in excess of that of glioblastoma. Similarly, the ependymoma series shows higher fractions of vessels that are surrounded by a basal membrane compared with the glioblastoma series.

Recently, antiangiogenesis studies in glioblastomas have shown some success in normalizing the vasculature as a means to sensitize the tumor to conventional treatment.[25, 26] Moreover, clinical trials using bevacizumab in the treatment of recurrent glioblastoma have shown progression-free survival rates that compare favorably with historical controls.[27-29] As we show here, the proangiogenic state of glioblastomas clearly supersedes that of ependymomas. In this context, ependymomas may not lend themselves very well to vessel-targeted therapy. However, these considerations are theoretical. In practice, at this time, the proangiogenic state of the tumor vasculature, as measured for instance by the angiopoietin balance, may not be a reliable predictor of the effect of antiangiogenesis treatment. Moreover, individual ependymomas show considerable overlap with the glioblastomas. Therefore, it is not impossible that the treatment of ependymomas or a subset of these tumors may benefit from antiangiogenesis as an addition to chemo- or radiotherapy.

Conclusions

Despite a lower proliferation fraction of endothelial cells in ependymomas compared with glioblastomas, the vascular density of both tumors is similar. The ependymoma vasculature, in general, has a higher maturation status than the glioblastoma vasculature, although these parameters show considerable overlap. Although successful vessel normalization through the use of antiangiogenic drugs may, in theory, be less likely in ependymomas than glioblastomas, any effect of such a therapy in ependymomas cannot be excluded beforehand. Because of the urgent need for additional therapeutic options in the treatment of pediatric intracranial ependymomas, further research into the feasibility of adding antiangiogenic drugs to the current treatment protocols is warranted.

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Chapter 5

Tumour vasculature and angiogenic profile of paediatric pilocytic astrocytoma; is it much different from glioblastoma?

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Abstract

Aims: Pilocytic astrocytomas are the most frequent brain tumours in children. Because of their high vascularity, this study aimed to obtain insights into potential angiogenic related therapeutic targets in these tumours by characterization of the vasculature and the angiogenic profile. In this study 59 paediatric pilocytic astrocytomas were compared with 62 adult glioblastomas, as a prototype of tumour angiogenesis.

Methods: Microvessel density, vessel maturity in terms of basement membrane and pericyte coverage, and turnover of both endothelial and tumour cells, and vascular endothelial growth factor (VEGF) expression were evaluated in tumour tissue, immunohistochemically stained with, respectively, CD34, collagen IV, smooth muscle actin, Ki67/CD34, caspase-3/CD34 and VEGF(-A-D). As an indicator for vessel stability the angiopoietin (ANGPT)-1/ANGPT-2 balance was calculated using Real Time RT-PCR.

Results: Pilocytic astrocytoma and glioblastoma showed similar fractions of vessels covered with basement membrane and pericytes. Overlapping ANGPT-1/ANGPT-2 balance and VEGF-A expression were found. Pilocytic astrocytoma had fewer but wider vessels compared with glioblastoma. Turnover of endothelial and tumour cells were relatively lower in pilocytic astrocytoma. Within pilocytic astrocytoma, higher ANGPT-1/ANGPT-2 balance was correlated with fewer apoptotic endothelial cells. Lower numbers of vessels were correlated with higher VEGF-A expression.

Conclusions: Despite the fact that pilocytic astrocytoma showed a different vessel architecture compared with glioblastoma, a critical overlap in vessel immaturity/instability and the angiogenic profile was seen between both tumours. These findings suggest encouraging possibilities for targeting angiogenesis (for instance with anti-VEGF) as a therapeutic strategy in pilocytic astrocytoma.

Introduction

Pilocytic astrocytomas are grade I astrocytomas (WHO 2007), typically occurring in children and young adults, and representing the most frequent brain tumours in children.[1] Although pilocytic astrocytomas are highly vascular but generally circumscribed and slowly growing tumours, they may infiltrate into surrounding brain parenchyma and involve leptomeninges, causing diverse symptomswhich also vary with tumour localization.[1, 2] Gross total resection is considered the treatment of choice and cure will often be achieved in those patients.[3, 4] For lesions in less favourable localizations, such as the diencephalic or thalamic regions, partial resection or biopsy only for histological diagnosis may be performed, possibly followed by postoperative chemotherapy and/or radiotherapy.[5] Despite a 5-year survival of approximately 90%,[6-8] morbidity can be serious, mainly because of the localization of the tumour (diencephalon and brain stem) and its subsequent chance of surgical morbidity and dismal effect of chemotherapy and/or radiation.[9] So to reduce morbidity as well as mortality in children with pilocytic astrocytoma, new therapeutic modalities are needed. Because pilocytic astrocytomas are highly vascular, tumour vasculature or specific angiogenic factors could be potential therapeutic targets in these tumours.

Angiogenesis, formation of new vessels from preexisting ones, has been investigated as a target in cancer treatment for many years now, and has formed an effective and well-tolerated treatment in, for example, recurrent high grade astrocytomas.[10, 11] Before tumour growth is obtained by angiogenesis, a subset of tumours initially grows by co-opting existing host vessels; tumour cells proliferate around existing blood vessels in the tissue. This co-opted host vasculature regresses, leading to a secondarily avascular tumour and massive tumour cell loss.[12] Next, under the influence of crucial factors such as vascular endothelial growth factor (VEGF) and angiopoietins the remaining tumour is 'rescued' by angiogenesis at the tumour margin, the so-called angiogenic switch.[12-15] By targeting angiogenesis, resulting in disruption of blood supply and hypoxia, it is hypothesized that the tumour can be attacked indirectly.[16] However, other theories may include anti-angiogenic therapy leading to vessel normalization for improved drug delivery and efficacy[17] or resulting directly in suppression of tumour cells which express VEGF receptors.[18]

In theory, these possible effects of anti-angiogenic therapy could be more or less important in different tumours, depending on tumour vasculature and expression of angiogenic factors.[19] As a consequence of an imbalance between pro- and anti-angiogenic factors, including VEGF and angiopoietins, various tumours are characterized by abnormal, immature vessels.[20, 21] Those vessels have an abnormal endothelial-cell lining, detached pericytes, and a basement membrane that is abnormally thick or abnormally thin.[17] Contrary to the extensively described angiogenic profile of high grade astrocytomas in adults, little is known about tumour vasculature and possible angiogenic activity in lowgrade paediatric astrocytomas, including pilocytic astrocytomas.

This study aimed to characterize tumour vasculature and the angiogenic profile of paediatric pilocytic astrocytoma to obtain insights into potential angiogenic related therapeutic targets in these tumours. We investigated microvessel density (MVD), vessel maturity and stability, and different angiogenic factors, including endothelial cell proliferation and VEGF expression. Vessel maturity and stability were analyzed in terms of basement membrane and pericytes coverage and the angiopoietin-1/angiopoietin-2 (ANGPT-1/ANGPT-2) balance using, respectively, morphometric techniques and Real Time RT-PCR. These data were interpreted in the context of previously described adult glioblastoma (WHO 2007, grade IV astrocytoma),[22, 23] often seen as a model for studying angiogenesis.

Materials and methods

Patient and baseline characteristics

From November 1985 until March 2007, a total of 71 children (0–17 years of age) were histologically diagnosed with a pilocytic astrocytoma in our hospital. All patients underwent stereotactic biopsy, partial or gross total resection. Because the quantity and/or quality of tumour tissue from 12 patients were insufficient, tumour tissue of a final total of 59 pilocytic astrocytoma patients was included in this study, and evaluated in the context of tumour tissue of 62 adult patients with primary glioblastoma.[22] Utilization of all human tissue was approved by the relevant institutional committees (University Medical Center Groningen).

Between the study population (n = 59) and the total pilocytic astrocytoma patient group (n = 71) no differences in baseline characteristicswere found, including age at diagnosis, gender, tumour localization, neurosurgical procedure and possible post-operative chemotherapy and/or radiotherapy. 'Age at diagnosis' was defined as the patient's age at date of histological diagnosis. Tumour localization was divided into supratentorial, optic nerve, infratentorial and spinal cord. In addition, progression free survival (PFS), 5-year PFS and 5-year survival were evaluated. 'PFS' was defined as the time between operation and tumour progression as registered at magnetic resonance imaging (MRI) or computed tomography (CT). Tumour progression was described as the appearance of the tumour at MRI or CT after macroscopic total resection, as well as tumour progression assessed by imaging after stereotactic biopsy or partial resection. The baseline characteristics of glioblastoma patients have been described previously.[22]

Evaluation of MVD

Two methods were used for MVD determination. First, the Chalkley point overlap morphometric technique in which double immunohistochemical stained slides for Ki67/CD34 (endothelial cell marker) were evaluated with the use of an objective of x20 and an ocular grid with 25 random points.[24] After the tumour was scanned to identify a maximum of five hot spots, the ocular grid was turned to maximize the overlap between points on the grid and the CD34 stained vessels. The number of overlapping points was counted for every hot spot. The Chalkley score for each slide was obtained by calculating the mean score of five hot spots.

Because in glioblastoma two important areas are distinguished, the perinecrotic and intermediate area, two Chalkley scores were assessed in this tumour.[22] The second method was traditional counting of vessels (x200 magnification) in five randomized areas per slide, 30 min stained for CD34 with use of the NexES IHC Staining Module (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was performed at 98°C.

Morphometric analysis of vessel maturity

Vessel maturity was evaluated by calculating the fraction of tumour vessels that was covered with basement membrane or pericytes. So vessels were counted in, respectively, automatic stained slides as described above for collagen IV (main component of vascular basement membrane,[25] ICN Biochemicals, Costa Mesa, CA, USA, 1:800) and Smooth Muscle Actin (SMA, pericyte marker, Dako, Glostrup, Denmark, 1:100) at x200 magnification and next these numberswere divided by the CD34 vessel count. Five randomized areas were evaluated per tumour sample.[23] Moreover, these slides were analyzed by morphometry as described previously by Zhou et al.[26] Digital images [Leica DFC 420 C digital camera (Leica Microsystems, Heerbrugg, Switzerland) and Zeiss Axioskop model 451485 microscope (Carl Zeiss, Sydney, Australia)] were quantified for collagen IV and SMA at x200 magnification in six random areas per tumour sample. The pixel area of the positive staining was measured using Leica QWin software (Leica Imaging Systems, Cambridge, UK). The data were presented as the percentage of marker-positive surface area, which is calculated with the formula: marker-positive area x100/area of the optical field.

Genetic evaluation of vessel stability

As an indicator for vessel stability, gene expression of ANGPT-1 and ANGPT-2 was determined in deparaffinized tumour tissue using Real Time RT-PCR, followed by calculation of the balance between ANGPT-1 and -2 with the formula: $2^{\Delta Ct}$, in which ΔCt is the mean Cycle threshold (Ct) value of ANGPT-2 minus the mean Ct value of ANGPT-1.[22] As such, a higher ANGPT-1/ANGPT-2 balance corresponds with relatively higher ANGPT-1 expression.

RNA-isolation was realized with use of the protocol described by Specht et al.,[27] and quantified using Nanodrop ND-1000 UV Spectrophotometer (NanoDrop Technologies,Wilmington, DE, USA). To remove contaminating DNA from RNA preparations and to subsequently remove the DNase and divalent cations from the sample, the TURBO DNA-free Kit (Applied Biosystems/Ambion, Austin, TX, USA) was used.[22] Next, RNA was again quantified using Nanodrop. Synthesis of cDNA was performed as described previously.[28] Using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the primers ANGPT-1 (Hs 00181613.m1), ANGPT-2 (Hs 00169867.m1), and GAPDH (Hs 99999905.ml), Real Time PCR was fulfilled in triplicate with an ABI7900HT Real Time sequence detection system in 384-well reaction plates.

Analysis of endothelial and tumour cell turnover and VEGF expression

Proliferation and apoptotic fractions of both endothelial and tumour cells were assessed in double-stained slides for, respectively, Ki67/CD34 and cleaved caspase-3/CD34 using an objective of x40 as described previously.[22] In pilocytic astrocytoma three randomized high power fields (HPFs) were evaluated instead of five HPFs in glioblastoma, representing a more heterogeneic tumour. The expression of VEGF-A, -B, -C and -D was immunohistochemical determined using a semi-quantitative staining intensity score.[22] No visible staining was scored as 0, low staining as 1, moderate as 2 and high intensity staining as 3. Evaluations of glioblastoma tissue were performed in both the perinecrotic and intermediate tumour area.[22] During all these analyses of tumour tissue, investigators were blinded to the patient data.

Statistical analysis

PP-plots and the Shapiro-Wilk tests were used to evaluate if data had Gaussians distributions. Statistical comparisons were made with a Student's t-test if data had a Gaussian distribution and a Mann-Whitney U-test was used if data were not normally distributed. Comparisons between more than two groups were performed using anova if data were normally distributed and the Kruskal–Wallis test if data were ordinal and not normally distributed. The chi-square test was used for nominal (categorical) data. The nonparametric Spearman correlation test was used to compute correlations. For all statistical analyses, a two-tailed P-value of less than 0.05 was considered significant.

Results

Patient characteristics of the pilocytic astrocytoma study group

Median age at diagnosis in the study group of 59 patients was 7.2 years (Table 1). Children without tumour progression appeared to have a median age at diagnosis of 8.8 (range 0.2–16.6) years, while the median age of children with tumour progression was 5.3 years (range 0.8–14.1, $P = 0.023$). The choice of the type of neurosurgical procedure was dependent on tumour localization. When tumours were located centrally in the brain, such as thalamic tumours, only diagnostic biopsy was performed. Partial resection was mostly performed in pilocytic astrocytoma of the chiasm and hypothalamus, and gross total resection in infratentorial cerebellar tumours. As expected, a significant difference was found between the three neurosurgical procedures in the assessment of tumour progression: after stereotactic biopsy in all children tumour progression was seen, and after partial and gross total resection it was, respectively, 69% and 19% (chi-square $P < 0.001$). Five patients died due to tumour progression. Because of the heterogeneity in the study group concerning treatment and prognosis, no correlations of angiogenic data with outcome were studied.

Table 1. Baseline patient characteristics of the pilocytic astrocytoma study group (n = 59)

Characteric	n (%)
Gender, male	37 (63)
Tumour localization	
Supratentorial	10 (17
Optic nerve	18 (31)
Infratentorial	28 (47)
Spinal cord	3 (5)
Neurosurgery	
Stereotactic biopsy	2 (3)
Partial resection	29 (49)
Gross total resection	27 (46)
Missing data	1 (2)
Chemotherapy	6 (10)
Radiotherapy	3 (5)
Tumour progression	27 (46)
5-year progression free survival	(50)
Death	5 (8)
5-year survival	(90)

Characteristic	Median (range)
Age at diagnosis, years	7.2 (0.2–17.0)
Follow up, years	7.5 (0.1–21.5)
Progression free survival, years	2.2 (0.1–15.0)

Pilocytic astrocytoma showed fewer but wider vessels

As described previously, MVD was determined using (i) the Chalkley point overlap morphometric technique, as well as (ii) the traditional method of counting CD34 stained vessels. The results (Table 2) show that the Chalkley score was significantly higher in pilocytic astrocytoma. Interestingly, however, glioblastoma showed a higher number of blood vessels when using traditional vessel counting. In theory, the two techniques should be correlated. Indeed, in both pilocytic astrocytoma and the intermediate area in glioblastoma, a higher vessel counting was correlated with a higher Chalkley score (respectively r: 0.348, P = 0.011 and r: 0.408, P = 0.001). The Chalkley point overlap technique uses an ocular grid and the score depends on the maximum number of points lying in the lumen of blood vessels or on the CD34 stained endothelial cells. It is thus dependent not only on the number of blood vessels, but also on the diameter of these vessels. From these measurements it can be concluded that pilocytic astrocytoma had fewer but wider vessels than glioblastoma, which showed more vessels with a smaller diameter (Figure 1).

Cerebellar pilocytic astrocytomas (n = 24), known as highly vascularized tumours, showed a higher Chalkley score (median 8.6, range 2.4–15.8) compared with the other pilocytic astrocytomas (n = 35, median 6.7, range 3.0–12.4, P = 0.012), while the number of vessels was equal in all pilocytic astrocytoma (P = 0.986). These results reflect that cerebellar tumours have relatively wider blood vessels.

Table 2. Microvessel density (MVD), vessel stability, turnover of endothelial cells (EC) and tumour cells (TC), and vascular endothelial growth factor (VEGF) expression between pilocytic astrocytoma and glioblastoma

Parameter	Pilocytic astrocytoma (n = 59)	Glioblastoma (n = 62)*	P value
MVD			
Mean Chalkley score (95% CI)	7.6 (6.9–8.3)	P: 4.6 (4.3–5.0)	<0.001
		I: 5.4 (5.0–5.8)	<0.001
Median CD34 vessel count (range)	39.0 (16.0–104.0)	56.2 (17.0–126.4)	0.001
Vessel stability			
Median ANGPT-1/ANGPT-2 (range)	0.55 (0.05–11.63)	0.27 (0.03–1.67)	0.001
Turnover of EC			
Median PFEC (range)	1.9 (0.0–7.9)	P: 6.8 (0.9–28.8)	<0.001
		I: 9.5 (0.0–25.0)	<0.001
Median AFEC (range)	0.0 (0.0–2.2)	P: 14.9 (0.2–67.6)	<0.001
		I: 2.6 (0.0–12.5)	<0.001
Turnover of TC			
Median PFTC (range)	2.3 (0.0–9.2)	P: 13.1 (3.3–48.8)	<0.001
		I: 21.1 (3.9–72.6)	<0.001
Median APTC (range)	0.0 (0.0–0.5)	P: 3.0 (0.2–30.6)	<0.001
		I: 0.6 (0.0–7.8)	<0.001
Immunohistochemical VEGF expression			
Mean VEGF-A (95% CI)	1.26 (1.15–1.38)	P: 1.30 (1.14–1.45)	0.706
		I: 0.82 (0.69–0.95)	<0.001
Mean VEGF-B (95% CI)	0.97 (0.92–1.01)	P: 1.34 (1.20–1.48)	<0.001
		I: 1.26 (1.13–1.39)	<0.001
Mean VEGF-C (95% CI)	0.86 (0.77–0.95)	P: 1.11 (0.93–1.30)	0.021
		I: 0.93 (0.77–1.10)	0.547
Mean VEGF-D (95% CI)	1.02 (0.96–1.08)	P: 1.57 (0.44–1.71)	<0.001
		I: 1.13 (0.98–1.28)	0.141

*Perinecrotic area (P): relatively hypoxic area with HIF-1a positively and VEGF-A up regulation; Intermediate area (I): normoxic tumour area with HIF-1a negative.

PFEC: proliferation fraction of endothelial cells; AFEC: apoptotic fraction of endothelial cells; PFTC: proliferation fraction of tumour cells; AFTC: apoptotic fraction of tumour cells; ANGPT: angiopoietin.

