Biological and molecular heterogeneity of supra- and infratentorial pilocytic astrocytoma: are there therapeutic consequences?

Ronald Nijmeijer

S1825437

Master Biology – Specialization Behaviour and Neurosciences

Supervisor: Prof. dr. U.L.M. Eisel

Molecular Neurobiology – Centre for Behaviour and Neurosciences

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Abstract

Pilocytic astrocytomas are the most commonly found low-grade gliomas in the central nervous system. Although our knowledge of PA biology is further complemented every day, a lot still remains unclear. Therefore, this review aims to provide an overview of the state of the art in the PA research field.

Being the most common driving factor in PA, KIAA1549:BRAF fusion is likely to be key player in constitutive activity of the MAPK and PI3K/mTOR pathways in cerebellar PA but not in noncerebellar PA, which are most commonly found along the optic pathway. These PAs are likely associated with the neurofibromatosis 1 syndrome, or are in fact other low-grade gliomas expressing the BRAF:V600E mutation. The molecular alterations associated with PA do not have confirmed diagnostic or prognostic value, in contrast to tumor location. The involvement of VEGF in PA is gaining interest, although findings are not consistent between different studies. A putative association of the KIAA1549:BRAF protein with VEGFR2 signaling is proposed. However, the need for a robust *in vitro* model of PA is urgent. The development of a PA cell line enables us to extend our understanding of PA biology in order to develop more specific novel targeted therapies.

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1. Introduction

In the pediatric population, low-grade gliomas are the most commonly found tumors in the central nervous system. This group is comprised of WHO-grade I and grade II tumors, of which grade I PA and grade II diffuse fibrillary astrocytoma are the most common subtypes. These tumors are generally indolent and do not undergo malignant transformation. Indeed, PAs follow an indolent course of growth, are non-infiltrative and even spontaneous regression of some PAs has been witnessed (Sievert and Fisher, 2009). Children with PA have a good prognosis: 90% of patients survive over 10 years (Burkhard et al., 2003). However, recurrence, progressive disease and treatment of the disease itself can cause significant morbidity and psychosocial and physical dysfunction (Jones et al., 2012; Ohgaki et al., 2005).

PAs are identified by GFAP staining and the presence of Rosenthal fibers (Louis et al., 2007). Other key histologic characteristics include the biphasic tissue architecture, bland nuclei and eosinophilic granular bodies (Rodriguez et al., 2013). Multiple histological classification markers have been proposed for PA, including the Ki67-proliferation index and different anaplastic features (Rodriguez et al., 2013). However, these candidate markers are not sufficiently conclusive. This is highly undesirable, since it is crucial to distinguish PA from high-grade astrocytomas because the latter requires more aggressive therapy.

It was long thought that inactivation of the tumor suppressor gene NF1 was the only genetic alteration associated with pediatric PA in context of the NF1 tumor predisposition syndrome. However, NF1 loss is not found in sporadic non-NF1-associated PAs. Instead, somatic genetic alterations of the kinase domain of the oncogenic BRAF gene drives oncogenic signaling in PA cells. These alterations include fusion of the BRAF gene with different fusion partners ('BRAF rearrangement') and activating point mutations in the kinase domain of the BRAF gene. The former leads to increased transcription of the fusion gene which contains the BRAF kinase domain, whereas the latter renders the intrinsic kinase activity in the fusion protein to be hyperactive. Further downstream, activation of the Erk1/2 kinase, a crucial component of the MAPK signaling cascade is a feature shared by NF1-associated and sporadic PA (Jones et al., 2012).

PAs are most commonly located at the dorsal-ventral axis of the tentorium, an extension of the dura mater which separates the cerebellum from the ventral occipital lobes. They arise at sites where piloid cells are present, which include most commonly the cerebellum, followed by the thalamic/hypothalamic region, midbrain and brainstem. It is suggested that both sporadic and NF1-associated PAs, occurring at different sites above or beneath the tentorium arise from a monoclonal pool of embryonic glial progenitor cells during foetal or perinatal life (Payton et al., 2011; Gutmann et al., 2013). Differently localized PAs are histologically mostly similar, however, they exhibit clinical and molecular heterogeneity (Belirgen et al., 2012; Sadighi and Slopis, 2013; Stokland et al., 2010).

Although our knowledge of PA biology is further complemented every day, a lot still remains unclear. Attempts on integrating clinical parameters, pathology and genetic alterations are yet to be made. In other pediatric brain tumors, such as medulloblastoma and ependymoma, this approach is proven to be helpful in stratifying tumors into clinically relevant subgroups (Rodriguez et al., 2013; Northcott et al., 2012; Taylor et al., 2012).

Therefore, this review aims to provide an overview of the state of the art in the PA research field and will try to postulate new hypotheses about the most common molecular alterations and their functional implication in PA. Recommendations are given as an attempt to guide research on PA towards an integrated diagnostic and prognostic model of PA.