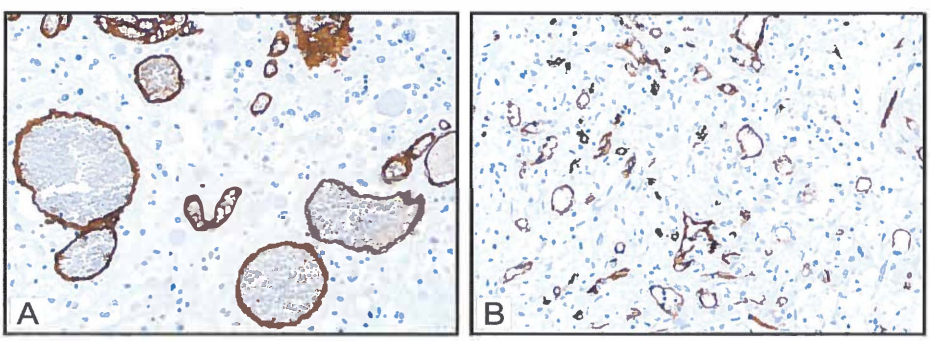

Figure 1. Photomicrographs of CD34-stained slides of pilocytic astrocytoma (**A**) and glioblastoma (**B**). Pilocytic astrocytoma showed fewer but wider vessels than glioblastoma, which showed more vessels with a smaller diameter. Original magnification: x200.

Overlap in vessel maturity and stability between pilocytic astrocytoma and glioblastoma

To investigate vessel maturity, we: (i) calculated the fraction of tumour vessels that was covered with basement membrane or pericytes; and (ii) used morphometry in which the surface area positive for collagen IV and SMA was assessed. Comparing pilocytic astrocytoma with glioblastoma, identical fractions of vessels covered with basement membrane and pericytes were found (Figure 2A). Moreover, although pilocytic astrocytoma showed statistically significant higher SMA percentages of positive surface area, clear overlap was seen between the two tumour types (Figure 2B).

Interestingly, only in pilocytic astrocytoma was a positive correlation found between pericytes and basement membrane coverage, suggesting relatively less immaturity in these tumours (r: 0.377, P = 0.005). Furthermore, the pericytes coverage that may shape and control a uniform vessel diameterwas positively correlated with the Chalkley score which partially depends on capillary diameter (r: 0.590, P < 0.001).

As described earlier, the ANGPT-1/ANGPT-2 balance measured with Real Time RT-PCR could also give an indication of vessel stability/maturity.[22] It has been proposed that ANGPT-1 recruits pericytes and potentiates microvessel stability and maturation by supporting interactions between endothelial cells and pericytes.[29-31] ANGPT-2, on the other hand, works as a destabilizing factor on blood vessels.[32, 33] Strikingly, pilocytic astrocytomas located in the cerebellum (*n* = 24) showed a significantly lower ANGPT-1/ANGPT-2 balance (median 0.39, range 0.05–1.24) compared with the other pilocytic astrocytomas (*n* = 35, median 0.68, range 0.21–11.63, P = 0.025), reflecting relatively more unstable vessels in cerebellar pilocytic astrocytoma. Compared with glioblastoma, the total pilocytic astrocytoma group showed a higher ANGPT-1/ANGPT-2 balance (Table 2). However, the subgroup of cerebellar pilocytic astrocytomas did not show a significant difference with glioblastomas (P = 0.450). No significant correlation was detected between the ANGPT-1/ ANGPT-2 balance and positive SMA surface area (r: 0.106; P = 0.585).

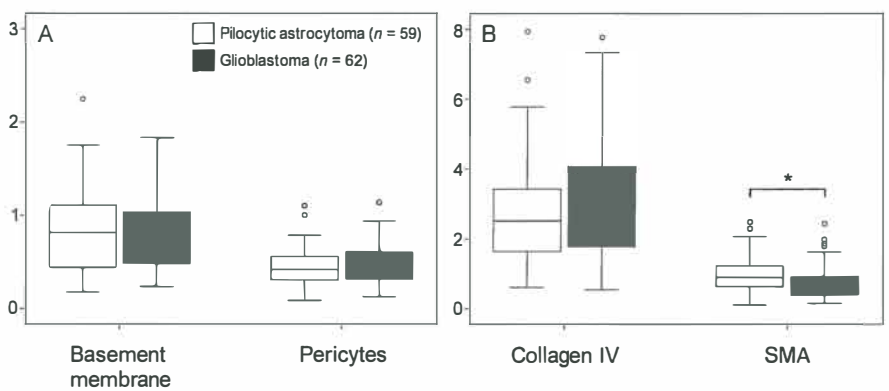

Figure 2. (**A**) Boxplots showing no difference between pilocytic astrocytoma and glioblastoma in fractions of vessels covered with basement membrane (collagen IV/CD34, P = 0.928) and pericytes (smooth muscle actin (SMA)/CD34, P = 0.406). (**B**) Boxplots showing the percentage positive surface area relative to the area of optical field, measured by morphometry software, between pilocytic astrocytoma and glioblastoma. Although pilocytic astrocytoma showed a statistically significant higher SMA positive surface area compared with glioblastoma (*P = 0.016), the boxplots show clearly an overlap in the results of the two tumour types.

Lower turnover of endothelial and tumour cells in pilocytic astrocytoma

The turnover of endothelial cells as parameter of angiogenic activity was significantly lower in pilocytic astrocytoma than in glioblastoma (Table 2). A similar difference was seen in the turnover of tumour cells. Within pilocytic astrocytoma, a higher ANGPT-1/ANGPT-2 balance, reflecting more vessel stability, was correlated with less apoptotic endothelial cells (r: -0.480, P = 0.007). No significance was found between a higher ANGPT-1/ANGPT-2 balance and less proliferating endothelial cells (r: -0.103, P = 0.590). Markedly, as characteristic for angiogenesis, more proliferating tumour cells were correlated with more proliferating endothelial cells within pilocytic astrocytoma (r: 0.636, P < 0.001). No differences were seen between the different tumour localizations.

Overlapping immunohistochemical VEGF expression between pilocytic astrocytoma and glioblastoma

The VEGF-A, which is induced by hypoxia, increases microvascular permeability, stimulates endothelial cell migration and division and induces angiogenesis.[34] Remarkable is the overlapping VEGF-A staining intensity scores between pilocytic astrocytoma and the hypoxic tumour area in glioblastoma (Table 2, Figure 3), suggesting a role for hypoxia induced VEGF expression in pilocytic astrocytoma. Consistent with this concept, lower numbers of vessels counted in CD34 stained pilocytic slides were correlated with higher expression of VEGF-A (r: -0.434, P = 0.001). Although no differences were found in VEGF expression between various tumour localizations, the coefficient of this correlation between the number of CD34 positive vessels and VEGF-A expression was stronger in cerebellar pilocytic astrocytomas (n = 24, r: -0.436, P = 0.042) which are mainly characterized by cyst formation compared with other tumour localizations (n = 35, r: -0.424, P = 0.022). In both pilocytic astrocytoma and the perinecrotic area in glioblastoma, VEGF-A expression was positively correlated with the fraction of vessels covered with pericytes (respectively r: 0.317, P = 0.026 and r: 0.314, P = 0.023).
In contrast to VEGF-A, the other members of the VEGF-family, including VEGF-B, -C and -D showed lower expression in pilocytic astrocytoma than in glioblastoma (Table 2, Figure 3), although (again) overlap between the two tumour types was observed.

Discussion

The present study demonstrated, besides differences in vessel architecture between pilocytic astrocytoma and glioblastoma, also a critical overlap in vessel immaturity/instability and angiogenic activity.
A fraction of the pilocytic astrocytomas showed a higher ANGPT-1/ANGPT-2 balance, stimulating vessel stability and leading to wider diameter blood vessels.[35, 36] The ANGPT-1/Tie-2 system has been described as a potent regulator of the enlargement of blood vessel calibre. One of the key determinants in this mechanism is apelin, which is up-regulated under the activation of the Tie-2 receptor by ANGPT-1.[37, 38] However, although cerebellar pilocytic astrocytoma showed the widest blood vessels, an interesting overlap with glioblastoma was found in the ANGPT-1/ANGPT-2 balance, demonstrating resemblance in vessel instability.

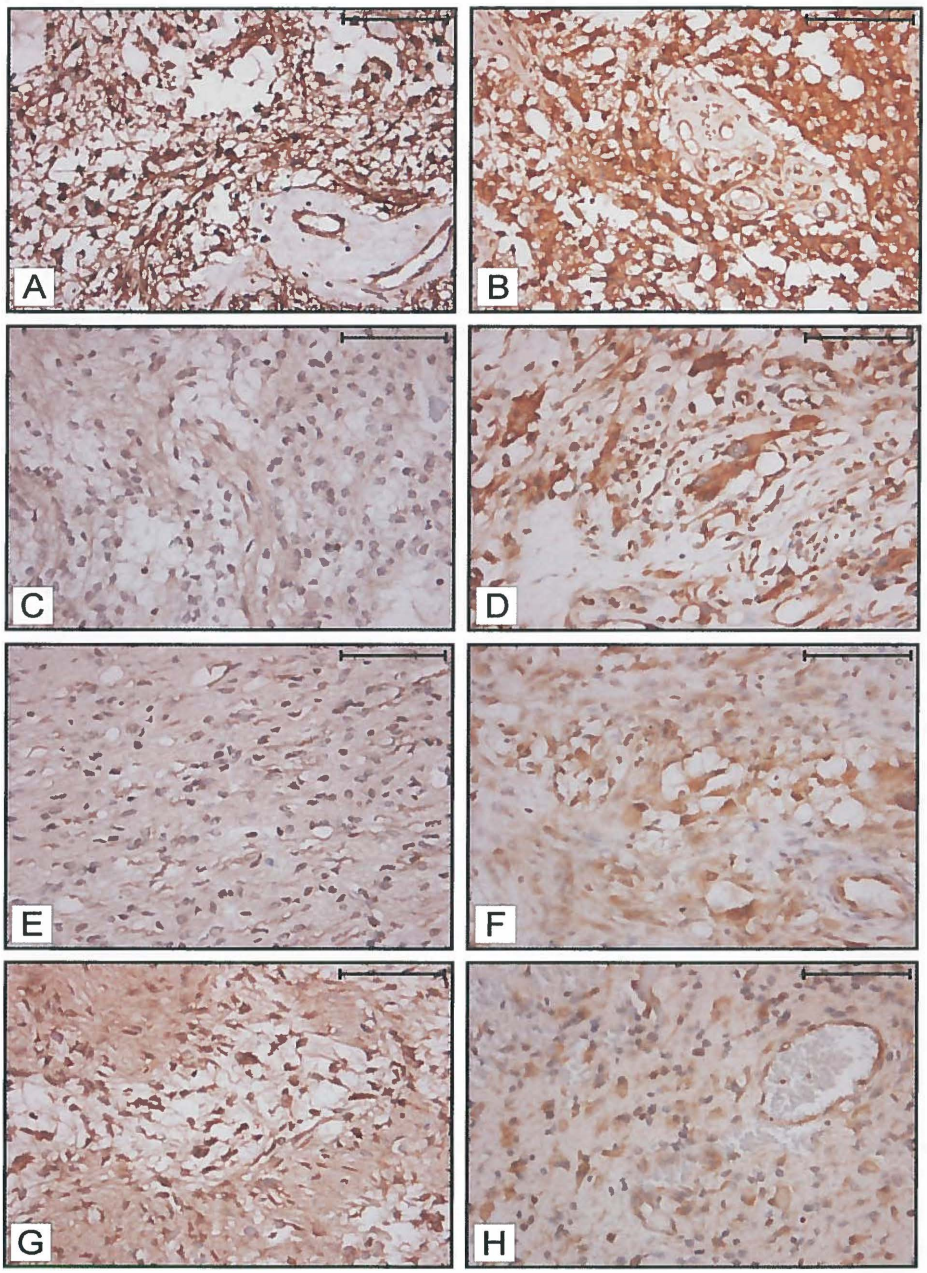

Figure 3. Photomicrographs of immunohistochemical stainings of pilocytic astrocytoma (**A, C, E** and **G**) and glioblastoma (**B, D, F** and **H**). Both pilocytic astrocytoma (A) and glioblastoma (B) show a relatively strong expression of vascular endothelial growth factor-A (VEGF-A) compared with the other growth factors (C-H). On average, the staining intensity of VEGF-B, -C and -D was stronger in glioblastomas than in pilocytic astrocytomas. Note that the tumour endothelium is also positive and could therefore be used as an intern control. The bars in the pictures represent 100 micro.

Indeed, besides ANGPT-1, also other factors including basic fibroblast growth factor and hypoxia could induce apelin expression and thereby increase vessel diameter.[37-39] As cerebellar pilocytic astrocytomas are characterized by cyst formation resulting in intratumoral hypoxia, this could possibly lead to apelin induction and increased blood vessel diameter.

Besides wider vessels, pilocytic astrocytomas showed more regularly shaped vessels.[40] This observation was confirmed with a positive correlation between pericytes that control uniformity in vessel diameter[41] and the Chalkley score partially depending on capillary diameter. ANGPT-2 seems to play a larger role in glioblastoma. It acts as an antagonist and inhibits ANGPT-1 induced phosphorylation of Tie-2 receptors in endothelial cells; as a result, ANGPT-2 initiates extensive angiogenesis resulting in pericyte drop-off and vascular destabilization.[32, 33] The association between angiopoietins and blood vessel shape has not been described previously in glioma.

Analyzing vessel maturity in pilocytic astrocytoma, a positive correlationwas found between pericytes and basement membrane coverage, suggesting a maturation step in which basement membrane is stimulated by pericyte recruitment in contrast to glioblastoma. However, this is only described for vasculogenesis, *de novo* formation of new blood vessels, but not for angiogenesis.[42] Interestingly, according to the vessel fraction covered with pericytes which corresponds with the vessel maturation index,[43, 44] both tumours showed a surprisingly equal degree of vessel immaturity. Nevertheless, to give detailed information about the presence or absence of pericytes, a single pericyte marker like SMA, may not be sufficient.[41]

Notably, the pro-angiogenic VEGF-A was similarly expressed in pilocytic astrocytoma compared with the relatively hypoxic perinecrotic tumour area in glioblastoma and even scored higher than in the intermediate tumour area. The two different areas in glioblastoma are a result of its aggressive growth pattern. Although pilocytic astrocytomas are slowly growing tumours, areas of hyaline and cystic degradation have been distinguished due to tumour growth and increased tissue pressure and characterized by high VEGF expression.[45] Moreover, the present study showed a negative correlation between the number of CD34 positive vessels in pilocytic astrocytoma and VEGF-A expression. As cerebellar pilocytic astrocytomas are particularly cystic and highly vascular tumours, this correlation was found to be stronger in these tumours. These findings support a critical role for hypoxia induced VEGF expression in pilocytic astrocytoma.

The VEGF-A stimulates endothelial cell proliferation and migration, its role on pericytes however is less clear and different studies present conflicting results. It is suggested that VEGF ablates pericyte coverage of nascent vascular sprouts, leading to vessel destabilization,[46] while it is also shown that VEGF could promote the recruitment of pericytes, thereby contributing to the maturation of newly formed blood vessels.[31, 47] This interaction between VEGF and pericytes was confirmed in the present study with a positive correlation between VEGF-A expression and the fraction of blood vessels covered with pericytes in both pilocytic astrocytoma and the perinecrotic area in glioblastoma. Furthermore, little is known about other members of the VEGF-family. In the present study, VEGF-B, -C and -D were expressed at relatively lower levels in pilocytic astrocytoma, although overlap between the two tumour types was observed. Within pilocytic astrocytoma, VEGF-B's probable function to stimulate angiogenesis[48, 49] was confirmed with a positive correlation between proliferating tumour cells and VEGF-B expression.

In our opinion, this is the first study that has investigated various aspects of tumour vasculature in terms of vessel maturity status and stability and the angiogenic profile in pilocytic astrocytoma, using quantitative techniques like morphometry. This method in which overlapping SMA and collagen IV positive surface area were found, is a new technique, introduced by Zhou et al. in the evaluation of anti-angiogenic therapy in glioma xenografts[26] and never used before in comparisons between human tumour tissues.

While it is suggested that (i) low grade astrocytomas only show vessel co-option instead of angiogenesis; and (ii) increased angiogenesis is associated with a higher malignant brain tumour grade,[15, 50] from our results it can be concluded that angiogenesis plays certainly a role in pilocytic astrocytoma. This is especially seen in overlapping VEGF-A expression between pilocytic astrocytoma and glioblastoma. Moreover, in high as well as low grade astrocytomas targeting the VEGF/VEGF receptor-2 (VEGFR-2) signalling pathway, which has been described recently, seems to be an effective therapeutic strategy.[11, 51] However, for low grade astrocytoma, a selected patient group was treated; ten children with multiple recurrent low-grade gliomas (mostly diencephalic tumours) who had failed several treatment regimes, received a combination of bevacizumab and irinotecan. Two pilocytic astrocytomas were included.[51]

Besides selection bias, more importantly the possible synergistic effect of bevacizumab and chemotherapy is unclear and probably involves multiple mechanisms.[19] In glioblastoma, for instance, it is thought that the efficacy seen with the combination of bevacizumab and irinotecan could be explained by an antitumour stem-cell effect of bevacizumab and anti-differentiated tumour cell effect of irinotecan.[11] In contrast to high grade gliomas which show increased VEGFR expression of tumour cells and surrounding tumour tissue, in pilocytic astrocytoma VEGFRs were confined to the endothelial cells.[44, 52] So other response mechanisms are probably more important in pilocytic astrocytoma.

Because our study demonstrated clear overlap in vascular abnormality between pilocytic astrocytoma and glioblastoma, normalization of tumour vasculature by targeting VEGF could be a crucial mechanism. In theory, vessel normalization results in decreased interstitial pressure, more mature blood vessels, less hypoxia and increased delivery of chemotherapy to the tumour.[17] Thus VEGF/VEGFR interaction could be an encouraging therapeutic target to control tumour growth and possibly reduce morbidity and mortality in children with pilocytic astrocytoma. However, different adaptive resistance mechanisms may arise in response to anti-angiogenic therapy, including up-regulation of alternative proangiogenic signalling pathways within the tumour.[53] For pilocytic astrocytoma, this is unclear and warrants further study. Moreover, possible toxicity related to angiogenic treatment strategies[10, 54, 55] needs to be carefully taken into account. Nonetheless, although cerebellar pilocytic astrocytomas showed the most obvious signs of vessel instability, anti-angiogenic therapy could be in particular suitable/needed for pilocytic astrocytomas in surgically inaccessible sites or in cases involving large unresectable tumours.

Acknowledgements

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Chapter 6

Pro- and anti-angiogenic VEGF-A isoforms in pediatric pilocytic astrocytoma and adult glioblastoma: possibilities for anti-angiogenic therapy in pilocytic astrocytoma?

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Abstract

Aims: The present study aimed to analyze VEGF-A isoforms in correlation with tumor vessel morphology in pediatric pilocytic astrocytoma and adult glioblastoma. Secondly, we aimed to determine the ratio between pro- (VEGF-A_{xxx}a) and anti-angiogenic (VEGF-A_{xxx}b) VEGF-A isoforms because VEGF-A_{xxx}b inhibits the effect of anti-VEGF-A antibodies like bevacizumab.

Methods: VEGF-A isoforms were analyzed in tumor tissue of 15 pilocytic astrocytoma and 12 glioblastoma patients using Real Time RT-PCR. Microvessel density and vessel maturity in terms of basement membrane and pericyte coverage were evaluated in immunohistochemically stained slides with CD34, collagen IV and smooth muscle actin respectively.

Results: All pro-angiogenic VEGF-A isoforms (VEGF-A_{121}a-$_{189}$a) were lower expressed in pilocytic astrocytoma compared with glioblastoma. However, analyzing these isoforms relative to VEGF-A_{165}a as it is predominantly expressed in normal brain, no significant differences were found between both tumors. VEGF-A isoforms were not correlated with differences in vessel architecture between pilocytic astrocytoma and glioblastoma. Longer isoforms were associated with more vessel maturity. Strikingly, both tumors showed overlapping VEGF-A_{xxx}a : VEGF-A_{xxx}b ratios with relatively higher VEGF-A_{165}a and VEGF-A_{189}a expression.

Conclusions: The interplay between various VEGF-A isoforms does not seem to be the sole explanation for the different vessel architecture in pilocytic astrocytoma and glioblastoma. Interestingly, a high overlapping pro- and anti-angiogenic VEGF-A isoform ratio was found between both tumors, suggesting possibilities for anti-angiogenic therapy not only in glioblastoma, but also in pilocytic astrocytoma.

Introduction

Pilocytic astrocytomas (WHO grade I) are the most frequent pediatric brain tumors.[1] Despite different therapeutic options including neurosurgery, chemotherapy and radiotherapy, still 10% of the children with a pilocytic astrocytoma die due to tumor progression within 5 years.[2] Furthermore, morbidity can be serious, mainly because of the tumor localization (diencephalon, brain stem, optic pathway) and its subsequent chance of surgical morbidity and dismal effects of chemotherapy and/or radiation.[2-4] New therapeutic strategies are warranted for these patients. Because pilocytic astrocytomas are highly vascular, anti-angiogenic therapy could be suitable and seems to be also an effective therapeutic option.[5] Moreover, this possible suitability for targeting angiogenesis, was also suggested in our previous study, in which a critical overlap in the angiogenic profile and vessel immaturity/instability was found between pilocytic astrocytoma and the extensively described glioblastoma (WHO grade IV). However, the highly vascular pilocytic astrocytoma showed fewer but wider vessels compared with glioblastoma which contained more and smaller vessels.[6] Various mechanisms, including the expression of vascular endothelial growth factor (VEGF) isoforms, could contribute to this difference in tumor vessel morphology. An increased insight in the tumor vasculature of pilocytic astrocytoma could result in a better understanding in new therapeutic strategies for these tumors, including anti-angiogenic therapy.

Angiogenesis, the development and remodeling of new blood vessels from an existing vasculature, is a fundamental regulatory process involved in tumor growth and progression.[7, 8] The most potent and dominant mediator in the mechanism of angiogenesis is VEGF-A which is up-regulated in several malignancies, including brain tumors.[9, 10] VEGF-A stimulates endothelial cell proliferation, migration, and increases microvascular permeability by activation of VEGF receptor 1 (VEGFR-1/ flt-1) and VEGFR-2 (KDR/flk1).[11, 12] The VEGF-A gene consists of eight exons that give rise to various isoforms of *i.e.* 121, 145, 148, 165, 183 and 189 amino acids through differential splicing (Figure 1A). All isoforms contain exons 1-5 and the terminal exon, exon 8. The heparin-binding domain encoded by exon 6 and 7 determine binding to the extracellular matrix, and therefore isoforms containing exon 6 (VEGF-A_{145}) or both exon 6 and 7 (VEGF-A_{183} and VEGF-A_{189}) are bound tightly to cell surface heparin-containing proteoglycans and the extracellular matrix, whereas those lacking the domain are diffusible.[11, 13-15] VEGF-A_{165} which contains only one heparin-binding region encoded by exon 7, is moderately diffusible, and VEGF-A_{121} which lacks the domain totally is highly diffusible (Figure 1B).[15, 16] This results in different contribution of the various isoforms to the process of tumor angiogenesis. The moderately diffusible VEGF-A_{165} was the first VEGF isoform described and is widely considered to be a potent enhancer of vascular permeability and the predominant regulator of angiogenesis. VEGF-A_{165} could fully rescue tumor growth.[17] VEGF-A_{165}-induced tumor vasculature consists of irregularly dilated and leaky vessels.[18] In contrast, the longer isoform VEGF-A_{189} which binds to heparin-containing proteoglycans with high affinity contributes more to local blood vessel growth and tumor development in the tumor microenvironment resulting in a highly dense tumor vasculature with small microvessels.[17-19] VEGF-A_{189}-induced microvessels penetrate deeply from the tumor rim to the tumor core and function better than VEGF-A_{165}-induced microvessels in maintaining the viability of tumors.[19]

The highly soluble isoform VEGF-A_{121} functions especially at more distal sites of the tumor.[19] However, conflicting results have been described about the potency of VEGF-A_{121} to induce angiogenesis. It has been suggested that VEGF-A_{121} only co-opts preexisting blood vessels and induces vasodilatation while some studies showed that $VEGF_{121}$ gave rise to much greater angiogenic activity than VEGF-A_{165} or VEGF-A_{189} and indeed increase angiogenesis.[10, 18, 20-22] VEGF-A_{121} show major defects in mural cell recruitment resulting in immature blood vessels that are fragile and leaky.[23] Important to notice is that these effects of the different VEGF-A isoforms have been studied in mouse models in which just one of the isoforms was overexpressed.[17-19, 23] However in crude tumor tissue of course all various isoforms are present.

Besides the pro-angiogenic isoforms (VEGF-A_{xxx}a) as described above, alternative 3' splice site selection in exon 8, could also give different C-terminal sequences, resulting in the more recently described anti-angiogenic isoforms (VEGF-A_{xxx}b), where xxx denotes the amino acid number (Figure 1A).[24-28] In many tumors, a switch is seen from a VEGF-A_{xxx}b-dominated milieu in normal tissue to a proliferative phenotype in which VEGF-A_{xxx}a isoforms dominate.[24, 26, 29, 30] In addition, the balance of other angiogenic factors including angiopoietins is also disrupted in tumors, leading to abnormal and immature vessels with a chaotic endothelial-cell lining, detached pericytes, and an abnormally thick or thin basement membrane.[31-33]

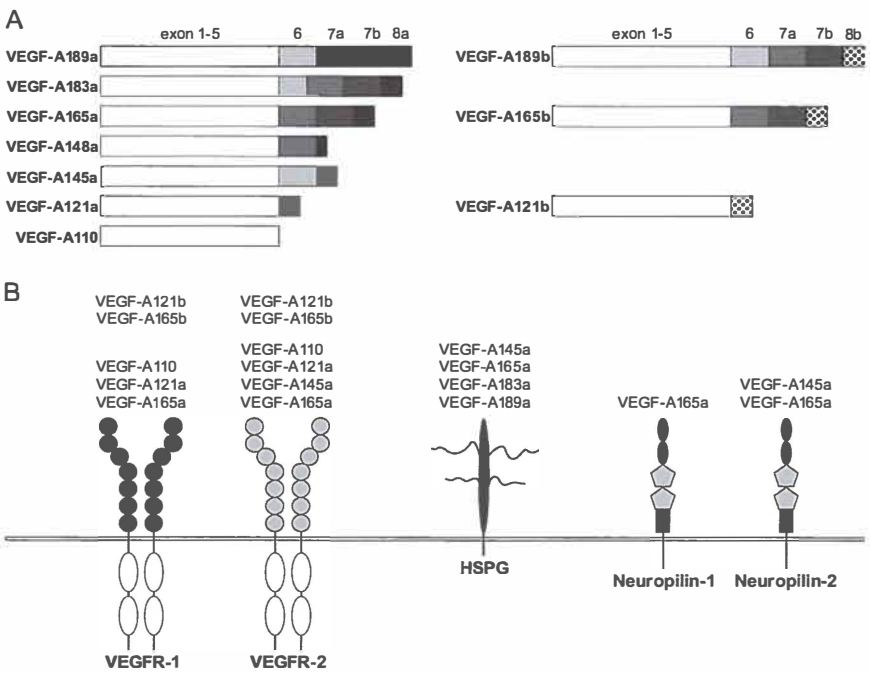

Figure 1. (**A**) Alternative splicing of the VEGF-A gene gives rise to multiple VEGF-A isoforms. An additional VEGF-A isoform of 110 amino acids results from proteolytic cleavage. Proximal splice-site selection in the terminal exon 8 leads to pro-angiogenic isoforms (VEGF-A_{xxx}a), whereas distal splice site selection gives rise to anti-angiogenic isoforms (VEGF-A_{xxx}b). (**B**) Interaction of pro- and anti-angiogenic VEGF-A isoforms with VEGF receptors (VEGFRs), heparin sulfate proteoglycans (HSPG) and neuropilin receptors.

As each isoform could contribute differently to the process of angiogenesis resulting in differences in blood vessel architecture, this study firstly aimed to analyze various VEGF-A isoforms in correlation with tumor vessel morphology in pediatric pilocytic astrocytoma and adult glioblastoma, as prototype of tumor angiogenesis. Interestingly, it is suggested that anti-angiogenic VEGF-A isoforms contain binding domains for the vast majority of anti-VEGF-A antibodies, including bevacizumab, and therefore inhibiting the effect of these drugs in tumors expressing significant levels of VEGF-A_{xxx}b. Tumors with relatively higher pro- than anti-angiogenic isoform expression will be probably more sensitive to anti-angiogenic therapy.[30, 34] Because of this possible clinical relevance, we secondly aimed to determine VEGF-A_{xxx}a : VEGF-A_{xxx}b ratios in both tumor types.

Materials and Methods

Patient samples

Tumor tissue was obtained from 15 pediatric patients who were diagnosed with pilocytic astrocytoma in the period 1996-2006 (Table 1) and adults with glioblastoma in 2001-2003 (n = 12). Both patient groups were derived from a larger study group of respectively 59 and 62 patients.[6, 35] However, we only included those patients with sufficient fresh frozen tumor tissue of high quality. Tissue material was histologically evaluated and graded according to WHO classification 2007.[1] Utilization of tumor tissue was approved by the relevant institutional committees (University Medical Center Groningen).

In the glioblastoma study group the median age at diagnosis using MRI or CT was 55.8 years (range 41.6 – 82.0) with a male predominance of 67%. Treatment included partial resection (n = 5) or gross total resection (n = 6). Nine patients received post-operative radiotherapy (up to a total of 60 Gy). None of the patients received any form of chemotherapy because chemotherapy was not part of the standard treatment for glioblastoma at that time in the Netherlands. The median overall survival time was 221 days with a range of 23 – 372 days. Because of the heterogeneity of both patient groups, no correlations were analyzed between clinical characteristics and biological tumor data.

Real Time RT-PCR of VEGF-A isoforms

Fresh frozen pilocytic astrocytoma (n = 15) and glioblastoma tissue (n = 12) was used for Real Time RT-PCR of different VEGF-A isoforms. RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany), and quantified with the Nanodrop ND-1000 UV Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). cDNA synthesis was performed using RevertAid first strand cDNA synthesis kit (Fermentas, Hanover, Maryland, USA) and again quantified. Real Time RT-PCR was performed in triplicate with SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California, USA) using ABI7900 HT Real-Time sequence detection system in 384-well reaction plates (Applied Biosystems, Foster City, California, USA). Primers were designed with Clone Manager® and used as previously described (Table 2).[27, 36]

As reference gene, β-actin (forward primer: GCTGTGCTACGTCGCCCTG, reverse: GGAGGAGCTGGAAGCAGCC) was used. TF-1 cells (acute myeloid leukemia cell line) were used as positive control for pro-angiogenic VEGF-A isoforms (VEGF-A_{xxx}a). This cell line was cultured in RPMI 1640 (Lonza) with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin (PAA Laboratories). Fresh frozen healthy kidney tissue functioned as positive control for anti-angiogenic isoforms (VEGF-A_{xxx}b). The ratio between pro- and anti-angiogenic isoforms (VEGF-A_{xxx}a : VEGF-A_{xxx}b ratio) was calculated using the formula $2^{\Delta Ct}$, in which ΔCt was VEGF-A_{xxx}b ΔCt minus VEGF-A_{xxx}a ΔCt. So a higher VEGF-A_{xxx}a : VEGF-A_{xxx}b ratio corresponds with relatively higher VEGF-A_{xxx}a expression reflecting a more pro-angiogenic status.

Table 1. Patient characteristics of the pilocytic astrocytoma study group (n = 15)

Characteric	*n* (%)
Gender, male	11 (73)
Tumour localization	
Supratentorial	3 (20)
Optic nerve	7 (47)
Infratentorial	5 (33)
Neurosurgery	
Partial resection	7 (47)
Gross total resection	8 (53)
Chemotherapy	1 (7)
Tumour progression	4 (27)
5-year progression free survival	(50)
Death	1 (7)
5-year survival	(83)
Characteristic	**Median (range)**
Age at diagnosis, years	11.1 (1.0 – 15.5)
Follow up, years	3.5 (1.1 – 10.8)
Progression free survival, years	3.6 (2.1 – 9.6)

Immunohistochemical analyses of MVD

Microvessel density (MVD) was determined using first the Chalkley point overlap morphometry technique and secondly traditional counting of blood vessels in paraffinic slides.[35] The first technique includes an evaluation of double immunohistochemical stained slides for Ki67/CD34 (endothelial cell marker) with the use of an ocular grid with 25 random points (x200 magnification). For each slide, five hot spots were analyzed by turning the ocular grid to maximize the number of overlapping points on the grid and the CD34 stained vessels. Next the mean score per slide was calculated representing the Chalkley score.

For glioblastoma two scores were assessed, both for the perinecrotic and intermediate area. Counting of blood vessels (x200 magnification) was performed in five randomized areas per slide, which were 30 min stained for CD34 with use of the NexES IHC Staining Module (Ventana Medical Systems, Tucson, AZ, USA). Antigen retrieval was performed at 98°C. As described previously, a higher Chalkley score corresponds with larger blood vessels, while CD34 vessel counting represents the number of vessels.[6]

Morphometric evaluation of vessel maturity

Vessel maturity was evaluated by two methods. In collagen IV and Smooth Muscle Actin (SMA) stained slides the fraction of tumor vessels that was covered with respectively basement membrane or pericytes was calculated in five randomized areas per tumor sample (x200 magnification). Furthermore, morphometry was used to analyze the percentage of marker-positive surface area of collagen IV and SMA in six random areas per tumor sample (x200 magnification), with use of the formula: marker-positive area x100/area of the optical field.[6]

During all these analyses of tumor tissue, investigators were blinded to the patient data. Because analyses of the tumor vasculature and different aspects of the angiogenic profile have been performed previously,[6, 35] we presented the data in correlation with the expression of the diverse VEGF-A isoforms. Results from this subgroup correspond with those derived from the larger, original study group.

Table 2. Primers used for Real Time RT-PCR of VEGF-A isoforms

Isoform	Primer sequence (5'-3')	Product size (bp)
VEGF-A_{121}a	Forward: GCGGATCAAACCTCACCAAG Reverse: TCGGCTTGTCACATTTTTCTTG	116
VEGF-A_{121}b	Forward: GAAAATCTCTCACCAGGAAA Reverse: CTGGATTAAGGACTGTTCTG	100
VEGF-A_{145}a	Forward: GAATGCAGACCAAAGAAAGATAGAG Reverse: TCGGCTTGTCACATACGCTCC	124
VEGF-A_{148}a	Forward: GACAAGAAAATCCCTGTGG Reverse: TCGGCTTGTCACATCTTGCAAC	120
VEGF-A_{165}a	Forward: GAGCAAGACAAGAAAATCCC Reverse: CCTCGGCTTGTCACATCTG	163
VEGF-A_{165}b	Forward: GAGCAAGACAAGAAAATCCC Reverse: GTGAGAGATCTGCAAGTACG	156
VEGF-A_{183}a	Forward: GAGATGAGCTTCCTACAGCAC Reverse: GGCCCACAGGGACGGGATTT	134
VEGF-A_{189}a	Forward: TCCTGGAGCGTTCCCTGTG Reverse: CCTCGGCTTGTCACATCTG	158
VEGF-A_{189}b	Forward: TCCTGGAGCGTTCCCTGTG Reverse: GTGAGAGATCTGCAAGTACG	152

Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics 19 software. Because the data had no Gaussian distribution, a Mann-Whitney U-test was used for comparisons between the pilocytic and glioblastoma study group. The nonparametric Spearman correlation test was used to analyze correlations between angiogenic data. For all analyses, a two-tailed P-value of less than 0.05 was considered statistically significant.

Results

Overlapping expression of pro-angiogenic VEGF-A isoforms in pilocytic astrocytoma and glioblastoma

Analyzing the pro-angiogenic VEGF-A isoforms, an overall lower expression level was seen in pilocytic astrocytoma compared with glioblastoma (Figure 2), suggesting possible lower angiogenic activity in pilocytic astrocytoma. However, studying the expression of the various pro-angiogenic isoforms relative to VEGF-A_{165}a as it is predominantly produced in normal cells and tissues under physiological conditions,[37, 38] interestingly no significant differences were found between both tumors (Figure 3). Pilocytic astrocytoma and glioblastoma both seem to show relatively higher expression of the freely diffusible isoform VEGF-A_{121}a and the longer isoform VEGF-A_{189}a (Figure 2 and 3). In theory, the relative higher expression of these two isoforms would lead to a mixture of large, immature and small, more mature blood vessels.[19, 23]

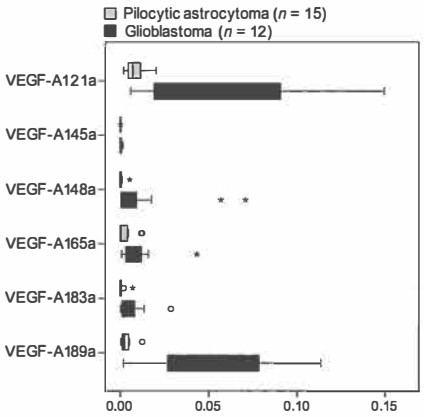

Figure 2. Boxplots showing lower gene expression of various pro-angiogenic VEGF-A isoforms in pilocytic astrocytoma compared with glioblastoma (VEGF-A_{121}a: P < 0.001, VEGF-A_{145}a: P = 0.004, VEGF-A_{148}a: P = 0.004, VEGF-A_{165}a: P = 0.001, VEGF-A_{183}a: P = 0.002, VEGF-A_{189}a: P < 0.001).