Research questions

- Which pathways are involved in PA and which mutations are associated with them?
- Do supra- and infratentorial pilocytic astrocytomas show different angiogenic profiles?
- Are there differences in behavior and clinical prognosis between supra- and infratentorial pilocytic astrocytomas and could they be correlated with molecular phenotype?
- What are the therapeutic consequences of different molecular phenotypes in PA?

2. The molecular phenotype of PA

This section provides a brief overview of the most common signaling pathways in PA. The role of the most common BRAF alterations and other oncogenic mechanisms in PA will be reviewed and discussed. Special attention is given to the newly evaluated role of VEGFs in tumor biology, which is associated with the highly vascular nature of PAs. Finally, hypotheses are suggested about the association of BRAF alterations and VEGF-mediated signaling.

2.1. Signalling pathways involved in PA

The subgroups identified in PA are defined by tumor localization and different molecular alterations. Accumulating studies on the molecular background of PA have shown that the functional significance of these alterations converges most commonly on deregulation of the mitogen-activated protein kinase (MAPK-) signaling cascade and the phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/Akt/mTOR-) pathway. Being the most common driving factor in PA, BRAF rearrangement and mutation are likely to be key players in constitutive activity of the MAPK pathway. It is difficult to say whether PAs show altered MAPK activity when compared to its low-grade counterparts, since no such comparison has been done yet. However, it is reported that PAs are a disease that is molecularly and clinically distinct from their grade II counterparts (Korshunov et al., 2009; Marko et al., 2010; Marko and Weil, 2012). It is likely that specific mutations in (one of) these pathways are the underlying cause of the distinct molecular and clinical phenotype of PA.

2.1.1. MAPK pathway

The MAPK signaling cascade is comprised of protein kinases that convey signals from the cell membrane to the nucleus. Functionally, this pathway is involved in cell proliferation, survival and differentiation, but also in cortical neurogenesis and development of multiple brain areas, including the midbrain and cerebellum (Samuels et al., 2008; Sato et al., 2004; Tatevossian et al. 2010). Especially the latter aspects are of importance with respect to the development of different types of cancer. Indeed, the MAPK pathway is known to be deregulated in third of all cancers (Dhillon et al., 2007). Neuronal stem and precursor cells, rather than post-mitotic glial cells, are involved in glial tumorigenesis. Taken together with the predisposed cerebellar localization of PA, this indicates the association of PA pathogenesis with the MAPK pathway (Jones et al., 2012).

The protein kinases comprising this pathway extend the signal by phosphorylating their target proteins, i.e. they attach a phosphate group to their target protein at specific serine, threonine or tyrosine amino acids (Tatevossian et al., 2011). Phosphorylation at these sites often activates the protein which then executes a similar function. These 'molecular switches' thrust the signal initiated by cell membrane-bound receptors from the cell surface to the nucleus, where target transcription factors are activated. Mutations in either membrane receptor, intermediate of the signaling cascade

or other associated factors, lead to constitutive pathway activity, which has been shown to be a highly oncogenic mechanism (Jones et al., 2012; Tatevossian et al., 2011).

2.1.2. PI3K/mTOR pathway

The phosphatidylinositol 3-kinase/protein kinase-B/mammalian target of rapamycin (PI3K/Akt/mTOR-) pathway is crucial in various cellular processes, including cell proliferation, differentiation, metabolism, apoptosis and survival (Polivka and Janku, 2014). In multiple inherited diseases, germ line mutations in this pathway have been shown to be associated with different types of cancers (Yuan and Cantley, 2008). In the developing brain, neuronal survival is a crucial property in different neuronal cell types and is mediated by a properly functioning PI3K/mTOR pathway (Brunet et al., 2001; Reed, 2000). In addition, besides MAPK signaling, activation of PI3K/mTOR may mediate increased proliferative activity (Rodriguez et al., 2011). Therefore, constitutive PI3K/mTOR activity is an important mechanism underlying tumorigenesis in the pediatric brain. Regarding the unresolved questions that remain in understanding the nature of PAs, better understanding of the interactions between components of this this pathway with MAPK components might be useful.

Activation of mTOR is initiated *via* two different routes, which converge on activation of PI3K. This kinase is phosphorylated by receptor tyrosine kinases (RTKs) either directly, or indirectly by the intermediate G-protein coupled Ras-kinase. PI3K then translocates to the plasma membrane where it induces phosphorylation of PIP2 to PIP3. Subsequently, PIP3-mediated activated Akt inhibits the inhibitory TSC1/TSC2 complex of mTOR by phosphorylation of TSC2 (Manning and Cantley, 2003), thereby indirectly inducing mTOR signaling. Increased mTOR activity leads to increased expression of its effectors, eukaryotic initiation factors (eIFs) and p70S6 kinase (S6K) (Engelman et al., 2006). Ultimately, phosphorylated eIFs and S6 proteins translocate to the nucleus where they initiate mTOR-mediated gene expression.