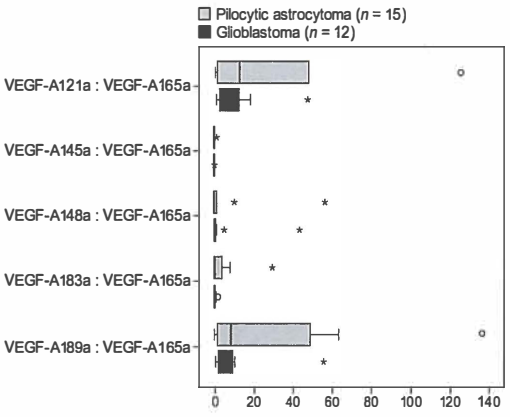

Figure 3. Boxplots showing various pro-angiogenic VEGF-A isoforms relative to VEGF-A_{165}a in pilocytic astrocytoma compared with glioblastoma (VEGF-A_{121}a: P = 0.399; VEGF-A_{145}a: P = 0.905; VEGF-A_{148}a: P = 0.067; VEGF-A_{183}a: P = 0.427; VEGF-A_{189}a: P = 0.614).

Pro-angiogenic VEGF-A isoforms versus vessel architecture in pilocytic astrocytoma and glioblastoma

As described previously, each pro-angiogenic VEGF-A isoform contributes in a unique way to the process of tumor angiogenesis, resulting in differences in vessel architecture. In line with this hypothesis, it has been expected that VEGF-A_{121}a and VEGF-A_{165}a gives rise to the more dilatated blood vessels in pilocytic astrocytoma, while in glioblastoma VEGF-A_{189}a contributes to their characteristic tumor vasculature with a high number of smaller and tortuous vessels. However, in both pilocytic astrocytoma and glioblastoma no significant correlations were found between VEGF-A isoform expression and vessel architecture. Indeed, VEGF-A isoforms do not seem to be the only determining factor in differences in vessel architecture between pilocytic astrocytoma and glioblastoma (Figure 4).

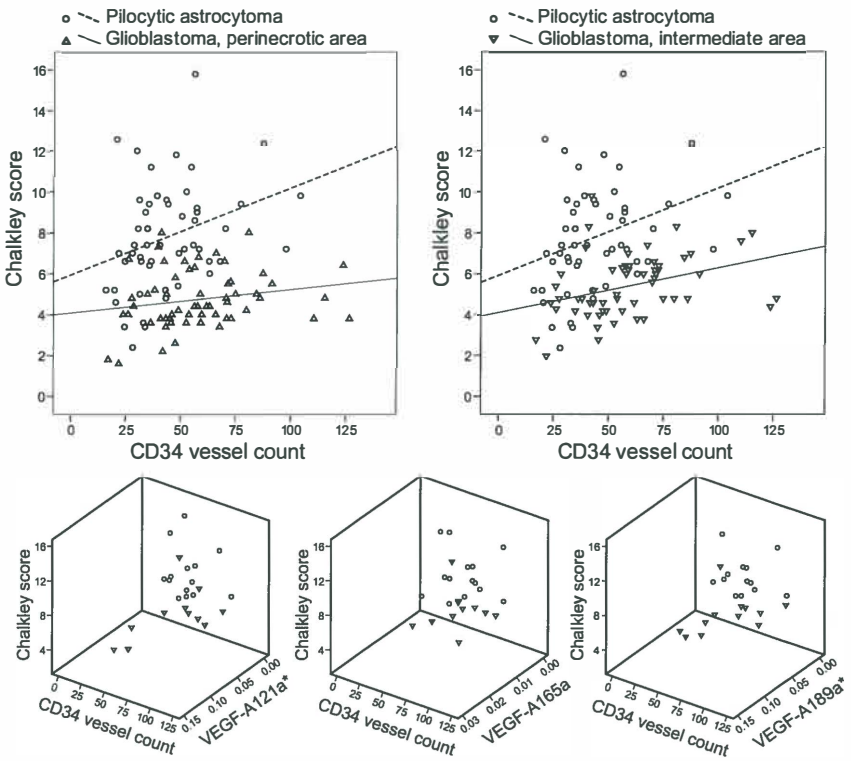

Figure 4. Scatterplots showing that differences in vessel architecture between pilocytic astrocytoma and glioblastoma do not seem to be only determined by expression of pro-angiogenic VEGF-A isoforms. *Exclusion of extreme value (VEGF-A_{121}a : 0.78 and VEGF-A_{189}a : 0.59).

Correlation between larger isoforms and more blood vessel maturity

Although pro-angiogenic VEGF-A isoforms were not correlated with vessel architecture, in both pilocytic astrocytoma and glioblastoma a clear correlation was seen between larger isoforms which are cell- and extracellular matrix-associated due to moderate heparin-surface glycoprotein interactions and a more mature vasculature.[23] In pilocytic astrocytoma, a positive correlation was found between VEGF-A_{183}a, VEGF-A_{189}a and the fraction of vessels covered with pericytes, as marker of vessel maturity (r: 0.714, P = 0.006; r: 0.621, P = 0.024). Taking pilocytic astrocytoma and glioblastoma together in the analyses, as both tumors showed a critical overlap in vessel (im) maturity,[6] VEGF-A_{189}a was also positively correlated with vessels covered with pericytes (r: 0.526, P = 0.010).

Overlapping pro- and anti-angiogenic VEGF-A isoform ratios in pilocytic astrocytoma and glioblastoma

A higher VEGF-A_{xxx}a : VEGF-A_{xxx}b ratio corresponds with relatively higher VEGF-A_{xxx}a expression, showing a more pro-angiogenic status. In normal tissue, anti-angiogenic isoforms are dominantly expressed, while in many tumors, a switch is seen towards a dominated pro-angiogenic isoform milieu.[24, 26, 29] Surprisingly, in both pilocytic astrocytoma and glioblastoma the median VEGF-A_{121} ratio was lower than 1 (Figure 5), possibly reflecting less VEGF-A_{121}a expression compared with VEGF-A_{121}b. The other ratios showed, as expected, relatively more expression of the pro-angiogenic isoforms of VEGF-A_{165} and VEGF-A_{189}. Notably, the ratios between pro- and anti-angiogenic VEGF-A isoforms did not differ significantly between pilocytic astrocytoma and glioblastoma (Figure 5).
Especially the VEGF-A_{165}a : VEGF-A_{165}b and VEGF-A_{189}a : VEGF-A_{189}b ratios are of crucial interest as it is hypothesized that tumors with relatively more VEGF-A_{xxx}a expression compared with VEGF-A_{xxx}b will be more sensitive to anti-angiogenic therapy. In the present study we provide clues that this might be the case in glioblastoma as well as pilocytic astrocytoma. It is suggested that tumors expressing significant levels of VEGF-A_{xxx}b will be less responsive to VEGF-A antibodies, including bevacizumab, because VEGF-A_{xxx}b inhibits the effect of this anti-VEGF-A antibody.[30, 34]

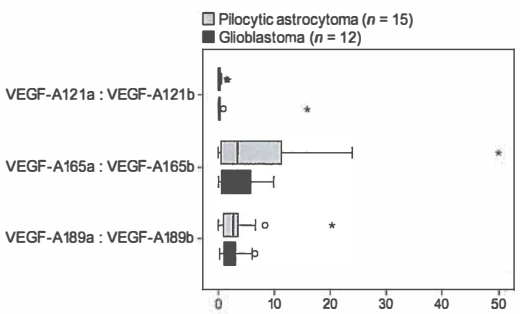

Figure 5. Overlapping VEGF-A isoform ratios between pilocytic astrocytoma and glioblastoma (VEGF-A_{121}a : VEGF-A_{121}b, P = 0.829; VEGF-A_{165}a : VEGF-A_{165}b, P = 0.486, VEGF-A_{189}a : VEGF-A_{189}b, P = 0.456).

Discussion

The present study showed relatively overlapping VEGF-A isoform expression between pediatric pilocytic astrocytoma and adult glioblastoma. VEGF-A isoforms do not seem to be the only determining factor in differences in vessel architecture between pilocytic astrocytoma and glioblastoma, but do correlate with vessel maturity, as longer isoforms were positively correlated with more vessel maturity in both tumors. Furthermore this study distinguished in the expression of VEGF-A isoforms between pro- and anti-angiogenic isoforms which has not been investigated previously in these tumors. Interestingly, a clear overlap was demonstrated between pilocytic astrocytoma and glioblastoma in the pro- and anti-angiogenic VEGF-A isoform ratios with relatively high VEGF-A_{165}a and VEGF-A_{189}a expression, suggesting possibilities for anti-angiogenic therapy not only in glioblastoma, but also in pilocytic astrocytoma.

So pilocytic astrocytoma seems to be more similar to the extensively studied glioblastoma, as probably has been thought. Firstly, analyses of the different pro-angiogenic VEGF-A isoforms (VEGF-A_{121}a, $_{145}$, $_{148}$, $_{183}$, $_{189}$) relative to VEGF-A_{165}a as it is predominantly expressed in brain under normal physiological conditions,[38] showed no significant differences between both pilocytic astrocytoma and glioblastoma. These findings correspond with the observations in our previous study in which pilocytic astrocytoma showed overlapping VEGF-A expression with glioblastoma using immunohistochemistry.[6] While under normal physiological conditions only low levels of VEGF-A_{121}a are observed in brain,[38] both pilocytic astrocytoma and glioblastoma showed relatively high VEGF-A_{121}a expression, suggesting a shift from VEGF-A_{165}a toward VEGF-A_{121}a as has been also described in prostate cancer.[39] VEGF-A_{121}a is also upregulated in colon cancer, where it is hypothesized to play an important role in this proliferative, angiogenic process due to its bioavailability.[14] Furthermore it has been discussed whether during malignant progression an angiogenic switch favoring the shorter diffusible isoforms occurs, for example in lung cancer.[40] Our study does not support this hypothesis for brain tumors as both low and high grade glioma showed high VEGF-A_{121}a expression, followed by relatively high expression of VEGF-A_{189}a, one of the longer isoforms.

The relatively high expression of these two isoforms was not associated with characteristics in tumor vessel architecture. The present study showed that the interplay between the various VEGF-A isoforms does not seem to be the sole explanation for differences in vessel architecture between pilocytic astrocytoma with dilatated blood vessels and glioblastoma with their highly dense tumor vasculature and small microvessels. In both tumors no significant correlation were found as has been showed in previous studies in which just one of the isoforms was overexpressed. These mouse models give insight into the specific isoform induced tumor vasculature.[17-19, 23] However, the influence of possible interactions between diverse isoforms on the vessel architecture is less clear, and plays probably a role in the present study.

Nevertheless, the VEGF-A isoforms are only one of the mechanisms that could influence vessel architecture, besides for example the angiopoietin/Tie-2 system which has been described as a potent regulator of blood vessel enlargement.[41, 42] Remarkable in clinical practice is the overlapping signal intensity of gadolinium-based dynamic contrast-enhanced MRI between the low grade pilocytic astrocytoma and glioblastoma. As it has been described that the signal intensity is both dependent on vessel density and vessel caliber,[43, 44] the wide blood vessels in pilocytic astrocytoma probably contribute to this high intensity while in glioblastoma it seems to be due to their higher number of smaller vessels.

Another finding in which pilocytic astrocytoma showed their similarity with glioblastoma, has been showed by a positive correlation between longer VEGF-A isoforms and vessel maturity in both tumors. The affinity of the VEGF isoforms to bind heparin has an overall effect on isoform spatial distribution; these variations then determine whether blood-vessel growth is organized and directed, or disordered.[15, 16, 18] VEGF-A_{189}a for example is associated with a much more mature vasculature, showing good levels of pericyte coverage and less hemorrhage, while VEGF-A_{121}a show major defects in mural cell recruitment resulting in immature blood vessels that are fragile and leaky.[23] In line with literature, the longer isoforms VEGF-A_{183}a and VEGF-A_{189}a were positively correlated with the fraction of vessel covered with pericytes. The protein product of VEGF-A_{183}a is six amino acids shorter than VEGF-A_{189}a as a consequence of an alternative splicing event within exon 6a.[45]

Furthermore, in both pilocytic astrocytoma and glioblastoma VEGF-A_{121}a was highly expressed, however surprisingly the anti-angiogenic isoform VEGF-A_{121}b expression exceeded the expression of the pro-angiogenic isoform. For VEGF-A_{165} and VEGF-A_{189}, relatively higher expression of the pro-angiogenic isoforms were found in both tumors, although the ratio was variable with some patients having no excess of VEGF-A_{xxx}a and others with high excess of VEGF-A_{xxx}a, a previously described phenomenon in colon cancer.[30] Overall, the effect of a shift from anti-angiogenic VEGF isoform expression under normal physiological conditions to pro-angiogenic expression in cancer is due to a combination of reduced VEGF-A_{xxx}b expression and increased VEGF-A_{xxx}a expression.[24, 26, 29, 30] The anti-angiogenic isoforms VEGF-A_{121}b and VEGF-A_{165}b bind even as VEGF-A_{121}a and VEGF-A_{165}a, to the conventional VEGFRs (Figure 1B), however resulting in inhibition of the process of angiogenesis.[26, 29, 46] So, the replacement of the terminal six amino acids with those coded by exon 8b is of crucial importance in converting the dominant pro-angiogenic growth factor into a positively anti-angiogenic molecule. Interestingly, while equal binding affinity is seen between VEGF-A_{xxx}a and VEGF-A_{xxx}b to VEGFR-2,[26, 29] VEGF-A_{xxx}b is not simply a classical competitive inhibitor. VEGF-A_{165}b for example does stimulate the phosphorylation of VEGFR-2 but qualitatively in a unique way; VEGF-A_{165}b is considerably poorer than VEGF-A_{165}a in inducing phosphorylation of the angiogenic positive regulatory site in VEGFR-2.[29, 47] Moreover contrary to VEGF-A_{165}a, the anti-angiogenic isoform is unable to bind the co-receptor neuropilin, contributing to the lack of angiogenic properties of VEGF-A_{165}b.[47]

Similarly, VEGF-A_{121}b and VEGF-A_{189}b are expected to prevent angiogenesis, although VEGF-A_{121} binds VEGFRs with lower receptor affinity than that of VEGF-A_{165}[14] and the VEGF-A_{189} isoform does not bind VEGFRs at all, but is available for cleavage by plasmin and subsequent release in the soluble form, VEGF-A_{110} which binds VEGFRs.[15, 48] However, Catena et al suggested a weak angiogenic instead of anti-angiogenic function of VEGF-A_{121}b, showing in VEGF-A_{121}b-overexpressing tumors a significant increase in angiogenesis compared with controls and VEGF-A_{165}b-overexpressing tumors.[49] Furthermore, the observations that VEGF-A_{121}b seems to act as a survival factor for endothelial cells[26] and the present study demonstrating a relatively high VEGF-A_{121}b expression compared with VEGF-A_{121}a in both pilocytic astrocytoma and glioblastoma, emphasized the complexity of the VEGF-A isoform biology and the carefulness of the interpretation of the results.
Finally, of particular interest is that the balance between pro- and anti-angiogenic isoforms seems to affect the sensitivity of tumors to anti-angiogenic drugs including bevacizumab. It was demonstrated that VEGF-A_{165}b binds to bevacizumab with similar affinity as VEGF-A_{165}a, and inhibits the bevacizumab effect on tumor growth. No effect of bevacizumab was seen in tumors expressing predominantly VEGF-A_{165}b.[30, 34] As we showed a crucial overlap in relatively more VEGF-A_{165}a and VEGF-A_{189}a expression between pilocytic astrocytoma and glioblastoma and as encouraging results were described with bevacizumab in glioblastoma,[50] pilocytic astrocytoma could also be suitable for anti-angiogenic therapy. In theory, especially patients with unresectable tumors and a high VEGF-A_{xxx}a : VEGF-A_{xxx}b ratio could possible benefit of bevacizumab. As such, morbidity and mortality in children with pilocytic astrocytoma could be reduced. In the future, combining multiple anti-angiogenic strategies will be of increasingly importance.

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Chapter 7

Less tumor engraftment after anti-VEGF therapy in pediatric low grade astrocytoma

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Proceedings of the AACR 2013 (abstract)

Pediatric low grade astrocytoma mouse models

Up to now, two spontaneous low grade astrocytoma mouse models have been described. The first *in vivo* model was developed based on the neurofibromatosis-1 associated optic glioma in young children.[1] So a genetically engineered mouse model of neurofibromatosis-1 was developed in which optic low grade glioma arise.[2] Secondly, a mouse model was developed in which a BRAF kinase domain was activated to induce pilocytic astrocytoma.[3] Besides these induced murine tumor models, no pediatric low grade astrocytoma xenograft mouse model have been described. To further investigate the possible therapeutic window for anti-angiogenic therapy in pediatric low grade astrocytoma, we performed the following study which is published in abstract form.

Abstract

Introduction: Low grade astrocytomas are the most frequent brain tumors in children. Although some children benefit from traditional treatments including neurosurgery, chemotherapy and radiotherapy, still children die due to tumor progression. Furthermore, morbidity can be serious. So new therapeutic strategies are warranted for these patients. Vascular endothelial growth factor (VEGF), the most critical angiogenic factor may be a potential therapeutic target. The present study aimed to analyze the effect of VEGF inhibition *in vitro* and *in vivo* in pediatric low grade astrocytoma.
Materials and methods: Three different pediatric low grade astrocytoma cell lines were used: Res-186 (WHO grade I astrocytoma), Res-259 and UW-467 (WHO grade II astrocytoma). Effects *in vitro* of the anti-VEGF monoclonal antibodies, bevacizumab (Avastin®, anti human VEGF) and B20-4.1.1 (anti human and mouse VEGF) were studied using a WST-1 cell viability assay and a cell proliferation assay with BRDU. Furthermore an intracranial pediatric low grade astrocytoma mouse model was developed in which Res-259 cells were orthotopically implanted in NOD-scid IL2Rg^{null} mice. After 6 weeks treatment intraperitoneal twice weekly with bevacizumab 15 mg/kg, B20-4.1.1 5 mg/kg or phosphate buffered saline as control group, mice were euthanized and tumor engraftment was studied in brain slides using Aperio's imagescope viewer.
Results: As expected, no effects were seen of anti-VEGF on pediatric low grade astrocytoma cell viability and cell proliferation. However *in vivo* results showed less tumor engraftment in bevacizumab (n = 10, 70%) and B20-4.1.1 (n = 9, 44%) treated mice compared with the control group (n = 10) in which in all mice tumor engraftment was observed. In contrast with bevacizumab which did not show significantly lower tumor mass compared with control (P = 0.062), in B20-4.1.1 treated mice significantly lower tumor mass was determined (P = 0.001).
Conclusion(s): This study showed less tumor engraftment after anti-VEGF therapy in a newly developed pediatric low grade astrocytoma mouse model. In these tumors anti-VEGF seems to work especially on tumor microenvironment. Suggesting that anti-VEGF could have a potential role in the therapeutic strategy for children with low grade astrocytoma, thoughtfulness on possible tumor escape mechanisms that may arise will be crucial.

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Chapter 8

Growth-factor-driven rescue to RTK inhibitors in pediatric low grade astrocytoma and ependymoma

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Abstract

It has been shown that single targeted kinase inhibition failed due to tumor resistance mechanisms. The present study will extend our previous observations that vascular endothelial growth receptor (VEGFR) 2, platelet derived growth factor receptor (PDGFR) β, Src, the epidermal growth factor receptor (ErbB) family, and hepatocyte growth factor receptor (HGFR/cMet) are potential drugable targets in low grade astrocytoma and ependymoma with investigations concerning growth-factor-driven resistance. This rescue mechanism was investigated in pediatric low grade astrocytoma and ependymoma cell lines treated with receptor tyrosine kinase (RTK) inhibitors *e.g.* sorafenib, dasatinib, canertinib and crizotinib. In the present study, flow cytometry analyses were performed showing high expression of VEGFR-1, fibroblast growth factor receptor (FGFR) 1, ErbB-1, HGFR and recepteur d'origine nantais (RON) and their respective inhibitors showed induced decrease of tumor cell viability, measured with WST-1 cell viability assays. EGF, HGF and FGF, which are normally expressed in brain (tumor) tissue, showed to be effective rescue inducing growth factors resulting in increased cell survival especially during treatment with dasatinib or sorafenib. Growth-factor-driven rescue was less prominent when canertinib or crizotinib were used. Rescue was underscored by activating downstream Akt and/or Erk phosphorylation. Combination treatment showed to be able to overcome the growth-factor-driven resistance. In conclusion, our study highlights the extensive importance of environmentally present growth factors in developing resistance mechanisms towards RTK inhibitors. It is of great interest to anticipate upon these results for the design of new therapeutic trials with RTK inhibitors in pediatric low grade astrocytoma and ependymoma.

Introduction

Low grade astrocytomas (WHO grade I-II) are the most frequent brain tumors in children. Ependymoma (WHO grade II-III) accounts for 6-12% of all pediatric brain tumors and is the fourth most common brain tumor in children.[1] Although the 5-year survival of patients with pilocytic astrocytoma is around 90%, morbidity can be serious mainly because of the tumor localization and the change of surgical morbidity.[2, 3] Moreover, despite developments in neurosurgery, chemotherapy and radiation, the 5-year survival of pediatric ependymoma is approximately 57%.[4] Therefore, a search for new targeted therapies has started. With kinome profiling we previously identified vascular endothelial growth factor receptor 2 (VEGFR-2), platelet derived growth factor receptor β (PDGFRβ), Src, the epidermal growth factor receptor family (ErbB1-4), and hepatocyte growth factor receptor (HGFR/cMet) as potential drugable targets in these brain tumors.[5] Potential interesting inhibitors for these targets are sorafenib, dasatinib, canertinib and crizotinib respectively (Figure 1). Today, still very limited data is published about the clinical use of inhibitors targeting these receptor tyrosine kinases (RTKs) in pediatric brain tumors, and even less is known in low grade astrocytoma and ependymoma.

Although in chronic myeloid leukemia, single kinase targeted therapy, for oncogenic activated BCR/Abl has proven successful,[6] more recent trials failed to show prolonged responses. The first results of single receptor tyrosine kinase targeted tumor therapies are disappointing. It is thought that tumor progression is the net result of signaling through various protein kinase mediated networks driving tumor cell proliferation and survival. The signaling networks can be reflected by oncogenic mutations, silence tumor suppressor mutations, epigenetic changes and stromal interaction, such as angiogenesis. Important growth factors that can bind RTKs in brain tumors mediating tumor cell survival include VEGF, EGF, HGF, FGF and PDGF. Altogether these changes result in specific signaling profiles which promote tumor cell growth (Figure 1).

It has been shown that single targeted kinase inhibition failed due to tumor resistance mechanisms through alternate routes of kinase pathway activation. For example, RTK upregulation has been observed following targeted inhibition of selective kinases. This kinome reprogramming circumvents inhibition of proto-oncogenic kinases as for instance described for oncogenic RAS.[7] Furthermore, inhibition of specific RTKs could trigger the tumor to upregulate RTK ligands, through autocrine tumor-cell production, paracrine contribution from tumor stroma or systemic production, eventually contributing to tumor resistance to the RTK inhibitor with a similar signaling output as shown recently by Wilson in oncogenic mutated cell lines.[8] The present study will extend our previous observations that VEGFR-2, PDGFRβ, Src, ErbB family, and HGFR/cMet are potential drugable targets in low grade astrocytoma and ependymoma, with investigations concerning growth-factor-driven resistance in pediatric low grade astrocytoma and ependymoma treated with RTK inhibitors *e.g.* sorafenib, dasatinib, canertinib and crizotinib.

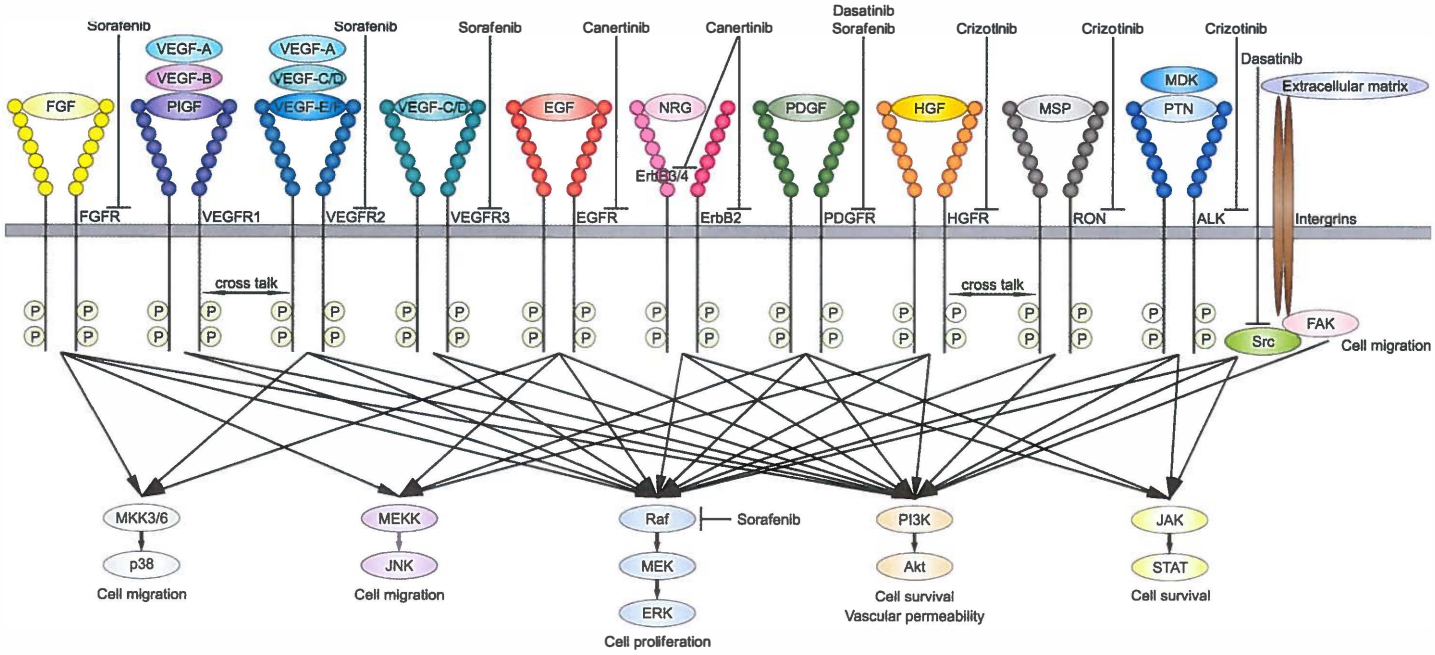

Figure 1. Schematic illustration showing important growth factors in brain tumors that can bind RTKs activating downstream pathways which contributes to tumor cell survival. These potential drugable targets can be inhibited by *e.g.* sorafenib, dasatinib, canertinib and crizotinib.

Materials and methods

Cell culture

Three pediatric low grade astrocytoma cell lines (WHO grade I: Res-186, grade II: Res-259 and UW-467) and one ependymoma cell line were evaluated (Res-196; Dr. Michael S. Bobola, Seattle Children's Hospital Research Institute, USA).[9] All cell lines were cultured in DMEM/F12 (Life Technologies, Carlsbad, CA, USA) containing 5% fetal calf serum (FCS). Cell cultures contained 100 units/ml penicillin and 100 ul/ml streptomycin (PAA Laboratories, Les Mureaux, France).

Flow cytometry analyses

Cells were blocked by PBS 1% BSA (Bovine Serum Albumin, Sigma Aldrich, St Louis, MO, USA), and stained with anti-VEGFR-1 (Sigma Aldrich), anti-VEGFR-2-PE (Sigma Aldrich), anti-VEGFR-3-APC (R&D Systems, Minneapolis, MN, USA), anti-EGFR (Abcam, Cambridge, UK), anti-ErbB2-PE (R&D Systems), anti-ErbB3-APC (R&D Systems), anti-ErbB4 (R&D Systems), anti-HGFR/cMet-FITC (R&D Systems), anti-RON-APC (R&D Systems), anti-FGFR-1 (Cell Signaling, Danvers, MA, USA), anti-FGFR-2 (R&D Systems), anti-PDGFRα-biotin (BD Biosciences, San Jose, CA, USA), anti-PDGFRβ-PE (BD Biosciences). Primary FGFR-2, ErbB4 antibodies were visualized using a Rabbit anti-Mouse PE-conjugated secondary antibody (Dako Cytomation, Glostrup, Denmark), and FGFR-1 with a FITC-conjugated Swine anti-Rabbit secondary antibody (Dako Cytomation). Primary EGFR was visualized using Alexa Fluor 488 conjugated anti-Rat secondary antibody (Cell Signaling) and PDGFRα antibody was visualized using streptavidin FITC (BD Biosciences). IgG-FITC/PE/APC and secondary antibodies were used as negative (isotype) controls. Expression was analyzed using LSRII (BD FACS DIVA software, BD Biosciences). The data was eventually developed using FlowJo software (Tree Star Inc., Ashland, OR, USA). Expression levels above 5% were considered as actual membrane protein expression, reaching above isotype controls.

Cell viability assays

WST-1 colorimetric viability assays were performed following the protocol described by the manufacturer (Roche, Basel, Switzerland). Cells were seeded in 96-wells plates at a density of 15 x 103 per well in medium (1% FCS) and after adhesion treated with sorafenib (0 – 8 uM), dasatinib (0 – 1000 nM), canertinib (0 – 10 uM) or crizotinib (0 – 12 uM) for 48h. Sorafenib, dasatinib, canertinib and crizotinib (LC laboratories, Woburn MA, USA) were dissolved in sterile dimethyl sulfoxide (DMSO) and stored at -20 °C. Growth factors including VEGF-A, EGF human, HGF human, FGF basic, PDGF-AB (all 100 ng/ml, Life Technologies) were added 1h after incubation by the inhibitor. After addition of the WST-1 cell survival reagent the absorbance was measured at 450 nm in a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). In every assay, per inhibitor, each concentration contains equal concentrations of DMSO. For each concentration 6 replicates were included. Similar conditions were maintained for different combination treatments.

Western blot analyses

1 x 10^6 cells were seeded in T25 flasks in 5 ml DMEM-F12 containing 1% FCS to which after adhesion sorafenib 8 uM, dasatinib 1 uM, canertinib 8 uM or crizotinib 9 uM was added. In the combination treatments, the second inhibitor was added at a lower concentration: sorafenib 2 uM, canertinib 4 uM or crizotinib 3 uM. After 1h, growth factors including VEGF-A, EGF, HGF, FGF or PDGF (all 100 ng/ml, Life Technologies) were added for another 1h. Cells were lysed in laemmli sample buffer (Bio-Rad Laboratories). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transported to nitrocellulose membranes. First, the membranes were incubated overnight with monoclonal primary antibodies for phospho-Erk (pErk) and total Erk (tErk), pAkt and tAkt, actin (Cell Signaling), followed by 1 hour incubation with HRP conjugated secondary antibodies (Dako Cytomation). Protein bands were visualized by chemiluminescence, on an x-ray film. Actin was used as loading control.

Results

EGF, HGF and FGF driven rescue to various RTK inhibitors

Pediatric low grade astrocytoma and ependymoma cell lines showed high expression of VEGFR-1, FGFR-1, EGFR, HGFR and RON (Figure 2A). Their respective inhibitors showed inhibitor induced decrease of tumor cell viability (Figure 2B). Effect of canertinib and crizotinib was also confirmed on RTK phosphorylation level studied with western blotting (data not shown). Interestingly, by addition of various growth factors, which are normally expressed in brain (tumor) tissue, inhibition by sorafenib, canertinib and crizotinib could be rescued partially, and even completely in dasatinib treated tumor cells (Figure 3). Especially EGF, HGF and FGF showed a strong rescue potential. Expression levels of the RTKs were not correlated with growth-factor-driven rescue in terms of increase in LC50. Eventually, as HGF was capable to induce rescue during canertinib and EGF during crizotinib, a combined treatment of these inhibitors could be promising in pediatric low grade astrocytoma and ependymoma (see below).

Growth factors overcome RTK-inhibited-phosphorylation of downstream targets

Before studying the possibilities of combined RTK inhibitors to overcome resistance, we assessed growth-factor-driven rescue on RTK-inhibited-phosphorylation of downstream targets to validate the previous rescue results on cell viability level. Analyses of the PI3K/Akt and MAPK/Erk downstream pathways, as the most commonly used survival signaling pathways by RTKs (Figure 1), indeed underscored EGF and HGF driven rescue during sorafenib and dasatinib treatment which resulted in phosphorylation of Akt and Erk (Figure 4A, B). HGF-driven rescue, positively effecting cell viability during canertinib, was also confirmed with overcoming the RTK-inhibited-phosphorylation of downstream targets. As intern negative control no Akt or Erk phosphorylation was found with FGF or PDGF during sorafenib, EGF during canertinib, and HGF during crizotinib treatment (Figure 4A, C, D).

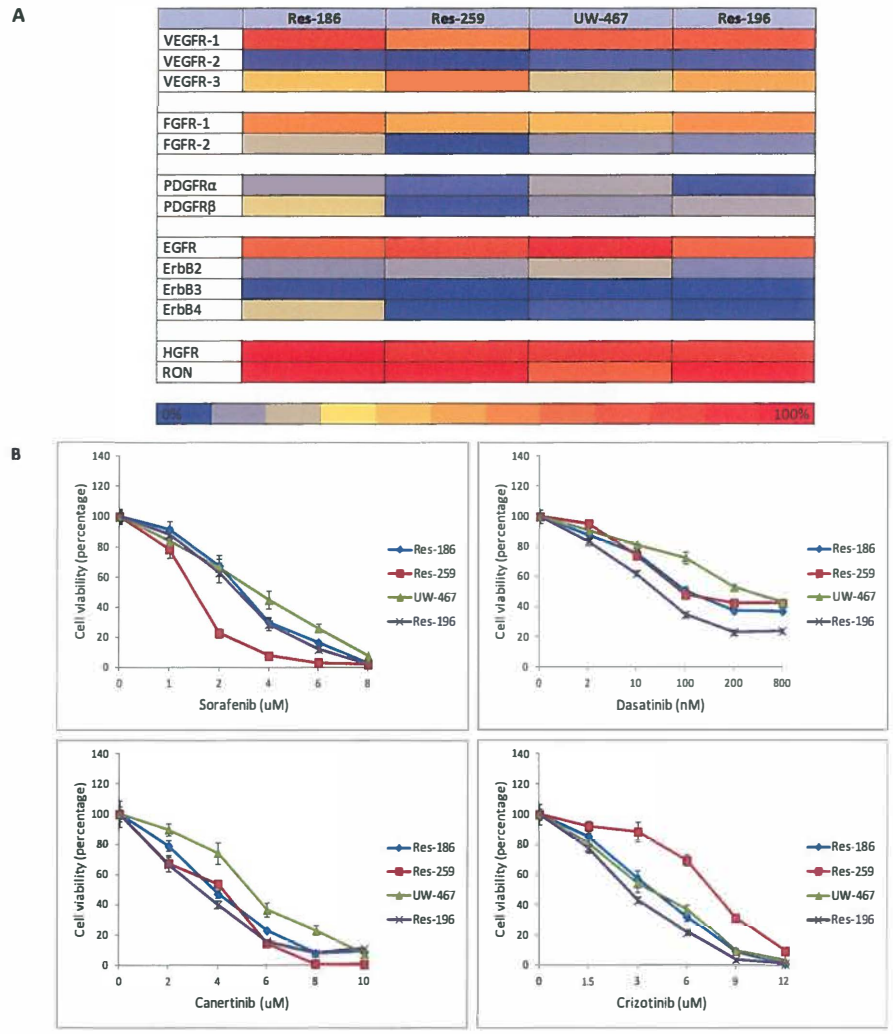

Figure 2. (**A**) Heat map showing expression levels in percentages of numerous growth factor receptors using flow cytometry analyses in pediatric low grade astrocytoma (Res-186, Res-259, UW-467) and ependymoma (Res-196) cell lines. (**B**) Cell viability assays demonstrating the effect of sorafenib, dasatinib, canertinib and crizotinib on pediatric low grade astrocytoma (Res-186, Res-259, UW-467) and ependymoma (Res-196) cell lines after 48h. Corresponding results in proliferation and apoptotic assays were found (data not shown).

A

Inhibitor	Cell line	VEGF-A	EGF	HGF	FGF basic	PDGF-AB
Sorafenib	Res-186		35%	25%		
Raf, VEGFR-2, -3	Res-259		28%	35%		
PDGFRβ, c-Kit	UW-467		36%	53%		
BRAF, FGFR-1	Res-196		24%	11%		6%
Dasatinib	Res-186		157%			
Src,BCR/Abl	Res-259		CR	CR	CR	
PDGFRα, -β, c-Kit	UW-467		CR	CR	CR	
	Res-196					
Canertinib	Res-186			10%		
Pan-ErbB	Res-259				15%	
	UW-467					
	Res-196			15%		
Crizotinib	Res-186					
cMet, ALK, RON	Res-259					
	UW-467					
	Res-196		23%			

B

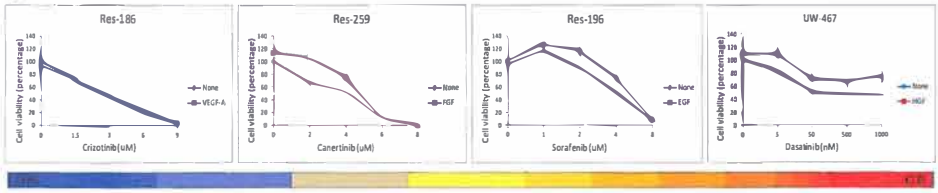

Figure 3. **(A)** Heat map showing the effect of growth factors on pediatric low grade astrocytoma and ependymoma cells treated with different TKIs. Percentages represent significant increase in LC50 of cells treated with inhibitor plus growth factor compared with inhibitor-treated cells only (purple squares, no rescue; yellow/orange, partial rescue; red squares, complete rescue). As an intern negative control no growth-factor-driven rescue was found of the particular RTK ligand of which the receptor is inhibited. **(B)** Cell viability assays demonstrating the diversity of growth factors on TKI-treated cells.