2.2. BRAF alterations in PA

Unlike any other gene, BRAF (v-raf murine sarcoma viral oncogene homolog B1) has been a recent subject of great interest in the neuro-oncology field (Horbinski, 2013). Its role in pediatric low-grade glioma is well established too, featuring the association between sporadic PA and BRAF alterations (Horbinski, 2013; Rodriguez et al., 2013; Sadighi and Slopis, 2013). BRAF is a serine/threonine–specific protein kinase in the MAPK pathway, which is activated downstream of Ras. BRAF activates tyrosine/threonine-specific MEK1/2, which in turn activates the mitogen-activated protein kinase (ERK) to trigger the oncogenic process (Forshew et al., 2009). Aberrant activity of BRAF, caused by rearrangement or mutation of the *BRAF* gene, is the major oncogenic driver in PA. However, to what extent the functional consequences of different BRAF alterations overlap and differ is poorly understood. The following provides an overview of different BRAF alterations, including genetic fusion and mutation (Fig 1).

2.2.1. KIAA1549:BRAF fusion

A tandem duplication of a region approximately 2Mb in size at chromosome 7q34, that spans from KIAA1459 to BRAF, was found to give rise to the KIAA1549:BRAF fusion gene (Jones et al., 2008; Nagase et al., 2000; Pfister et al., 2008; Sievert et al., 2009). The tandem duplication transposes the 5′ half of KIAA1549 with the 3′ half of BRAF, resulting in a fusion gene expressed under the KIAA1549 promoter (Sievert et al., 2009). Remarkably, KIAA1549:BRAF fusion appears to be highly specific for PA (Horbinski, 2013). However it is not completely understood which mechanisms underlie this specificity. Two studies have identified hallmark characteristic elements of non-homologous end-joining (NHEJ) DNA repair processes that are present at the fusion sites (Cin et al., 2011; Lawson et al., 2011). The high specificity of 7q34 duplication might be explained by this underlying oncogenic recombination mechanism (Jones et al., 2009).

Despite chromosomal similarity amongst tumors carrying the KIAA1549:BRAF fusion, the nucleotide positions of the break points vary, resulting in an heterogeneous exonic composition of the fusion gene (Ichimura et al., 2012). The 3 most common variants of the fusion gene are KIAA1549 exons 1–16 and BRAF exons 9–18 (K:B16_9) (60% of all KIAA1549-BRAF); K:B15_9 (30%) and K:B16_11 (10%). Still, these fusion variants are likely to be functionally similar, since KIAA1549:BRAF fusion leads to loss of the BRAF N-terminal, harboring the BRAF autoregulatory domain, but not the BRAF C-terminal, which contains the BRAF kinase domain (Jones et al., 2012). Increased transcription rates of the KIAA1549:BRAF fusion gene cause higher levels of active BRAF kinase and lead ultimately to constitutive activation of the MAPK pathway (Horbinski et al., 2013; Ichimura et al., 2012). In line with this is the finding of increased phosphorylated ERK protein in tumors with the tandem duplication at 7q34 (Sievert et al.,2009).

The KIAA1549 gene encodes a protein containing two trans-membrane domains, which are retained in the KIAA1594:BRAF fusion products (Horbinski, 2012). Therefore, as the fusion partner of BRAF, the KIAA1549 gene might not only provide an efficient promoter for the fusion gene, but also renders the fusion products functionally relevant in mediating intracellular signaling.

2.2.2. FAM131B:BRAF fusion

A ~2,5 Mb-interstitial deletion of the BRAF N-terminal inhibitory domain transposes the BRAF N-half to the C-half of the FAM131B gene, resulting in constitutively activated BRAF kinase. Similar to KIAA1549:BRAF, the FAM131B:BRAF fusion protein constitutively phosphorylates MEK and is able to transform NIH-3T3 cells (Cin et al., 2011).

Remarkably, very few exons from the FAM131B gene are fused with BRAF. This might imply that this gene provides a promoter and stable carrier, but not a functional segment for the BRAF kinase domain (Jones et al., 2012).

2.2.3. SRGAP3:RAF1 fusion

A tandem duplication at chromosome 3p25 gives rise to fusion of exons1-12 of the SRGAP3 gene to exons 10-17 of the RAF1 gene. The fusion protein contains a major part of the *RAF1* C-terminal amino acids, including the entire RAF1 kinase domain (Jones et al., 2009). The fusion protein constitutively phosphorylates MEK, leading to activated MAPK signaling (Forshew et al., 2009).