Discrepancies between growth-factor-driven rescue on cell viability level and downstream phosphorylation status

Notably, is the apparent discrepancy of HGF-driven phosphorylation by Akt and Erk without increased cell viability during canertinib in Res-259 cells, because there was HGF-driven rescue observed on cell viability level however at a lower dose, before the LC50. Also, in dasatinib treated Res-186 cells, HGF was unable to rescue cells at cell viability level despite the expression of the RTK and observed phosphorylation of Akt and Erk (Figure 4). This could possibly be explained by the various downstream targets of dasatinib with the consequence that activation of Akt and Erk is insufficient for cell viability rescue. Vice versa, FGF-driven rescue was not confirmed with Akt or Erk phosphorylation possibly due to involvement of other downstream pathways (Figure 1), that were not evaluated in this study.

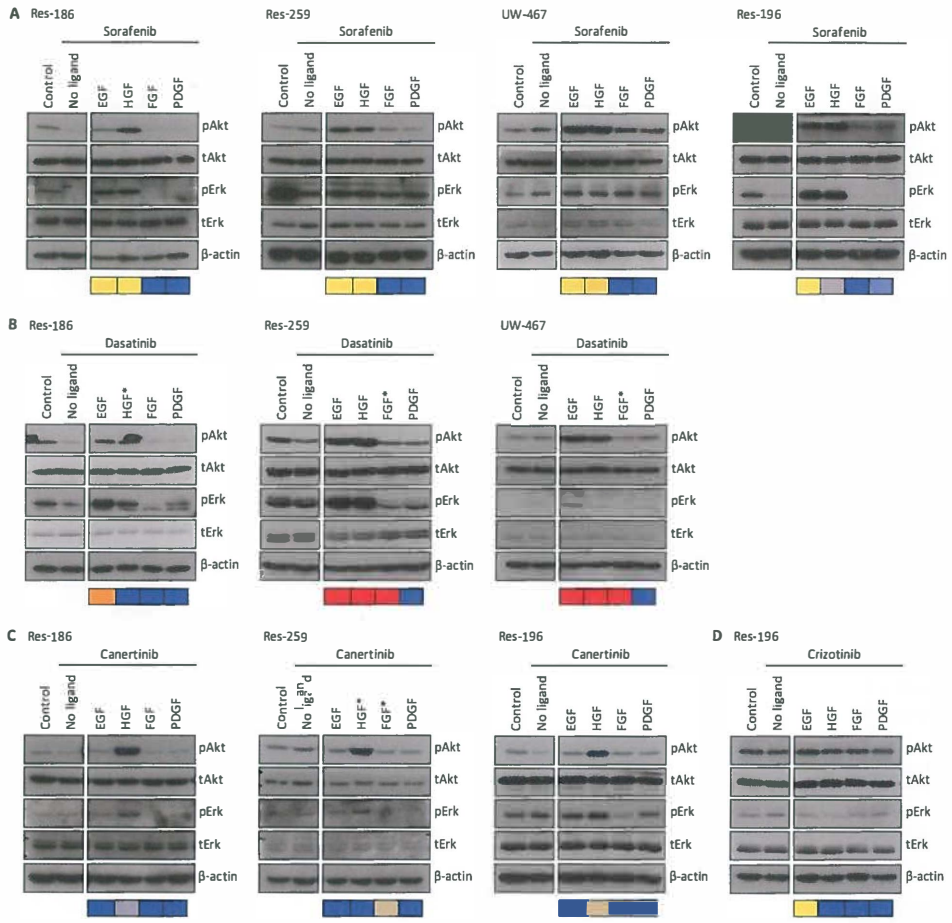

Figure 4. Immunoblots showing effects of growth factors (100 ng/ml) on Akt and Erk phosphorylation (p) after sorafenib **(A)**, dasatinib **(B)**, canertinib **(C)**, crizotinib **(D)** in pediatric low grade astrocytoma and ependymoma cells (2h). Growth-factor-driven rescue on cell viability as given in Figure 3 is indicated underneath the blots: purple squares, no rescue; yellow/orange squares, partial rescue; red squares, complete rescue. * Discrepancies between growth-factor-driven rescue on cell viability level and downstream phosphorylation status.

Combined RTK treatment can overcome growth-factor-driven rescue

Interestingly, EGF-driven rescue during sorafenib and HGF- and FGF-driven rescue during canertinib could be overcome by combining specific TK inhibitors. So by addition of canertinib to sorafenib treatment, EGF-driven rescue could be overcome and tumor cell survival was disrupted. In turn, sorafenib can overcome FGF-driven rescue during canertinib treatment (Figure 5). Actually as rescue by different growth factors was found in sorafenib, including both EGF and HGF, more than one RTK would have to be added. However, canertinib seems therapeutically more attractive showing less growth-factor-driven rescue, and was indeed successfully combined with sorafenib or crizotinib in both ependymoma and low grade astrocytoma cells.

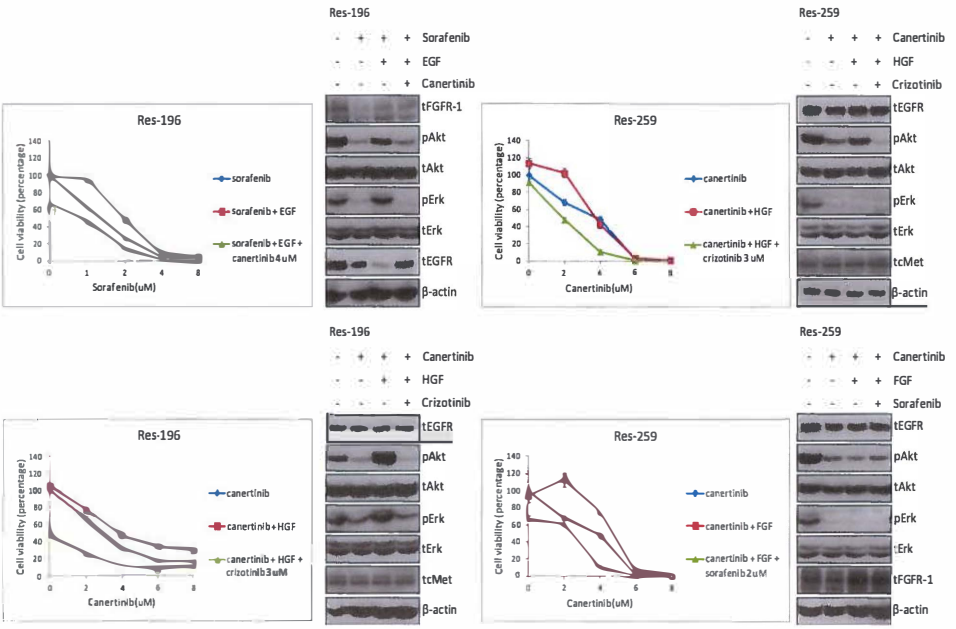

Figure 5. Cell viability assays demonstrating additional effects of combination treatment in pediatric low grade astrocytoma (Res-259) and ependymoma (Res-196) compared with single treatment (48h). Immunoblots showing effects of growth factors (100 ng/ml) on RTK, Akt and Erk phosphorylation (p) in pediatric low grade astrocytoma and ependymoma cells (2h).

Discussion

The present study showed growth-factor-driven rescue to various RTK inhibitors in pediatric low grade astrocytoma and ependymoma cell lines. Previously, we described several potential interesting drugable therapeutic targets upon kinome profiling *e.g.* VEGFR-2, PDGFRβ, Src, ErbB family members, and HGFR/cMet.[5] Drugs such as sorafenib, dasatinib, canertinib and crizotinib respectively were used to inhibit these targets. *In vitro* cell viability decreased upon the single use of one of these inhibitors as expected. In normal developing brain and therefore, also in brain tumors several growth factors including VEGF, EGF, HGF, FGF and PDGF, are commonly expressed.[10-17] Interestingly, the growth factor receptors are found often to be present on low grade astrocytoma and ependymoma cells.

Therefore, the present study investigated the rescue of ligand exposure to these RTK inhibitors. EGF and HGF showed to be effective rescue inducing growth factors in these resistance mechanisms acting via their RTKs resulting in increased cell survival and down-stream signaling. Combination treatment showed enhanced decrease in cell viability although growth factors were present. In ependymoma cells the combination of canertinib and crizotinib showed the best potential strategy, whereas in low grade astrocytoma cells the combination of both canertinib and crizotinib or canertinib and sorafenib demonstrated the best results. These findings highlight the potential growth factor induced cross talk in RTKs that are co-expressed on these low grade astrocytoma and ependymoma cells.

This is the first study to describe a RTK profile in relation to ligand exposure in low grade astrocytoma. In pediatric ependymoma a positive correlation between higher coexpression of ErbB2 and ErbB4 and tumor proliferative activity has been described.[18] Furthermore, ligand-dependent activation of ErbB receptor-signaling in cultured ependymoma cells resulted in Akt phosphorylation and cellular proliferation that was significantly blocked in a dose-dependent manner using WAY-177820, an inhibitor of ErbB2 tyrosine kinase activity.[18] The present study showed especially EGFR expression compared with the other ErbB family members. Moreover, EGF was shown to be a potential ligand to overcome resistance during other RTK inhibitors in both low grade astrocytoma and ependymoma. The rescue by growth factors is in line with previous reports in other tumor types. Particularly, HGF/cMet-induced resistance to RTK inhibitors is observed in diverse tumor types, including adult glioblastoma.[19] Further, PDGF upregulation in reaction to VEGFR inhibition and increased FGF/FGFR signaling during BRAF or EGFR inhibition may potentially contribute to tumor resistance.[20-23] Recently, tumor-cell-secreted VEGF has been demonstrated as one of the growth-factor-driven rescue mechanisms through activation of VEGFR-2 and the PI3K/Akt pathway.[24] As VEGFR-2 expression is in pediatric low grade astrocytoma and ependymoma mainly limited to endothelial cells,[25] we expect that VEGF will be able to enhance tumor growth only indirectly by inducing angiogenesis. This study underscores the previous result because the cell viability did not differ.

Until now, mainly pediatric high grade astrocytomas and just a few ependymomas were included into phase I studies analyzing erlotinib, an ErbB1/EGFR TK inhibitor as a single agent and in combination with chemotherapy or radiation.[26-28] Erlotinib was well tolerated in children, as were other ErbB family inhibitors including gefitinib and lapatinib.[26-30] The only published phase II study showed no increase in progression free survival or overall survival with gefitinib and radiation in malignant pediatric brain tumors.[30] Currently, erlotinib is under investigation in pediatric low grade astrocytoma and ependymoma in phase I and II trials respectively. As the ErbB TK family comprises four members, canertinib, a new pan-ErbB TK inhibitor showing anti-proliferative and pro-apoptotic effects on tumor cells,[31] could be more interesting. Up to now, canertinib has not been investigated in pre- or clinical pediatric brain tumor studies.

Sorafenib has been described in clinical trials, yet only restricted to adult brain tumors. Limited activity was reported of sorafenib in recurrent glioblastoma and in the first-line therapy for glioblastoma.[32-34] In pediatric low grade astrocytoma, sorafenib is topically analyzed. Although this could be interesting as alterations affecting the BRAF oncogene represent the main genetic defects in pilocytic astrocytoma (WHO grade I),[35, 36] we showed growth-factor-driven rescue during sorafenib in pilocytic astrocytoma cells, suggesting less potential for single application of this RTK inhibitor. Dasatinib and crizotinib are recently introduced in pediatric brain tumor clinical trials which are still ongoing.

As especially EGFR and HGFR were highly expressed in pediatric brain tumor cells, these findings provide a rational explanation of the limited efficacy of single targeted treatment in clinical trials. Moreover the successful combination treatments to overcome these resistance mechanisms support the importance for multi targeted therapy in low grade astrocytoma and ependymoma. Although mutations are of great importance in tumorigenesis and tumor progression, specific RTK inhibitors often result in a heterogeneous clinical benefit in various clinical trials as mentioned above. Our study highlights the extensive importance of environmentally present growth factors in developing resistance mechanisms towards RTK inhibitors. It is of great importance to anticipate upon these results for the design of new therapeutic trials with RTK inhibitors.

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Chapter 9

Summary, general discussion and future perspectives

Summary

Brain tumors are the most frequent pediatric tumors and are still the leading cause of cancer morbidity and mortality among children, despite different therapeutic modalities including neurosurgery, chemotherapy and radiation. As angiogenesis plays a crucial role in tumor growth and progression, anti-angiogenic therapy could be suitable in pediatric brain tumors. To obtain insight into the possible therapeutic window for anti-angiogenic therapy in these tumors, the research described in the present thesis analyzed the angiogenic profile, tumor vasculature and growth-factor-driven rescue to angiogenic inhibitors in pediatric brain tumors in the context of adult glioblastoma, as the commonly used model for angiogenesis in brain tumors. Started with a clinical point of view on anti-angiogenic therapy in pediatric brain tumors, more biological tumor characteristics were evaluated in (pediatric) brain tumor tissue, followed by analyses of growth-factor-driven rescue to receptor tyrosine kinase (RTK) inhibitors in pediatric brain tumor cell lines.

In **chapter 2** published clinical trials of anti-angiogenic therapy in pediatric brain tumors were reviewed and an overview of recently started clinical trials was provided. As many of these clinical trials were in the phase I setting and new agents are upcoming, it seems that we are still at the beginning of the development and optimalization of anti-angiogenic therapy in pediatric brain tumors. Although some encouraging results have been described of agents targeting VEGF/VEGFR signaling or the ErbB family, results were not convincing in the presentation of an anti-angiogenic strategy that significantly increases overall survival in pediatric brain tumor patients. This could be due to possible tumor escape mechanisms, including growth-factor-driven tumor resistance. Theoretically, this resistance mechanism could be overcome by multi targeted therapy. Overall, more insight is required to be highly conclusive about the efficacy of anti-angiogenic therapy with currently upcoming potential anti-angiogenic agents in pediatric brain tumors.

Chapter 3 evaluated aspects of the angiogenic profile and tumor vasculature in the context of therapeutic outcome in 62 adult patients with primary glioblastoma as prototype of angiogenesis in brain tumors. Microvessel density, turnover of endothelial and tumor cells, and VEGF-A-D expression were analyzed in tumor slides immunohistochemically stained with respectively Ki67/CD34, cleaved caspase-3/CD34, and VEGF-A-D. The ANGPT-1/ANGPT-2 balance as indicator for vessel stability was determined using Real Time RT-PCR. As VEGF-D was higher expressed in the relatively hypoxic perinecrotic tumor area, a possible role for hypoxia driven VEGF-D was suggested, comparable to VEGF-A. Interestingly, the ANGPT-1/ANGPT-2 balance was identified as a prognostic marker in primary glioblastoma. Angiopoietins are strongly associated with morphological and functional changes in tumor vasculature and are crucial in the process of tumor angiogenesis.[1-3] So the prognostic finding of the ANGPT-1/ANGPT-2 balance supported the need for further studies into the feasibility of anti-angiogenic therapy in primary glioblastoma, with a special focus on the normalization of tumor vasculature.

In the context of glioblastoma, often seen as a prototype for tumor angiogenesis, in **chapter 4** the angiogenic profile and tumor vasculature including vessel maturity status was investigated in 27 pediatric intracranial ependymomas (WHO grade II-III). Despite a low endothelial cell turnover in ependymomas, microvessel density and VEGF-A expression were similar to glioblastomas. The ependymoma vasculature, in general, showed more vessel maturity in terms of basement membrane and pericyte coverage than glioblastoma, although these parameters were overlapping. In theory, in ependymoma vessel normalization through anti-angiogenic agents may be less successful than in glioblastomas. However, as individual ependymomas showed considerable overlap with glioblastoma, ependymomas or a subset of these tumors may benefit from anti-angiogenic therapy as an addition to chemo- or radiotherapy.

Pediatric pilocytic astrocytomas (WHO grade I) showed besides differences in vessel architecture, a surprisingly critical overlap in vessel immaturity/instability and the angiogenic profile with glioblastoma (**chapter 5**). Moreover, a high overlapping pro- and anti-angiogenic VEGF-A isoform ratio was found between both tumors (**chapter 6**), suggesting encouraging possibilities for targeting angiogenesis as a therapeutic strategy, not only in glioblastoma, but also in pilocytic astrocytoma. Differences in vessel architecture could not be exclusively attributed to the VEGF-A isoforms.

Subsequently, **chapter 7** showed less tumor engraftment after anti-VEGF therapy in a newly developed pediatric low grade astrocytoma mouse model. In pediatric low grade astrocytoma VEGFR-2 expression is mainly limited to endothelial cells.[4] So as expected no effects of anti-VEGF were observed on tumor cell viability and proliferation level whereas anti-VEGF seems to work especially on tumor microenvironment. Suggesting again that anti-angiogenic therapy could have a potential role in the therapeutic strategy for children with low grade astrocytoma. Clinical trials did not show that consequent positive results for anti-angiogenic therapy. Recently, some studies described tumor resistance or rescue mechanisms when specific targets are inhibited.
These tumor escape mechanisms have been described not only in VEGF targeted therapies but also during different RTK inhibitors which target both angiogenic and oncogenic pathways.[5] These resistance mechanisms may include alternate routes of kinase pathway activation through kinome reprogramming or upregulation of other RTK and/or their ligands eventually contributing to tumor rescue to the RTK inhibitor with a similar signaling output.[6, 7] RTK ligands such as VEGF, EGF, HGF and PDGF are normally expressed in neuronal development and are expected to be present in the tumor microenvironment. Possibly due to these resistance mechanisms, clinical trials evaluating RTKs show disappointing results with single targeted therapy as reviewed in chapter 2.

Chapter 8 showed growth-factor-driven rescue of mainly EGF, HGF and FGF to RTK inhibitors (sorafenib, dasatinib, canertinib, crizotinib) in pediatric low grade astrocytoma and ependymoma cell lines. These results were found on cell viability level as well as phosphorylation level of downstream targets. These findings highlight the potential signal cross talk in RTKs that are co-expressed on these low grade astrocytoma and ependymoma cells. As the growth-factor-driven rescue could be overcome by addition of the relevant RTK inhibitor, this study is in line with chapter 2 preferring multi targeted therapy in pediatric brain tumors.

General discussion and future perspectives

The angiogenic profile and tumor vasculature in pediatric brain tumors have been analyzed in the context of the highly vascular adult glioblastoma as prototype of angiogenesis. As pediatric intracranial ependymoma and in more extent pilocytic astrocytoma showed a crucial overlap in the angiogenic profile and vessel immaturity/instability with glioblastoma, anti-angiogenic therapy achieving vessel normalization could be a suitable therapeutic strategy in combination with chemotherapy not only in glioblastoma but also in these pediatric brain tumors. However this thesis also highlights a tumor resistance mechanism, including growth-factor-driven rescue, that may arise in response to anti-angiogenic therapy. These tumor resistance mechanisms can be present during different angiogenic strategies, like anti-VEGF therapy to purpose vessel normalization but also during receptor tyrosine kinase (RTK) inhibition targeting both angiogenic and oncogenic pathways.

As results in clinical trials evaluating anti-angiogenic therapy in pediatric brain tumors were not convincingly presenting an angiogenic treatment that significantly increases overall survival, this indeed could possibly be due to growth-factor-driven rescue as one of the tumor escape mechanisms. Concurrently, we showed that growth-factor-driven rescue could be overcome by addition of the relevant RTK inhibitor. To further increase insight into these mechanisms and to test potential angiogenic agents, preclinical analyses *in vivo* or alternative *in ovo* and *ex vivo* models are highly needed. As such, translation of angiogenic agents into clinical trials will be more rational. Moreover by inclusion of a preclinical model simultaneously with a clinical trial, this model can be further optimized. Eventually, a potential preclinical model can be used in future clinical trials to perform prior analyses of tumor tissue derived from stereotactic biopsy, predicting tumor response and identifying the pediatric patients who will respond among the included trial patients.

Pediatric low grade astrocytoma mouse models

Up to now, two spontaneous low grade astrocytoma mouse models have been described: a genetically engineered mouse model of neurofibromatosis-1 developing optic low grade glioma and a mouse model in which a BRAF kinase domain was activated to induce pilocytic astrocytoma.[8, 9] However a xenograft model with human tumor cells may be relevant too for preclinical testing of potential anti-angiogenic agents. So in addition, we started a pediatric low grade astrocytoma xenograft mouse model, in which Res-259 cells (WHO grade II) were orthotopically implanted in NOD-scid IL2Rg^{null} mice. Due to the inconsistently shaped tumor engraftment varying from a massive tumor bulk to tumor engraftment around the brain, analyses of the tumor vasculature including vessel (im)maturity or possible tumor escape mechanisms like upregulation of alternative angiogenic factors or increased invasiveness could not be performed. Moreover, anti-angiogenic response monitoring of radiolabeled bevacizumab using positron emission tomography (PET) was not feasible due to small tumor size. The intracranial tumors were highly 2 mm in diameter while PET visualized subcutaneous tumors can reach diameters of 8 mm.[10] Although preliminary results were derived, this *in vivo* model warrants optimalization before it can be used for further drug testing.

In ovo and *ex vivo* models

An *in ovo* brain tumor model for studying angiogenesis is the chorioallantoic membrane (CAM) of the chicken embryo on which glioblastoma cells were deposited, resulting in tumor growth.[11, 12] It has been suggested that tumor protein expression in the CAM model is comparable to that of an *in vivo* model. Effect of anti-angiogenic agents can be demonstrated on tumor growth and blood vessel development.[12, 13] Especially the possibility to analyze different aspects of the vasculature, including vessel growth and vessel architecture such as vascular density and branching points makes the CAM an elegant model to study angiogenesis. Up to now the CAM model has not yet been used to analyze anti-angiogenic agents in pediatric brain tumors.

More recently, an *ex vivo* 3D brain tumor model has been published in which adult and pediatric glioblastoma cells were cultured till aggregates using a 3D rotary cell culture system (RCCS), originally developed by the National Aeronautics and Space Administration (NASA).[14, 15] This study demonstrated that these 3D RCCS cultures had profound effects on the genetic, epigenetic and metabolic profiles of the cultured glioblastoma cells, suggesting an intermediate phenotype between that of 2D cultures and primary tumors. The macroscopic RCCS aggregates recapitulated the heterogeneous morphology of glioblastoma with a distinct proliferating rim, necrotic core and oxygen tension gradient, allowing drug testing in a more relevant *ex vivo* system. As pediatric low grade astrocytoma cells (Res-186: WHO grade I and Res-259: WHO grade II) could be successfully cultured in this 3D system (personal collaboration), our data can be enriched by this method. Moreover, the RCCS makes it possible to analyze other modes of vessel formation in tumors, including vasculogenic mimicry in which a proportion of RCCS brain tumor aggregates exhibit a vasculogenic phenotype and angiogenic gene expression profile in the absence of any form of co-culture. In addition, the RCCS can be used as a system for maintaining viable explants of primary tumor directly from neurosurgery. This in combination with increased experiences culturing primary brain tumor cells may allow target discovery analyses and drug testing to predict tumor response in the particular patient.

In relatively little time, analyses in both *in ovo* and *ex vivo* models could be performed, as the CAM model takes 1-2 weeks and aggregates in the RCCS can be analyzed within 3 weeks compared with 6 weeks in the *in vivo* mouse model. These models could present an easier and less expensive alternative for *in vivo* models, reducing the use of animals. Ideally, a preclinical model should be as close as possible to the patient's situation to give the best chances to be successful in clinical trials, so taking also crucial aspects into account including the microenvironment and the presence of either an intact or leaky blood brain barrier that could influence the delivery of targeted therapy to brain tumors. A mouse model seems to be more representative for the human situation including brain microenviroment and a systemic blood flow. However the environmental cells are murine and as the low grade astrocytoma cells could only result in tumor engraftment in immune compromised mice, the systemic factor is limited. In the RCCS it is possible to include environmental cells, however if glioblastoma cells were co-cultured with endothelial cells, the aggregates showed less tumor characteristics.[14]

Conclusions

Today, brain tumors are still the leading cause of cancer morbidity and mortality among children. As angiogenesis is highly crucial in tumor growth and progression, this mechanism has been extensively investigated and different anti-angiogenic therapies have been developed. However these studies are limited in pediatric brain tumors. So the present thesis obtained to increase insight into the angiogenic profile, tumor vasculature and growth-factor-driven resistance to angiogenic inhibitors in pediatric brain tumors. This thesis suggested a therapeutic window for anti-angiogenic therapy in these tumors at the one site and at the other site highlighted the possible tumor escape mechanisms that can arise in response to anti-angiogenic therapy. To further increase insight into these mechanisms and to test potential angiogenic agents, preclinical analyses in *ex vivo* and *in vivo* models need to be optimized, making translation into clinical trials more rational. It is advisable to include a preclinical model simultaneously with a clinical trial to contribute to its optimalization and in future clinical trial function as a predictive tumor response model. At the end, more individualized anti-angiogenic therapy will be a worthwhile future perspective in pediatric brain tumors.

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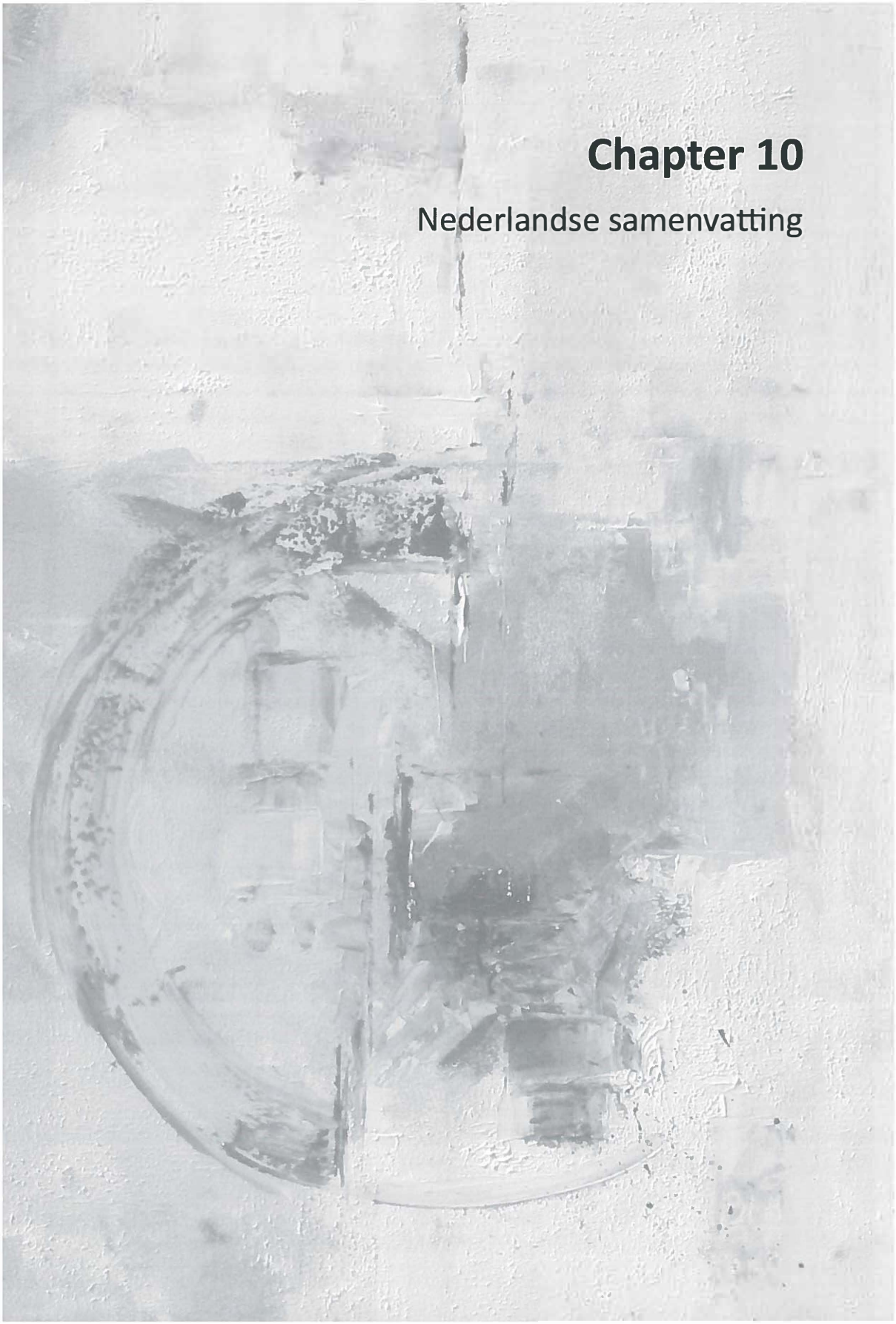
Chapter 10

Nederlandse samenvatting

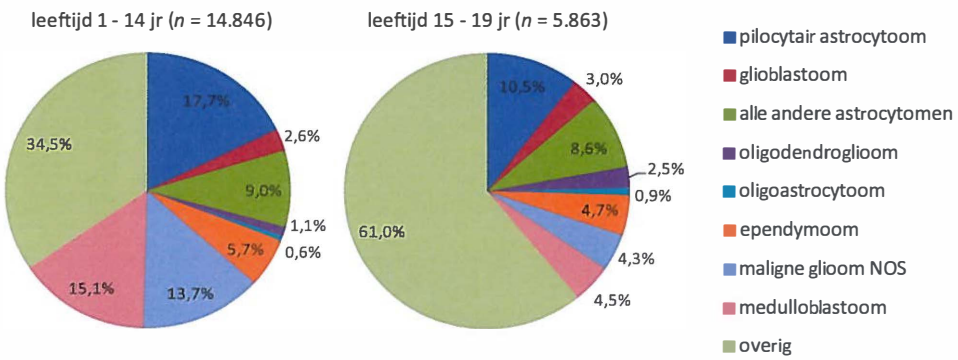


Hersentumoren

Elk jaar wordt in Nederland bij circa 1200 mensen een hersentumor vastgesteld. Hersentumoren beslaan 1,4% van alle soorten kanker bij volwassenen en zijn verantwoordelijk voor 2,3% van de sterftegevallen door kanker. Bij kinderen daarentegen zijn hersentumoren de meeste voorkomende tumoren en ondanks vele behandelingsmogelijkheden vormen hersentumoren nog steeds de grootste doodsoorzaak op de kinderleeftijd door kanker. Bovendien kan ernstige morbiditeit optreden, vooral bij ongunstige tumorlokalisaties met een aanzienlijke kans op chirurgische morbiditeit en nadelige effecten van chemotherapie en bestraling. Zo kunnen neurologische en endocriene restverschijnselen optreden en kan een gestoorde persoonlijke en sociaal-emotionele ontwikkeling plaatsvinden. Op deze wijze kunnen de kinderen die genezen van een hersentumor moeilijkheden ondervinden op maatschappelijk vlak waardoor gezond ouder worden bemoeilijkt wordt. Nieuwe behandelingsstrategieën zijn dus nodig om zowel mortaliteit als morbiditeit terug te dringen en daarmee 'healthy ageing' te bevorderen.

Aangezien angiogenese, oftewel bloedvatnieuwvorming, cruciaal is voor tumorgroei en -progressie, zou remming van angiogenese een geschikte therapie kunnen zijn. Echter in het betreffende onderzoeksveld zijn slechts een beperkt aantal studies gepubliceerd over angiogenese en tumorvasculatuur in kinderhersentumoren ten opzichte van volwassen hersentumoren. Om inzicht te krijgen in de therapeutische mogelijkheden van het remmen van angiogenese in kinderhersentumoren, wordt in dit proefschrift specifiek onderzoek gedaan naar het angiogene profiel in kinderhersentumoren. Analyses van kinderhersentumoren worden vergeleken met het angiogene profiel in het glioblastoom (WHO graad IV), de meest voorkomende hersentumor bij volwassenen. Glioblastomen zijn een van de meest vaatrijke tumoren en worden daarom ook wel gezien als een model voor de bestudering van angiogenese.

Op de kinderleeftijd komen laaggradige astrocytomen, waaronder het pilocytair astrocytoom (WHO graad I) en het diffuse astrocytoom (WHO graad II), het meeste voor, gevolgd door het medulloblastoom (WHO graad IV) en het ependymoom (WHO graad I-III) (Figuur 1).



Figuur 1. Histologische verdeling van tumoren van het centrale zenuwstelsel op de kinderleeftijd.

Angiogenese en anti-angiogene therapie

Angiogenese is van essentieel belang voor de bloedvoorziening van tumoren waarbij voedingstoffen aangevoerd worden en afvalstoffen die gedurende de tumorgroei ontstaan, uitgescheiden kunnen worden. Cruciale factoren in het proces van angiogenese zijn onder andere angiopoietine (ANGPT) en vasculair endotheel groeifactor (VEGF) die via binding aan specifieke tyrosine kinase receptoren, respectievelijk Tie2 en VEGF receptor, angiogenese kunnen reguleren. Ondanks het feit dat tumoren veel bloedvaten bevatten, worden tumoren vaak gekenmerkt door een relatief laag zuurstofgehalte (hypoxie), een zuur milieu en verhoogde interstitiële druk dat te wijten is aan een abnormaal vaatbed met onrijpe en lekke bloedvaten. Deze abnormale tumorvasculatuur kan het transport en/of de verdeling van chemotherapeutica naar en in de tumor negatief beïnvloeden waardoor de tumor zich ondanks de behandeling kan handhaven of zelfs groei kan vertonen.
Oorspronkelijk werd gedacht dat anti-angiogene therapie zou leiden tot remming van nieuwe bloedvatvorming en/of vernietiging van de reeds bestaande vaten waardoor toevoer van voedingsstoffen naar de tumor voorkomen kon worden. In de loop van de tijd zijn ook andere theorieën beschreven over de mogelijke werking van anti-angiogene therapie. Eén van de theorieën gaat in op de eerder beschreven vaatabnormaliteiten in tumoren. Er wordt gesuggereerd dat in tumoren het abnormale vaatbed te wijten is aan een disbalans tussen pro- en anti-angiogene factoren in het voordeel van de pro-angiogene factoren, waaronder VEGF. Door remming van bijvoorbeeld VEGF met anti-angiogene therapie kan de balans hersteld worden, resulterend in vaatnormalisatie met een verbeterde bloedstroom. Op deze wijze kan chemotherapie de tumor beter bereiken en zal radiotherapie in effectiviteit toenemen doordat er sprake is van een hoger zuurstofgehalte in de tumor. Daarnaast wordt beschreven dat anti-angiogene therapie die specifieke receptor tyrosine kinases remmen, de zogenoemde RTK remmers, niet alleen effectief zijn via angiogene routes om de tumor indirect te bestrijden, maar ook direct gericht zijn op de remming van tumorcelgroei. Echter, als reactie op anti-angiogene therapie kunnen tumor resistentiemechanismen optreden waarbij bijvoorbeeld andere groeifactoren hoger tot expressie komen of tumoren een meer invasief karakter laten zien. Bovendien kunnen tumoren via alternatieve routes ontsnappen aan de gegeven therapie.

Samenvatting

Het proefschrift geeft inzicht in de mogelijke therapeutische geschiktheid van anti-angiogene therapie in kinderhersentumoren. Het onderzoek dat in dit proefschrift wordt beschreven, analyseert het angiogene profiel, de tumorvasculatuur en groeifactor gedreven tumor ontsnappings-mechanismen aan receptor tyrosine kinase (RTK) remmers in kinderhersentumoren.

Voordat wordt ingegaan op de biologische aspecten van angiogenese in kinderhersentumoren, geeft **hoofdstuk 2** een overzicht van reeds gepubliceerde klinische studies over anti-angiogene therapie in kinderhersentumoren en recent gestarte klinische trials. Omdat veel van deze klinische trials nog fase I studies betreffen en nieuwe anti-angiogene middelen in opkomst zijn, lijkt het alsof we nog steeds aan het begin staan van de ontwikkeling en optimalisering van anti-angiogene therapie in kinderhersentumoren.

Hoewel soms veelbelovende resultaten worden beschreven van middelen die VEGF/VEGF receptor signalering remmen of gericht zijn op de ErbB familie, blijven overtuigende resultaten van een anti-angiogene strategie die de overleving van kinderen met een hersentumor significant verbeterd uit. Een verklaring hiervoor zouden mogelijke tumor ontsnappingsmechanismen kunnen zijn, zoals groeifactor gedreven tumorresistentie. Theoretisch zou een dergelijke tumorresistentie overtroffen kunnen worden met een behandeling gericht op verschillende groeifactoren en/ of receptoren, oftewel 'multi targeted' therapie. Om daadwerkelijk conclusief te zijn over de effectiviteit van anti-angiogene therapie in kinderhersentumoren is meer onderzoek nodig in de therapeutische werkingsmechanismen en mogelijke tumor ontsnappingsmechanismen zeker met de nu opkomende potentiële anti-angiogene middelen.

Hoofdstuk 3 evalueert aspecten van het angiogene profiel en de tumorvasculatuur gerelateerd aan de overleving in 62 volwassen patiënten met een primair glioblastoom als een angiogenese model in hersentumoren. Vaatdichtheid, turnover van endotheel- en tumorcellen en VEGF-A-D expressie werden geanalyseerd in tumorcoupes die immunohistochemisch gekleurd waren met respectievelijk Ki67/CD34, cleaved caspase-3/CD34 en VEGF-A-D. De ANGPT1/ANGPT2 balans als maat voor vaatstabiliteit werd bepaald met behulp van Real Time RT-PCR. Aangezien VEGF-D hoger tot expressie kwam in het relatief zuurstofarme perinecrotische tumorgebied werd er een mogelijke rol gesuggereerd voor hypoxie geïnduceerde VEGF-D expressie, evenals VEGF-A dat hoger tot expressie komt door hypoxie. Een interessante bevinding was dat de angiopoietine balans werd geïdentificeerd als een prognostische marker in primaire glioblastomen. Angiopoietines zijn sterk geassocieerd met morfologische en functionele veranderingen in de tumorvasculatuur en zijn cruciaal in het proces van tumorangiogenese. De prognostische bevinding van de ANGPT1/ANGPT2 balans steunt de noodzaak voor verder onderzoek naar de geschiktheid van anti-angiogene therapie in primaire glioblastomen, met speciale aandacht voor vaatnormalisatie van de tumorvasculatuur.

In de context van glioblastomen, vaak gezien als een prototype van tumorangiogenese, werd in **hoofdstuk 4** het angiogene profiel en de tumorvasculatuur met vaatmaturiteitsstatus bestudeerd in 27 intracraniële ependymomen bij kinderen (WHO graad II-III). Ondanks een lage turnover van endotheelcellen in ependymomen waren vaatdichtheid en VEGF-A expressie gelijkwaardig aan glioblastomen. Over het algemeen liet de ependymoom vasculatuur minder vaatimmaturiteit zien in termen van basaalmembraan- en pericytenbedekking vergeleken met glioblastomen, hoewel deze parameters wel overlappend waren. In ependymomen zal vaatnormalisatie door anti-angiogene middelen mogelijk minder succesvol zijn dan in glioblastomen. Echter gezien individuele ependymomen wel een aanzienlijke overlap vertoonden met glioblastomen, zouden patiënten met een ependymoom of een subgroep van de patiënten mogelijk wel voordeel hebben van anti-angiogene therapie als een toevoeging op chemo- of radiotherapie.

Pilocytaire astrocytomen bij kinderen (WHO graad I) lieten naast verschillen in vaatarchitectuur, een verrassend essentiële overlap zien in vaatimmaturiteit/-instabiliteit en het angiogene profiel met glioblastomen (**hoofdstuk 5**). Verdere analyse van VEGF isovormen die het proces van angiogenese dusdanig kunnen beïnvloeden met verschillen in vaatarchitectuur tot gevolg, vond plaats in **hoofdstuk 6**. Daarbij werden zowel pro- als anti-angiogene isovormen bestudeerd, waarbij gesuggereerd wordt dat de anti-angiogene isovormen kunnen binden aan anti-angiogene middelen, waaronder bevacizumab (anti-VEGF) en daarmee het effect van dergelijke middelen kunnen remmen. Tumoren met een relatief hoge expressie van pro-angiogene isovormen ten opzichte van anti-angiogene factoren zijn mogelijk gevoeliger voor anti-angiogene therapie. Uit hoofdstuk 6 bleek dat het verschil in vaatarchitectuur tussen pilocytaire astrocytomen en glioblastomen niet alleen toegeschreven kon worden aan de expressie van de verschillende VEGF-A isovormen. In lijn met hoofdstuk 5 werd een opmerkelijk overlappende pro- en anti-angiogene VEGF-A isovorm ratio gevonden tussen beide tumoren in het voordeel van de pro-angiogene isovormen. Deze resultaten geven aan dat er mogelijkheden zijn voor het remmen van angiogenese als therapeutische strategie, niet alleen in glioblastomen, maar ook in pilocytaire astrocytomen.

Tevens liet **hoofdstuk 7** minder tumorgroei zien na behandeling met anti-VEGF in een nieuw ontwikkeld laagggradig astrocytoom muismodel. Omdat in laaggradige astrocytomen, VEGF receptor 2 vooral tot expressie komt in endotheelcellen en niet in tumorcellen werd naar verwachting geen direct effect gezien van anti-VEGF op tumorceloverleving of -proliferatie. Aangezien wel effect werd gemeten in het diermodel lijkt de omgeving van de tumor van groot belang te zijn bij de effectiviteit van anti-VEGF in laaggradige astrocytomen. Ook deze studie suggereert weer dat anti-angiogene therapie een potentiële rol zou kunnen vervullen in de therapeutische strategie voor kinderen met een laaggradig astrocytoom. Klinische trials lieten echter geen eenduidig positief resultaat zien van anti-angiogene therapie. Recente studies beschrijven mogelijke tumor ontsnappingsmechanismen die kunnen optreden indien specifieke angiogene factoren worden geremd.

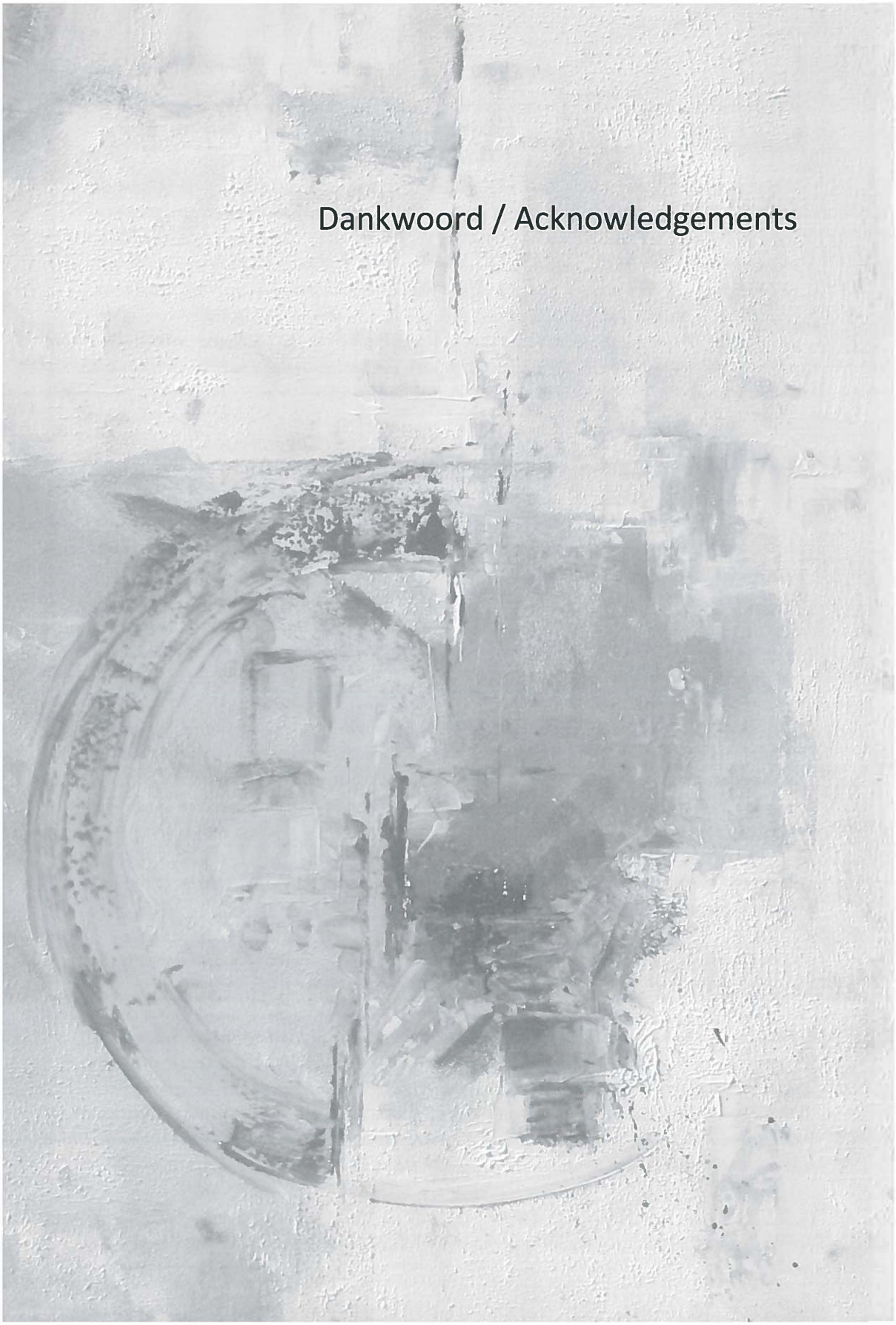
Deze tumor ontsnappingmechanismen worden niet alleen beschreven tijdens remming van VEGF maar ook tijdens verschillende receptor tyrosine kinase (RTK) remmers die zowel angiogene als oncogene routes remmen. Dergelijke resistentiemechanismen kunnen alternatieve routes includeren van kinase pathway activatie door kinoom reprogrammering of upregulatie van andere receptor tyrosine kinases of hun liganden wat uiteindelijk kan bijdragen aan tumor resistentie tegen RTK remmers met eenzelfde signaaloutput. RTK liganden zoals VEGF, epidermale groeifactor (EGF), hepatocytaire groeifactor (HGF) en fibroblast groeifactor (FGF) komen tot expressie in de normale neuronale ontwikkeling en worden verwacht aanwezig te zijn in het tumormilieu. De betreffende resistentiemechisnen zouden een mogelijke verklaring kunnen zijn waarom teleurstellende resultaten worden gezien bij 'single targeted' behandelingen zoals beschreven in hoofdstuk 2.

Hoofdstuk 8 laat groeifactor gedreven tumorresistentie zien van vooral EGF, HGF en FGF bij gebruik van RTK remmers (sorafenib, dasatinib, canertinib, crizotinib) in laaggradige astrocytoom- en ependymoomcellijnen. Deze resultaten worden zowel gevonden op het niveau van tumorceloverleving als op fosforylatieniveau van downstream targets. De bevindingen benadrukken de potentiële signaal cross talk in RTKs die tot co-expressie komen in deze laaggradige astrocytoom- en ependymoomcellijnen. Groeifactor gedreven tumorresistentie kon overtroffen worden met toevoeging van de relevante RTK remmer. Dit pleit voor 'multi targeted' therapie in kinderhersentumoren wat overeenkomt met de bevindingen uit hoofdstuk 2.

Conclusies en toekomstperspectieven

Vandaag de dag vormen hersentumoren nog steeds de nummer één doodsoorzaak bij kinderen met kanker. Omdat angiogenese van groot belang is bij tumorgroei en progressie, is dit mechanisme uitvoerig onderzocht en zijn verschillende anti-angiogene therapieën ontwikkeld. Echter zijn deze studies in kinderen slechts beperkt. Dit proefschrift vergroot het inzicht in het angiogene profiel, de tumorvasculatuur en groeifactor gedreven tumorresistentie bij angiogene remmers in laaggradige astrocytomen en ependymomen. Enerzijds suggereert dit proefschrift een therapeutische geschiktheid voor anti-angiogene therapie in deze tumoren maar anderzijds wordt ook de keerzijde benadrukt, namelijk de mogelijke tumor ontsnappingmechanismen die kunnen optreden als reactie op anti-angiogene therapie. Om het inzicht verder te vergroten in deze mechanismen en om potentiële angiogene middelen te testen, zijn goede preklinische analyses nodig ter optimalisatie, waardoor de vertaling naar klinische trials meer rationeel kan plaatsvinden. Gestreefd zou moeten worden naar een situatie waarin preklinische modellen simultaan lopen met een klinische trial zodat het preklinische model geoptimaliseerd kan worden en mogelijk in toekomstige klinische trials een voorspellende rol kan vervullen. Uiteindelijk zal in de toekomst een meer geïndividualiseerde anti-angiogene therapie zeer waardevol zijn in de behandeling van kinderhersentumoren.

Dankwoord / Acknowledgements



Dankwoord

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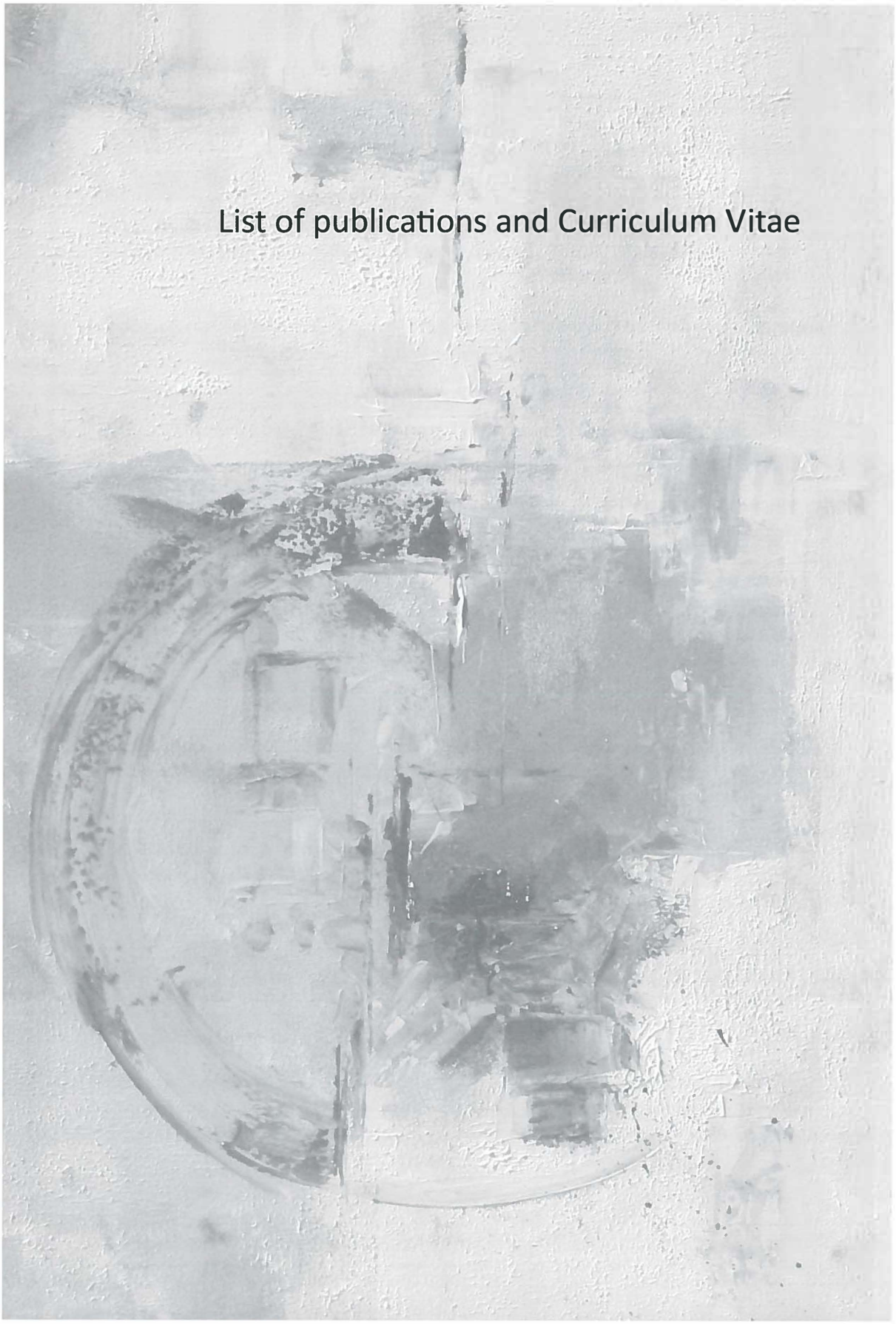

List of publications and Curriculum Vitae

List of publications

Articles

Sie M, den Dunnen WFA, Lourens HJ, Meeuwsen-de Boer TGJ, Scherpen FJG, Kampen KR, Hoving EW, de Bont ESJM. Growth-factor-driven rescue in pediatric low grade astrocytoma and ependymoma. *Mol Cancer Res; manuscript submitted.*

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* These authors contributed equally.

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15th International Student Congress of Medical Sciences, 2008, Groningen, The Netherlands. 'The angiopoietin-1/angiopoietin-2 balance as a prognostic marker in primary glioblastoma multiforme.' (*oral presentation*)

Grants

Jan Kornelis de Cock Stichting Grant 2013. Anti-angiogenic therapy in pediatric brain tumors.

Ubbo Emmius Fonds-Junior Scientific Masterclass (UEF-JSM) Van der Meer-Boerema Stichting Talent Grant 2011. Angiogenesis in pediatric brain tumors.

Jan Kornelis de Cock Stichting Grant 2011. The effect of VEGF/VEGF-receptor targeting on pediatric low grade astrocytoma *in vivo*.

Jan Kornelis de Cock Stichting Grant 2009. Angiogenesis in pilocytic astrocytoma.

Curriculum Vitae

Mariska Sie werd geboren op 18 december 1985 te Groningen. Na het behalen van het VWO-diploma aan het H.N. Werkmancollege te Groningen in 2004, startte zij met de studie Geneeskunde aan de Rijksuniversiteit Groningen. In het tweede jaar van de studie Geneeskunde nam zij deel aan diverse extracurriculaire programmaonderdelen van de Junior Scientific Masterclass. Aansluitend startte zij als derdejaarsstudent in 2006 met het verrichten van wetenschappelijk onderzoek bij de afdeling Kinderoncologie en Pathologie onder begeleiding van prof. dr. E.S.J.M. de Bont en dr. W.F.A. den Dunnen in samenwerking met M. Wagemakers en prof. dr. J.J.A. Mooij van de afdeling Neurochirurgie van het Universitair Medisch Centrum Groningen (UMCG).

In 2009 begon zij met het MD/PhD traject waarbij wetenschappelijk onderzoek onder begeleiding van prof. dr. E.S.J.M. de Bont en dr. W.F.A. den Dunnen gecombineerd werd met coschappen, hetgeen uiteindelijk resulteerde in dit proefschrift. Voor een korte onderzoeksstage ging zij in 2012 naar het Children's Brain Tumour Research Centre, University of Nottingham (United Kingdom) en werd begeleid door prof. dr. R.G. Grundy, dr. R. Rahman en S.J. Smith.

Tijdens haar studie is zij actief geweest in de Jaarvertegenwoordiging van het 2e en 3e jaar van de geneeskundestudievereniging Panacea. Tevens was zij als docent tutor 3 jaar verbonden aan de Faculteit der Medische Wetenschappen van de Rijksuniversiteit Groningen.

In de masterfase van haar studie liep zij de junior coschappen in het UMCG. De senior coschappen werden voltooid in de Isala Klinieken te Zwolle, het Diaconessenhuis Meppel, het Ziekenhuis Bethesda te Hoogeveen en bij de Stichting Regionale GezondheidsDienst te Paramaribo en Lelydorp (Suriname). Zij sloot haar studie af met een semi-arts stage bij de afdeling Keel Neus Oor Heelkunde onder begeleiding van dr. R.A. Feijen en bij de afdeling Neurochirurgie onder begeleiding van prof. dr. R.J.M. Groen in het UMCG. In 2012 ontving zij de artsenbul en in 2013 startte zij als ANIOS bij de afdeling Neurochirurgie van het UMCG.