SRGAP3 is known for its negative regulation of Rac1, a Rho-GTPase mediating growth and survival of tumor cells through regulation of signaling components downstream of Ras, which include activation of Erk and PI3K/Akt (Davis et al., 2013; Endris et al., 2011; Wang et al., 2010; reviewed by Vega and Ridley, 2008). Increased Rac1 activity causes hyper activation of Erk and Akt (Wang et al., 2010) which leads to cellular hyper proliferation. A similar effect was seen when Rac1 activity was blocked by overexpression of SRGAP3 (Yang et al., 2006).

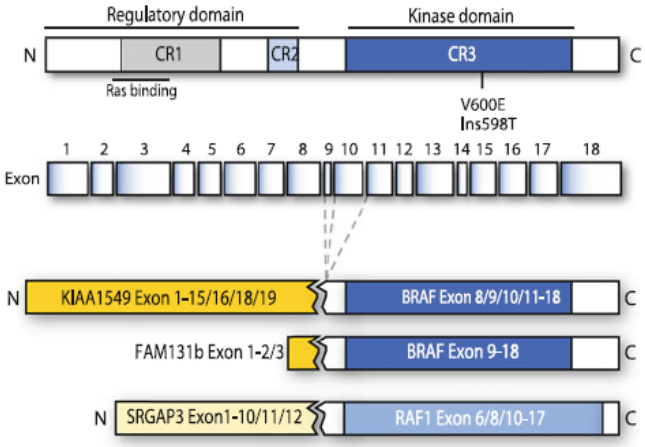

Fig 1. Schematic representation of the genomic and protein structure of human BRAF and the fusion products detected in PA. The gene fusions with their fusion partners and break points in all cases result in a loss of the autoregulatory domain. This, as well as the V600E point mutation and the Ins598T insertion in the full length protein, results in constitutive activity of the kinase domain. CR1-3 conserved region 1–3. *Adapted from: Jones et al. (2012) Cell Mol Life Sci (69)*

2.2.4. RAF fusion partners and their functional implications

RAF components in aforementioned gene fusions are clearly important in constitutive MAPK activation and the function of the retained kinase domain is well understood. Although both genetic rearrangement and mutation of RAF kinase segments lead to similar constitutive activity of the fusion protein, resulting tumors do show differences in behavior, localization and prevalence. This might imply that the effects of different RAF alterations are not completely similar (Horbinski, 2013; Tatevossian et al., 2011). Therefore, it might be worthwhile to address the possible roles of the most common RAF fusion partners, including KIAA1549, FAM131B and SRGAP3, in fusion protein localization and functioning.

The SRGAP3 protein is widely expressed in the developing brain and is in particular involved in axonal guidance during neurogenesis (Bacon et al., 2009; Endris et al., 2011). It contains a Fes/Cip4 homology (FCH) domain, which is retained in the SRGAP3:RAF1 fusion protein. Proteins containing FCH domains are involved in cytoskeletal organization, vesicular transport and endocytosis. It is suggested that this FCH domain localizes SRGAP3 to the cell membrane where it initiates a signaling cascade which ultimately leads to inhibition of the Rac1 signaling complex. Indeed, presence of the SRGAP3:RAF1 fusion gene implies loss of SRGAP3-mediated Rac1

inhibition and a gain of function of the RAF1 kinase domain, leading to dual hyper phosphorylation of MEK1/2. However, it is still unclear whether the functional property of the FCH domain is retained in the SRGAP3:RAF1 fusion protein.

It is unclear which function(s) the protein family 131 serves in both normal and tumor cells. However, a putative highly-conserved function might be suggested based on a number of characteristics featured by the aforementioned FAM131B protein. First, the FAM131B gene is expressed throughout all main tissues in the human body, with relative highest expression in the brain. Second, FAM131B gene orthologs have been identified in other species, of which the mouse shows highest similarity. These findings imply a putative housekeeping role of FAM131B, especially in the brain.

Additionally, a study done *in vitro* in monkey fibroblast-like cells, showed that the FAM131B protein is subjected to N-myristoylation, an irreversible post-translational lipid modification at the N-terminal, which enables protein-membrane and protein-protein interations (Suzuki et al., 2010). N-myristoylated proteins are associated with regulation of cellular structure and function, and likely comprise components of key signaling pathways, such as protein kinases, guanine nucleotide-binding proteins, phosphatases and Ca^{2+}-binding proteins (Suzuki et al., 2010). Activation states of Ras GTPases are under influence of such proteins (Ahearn et al., 2011). Therefore, FAM131B might possess a putative role in regulating the Ras molecular switch and thereby control activity of multiple signaling pathways, including MAPK and mTOR. This is in line with the notion that only a small portion of the FAM131B N-terminal is retained in the FAM131B:BRAF fusion protein. N-myristoylation of the FAM131B terminal might localize the fusion protein to the cell membrane where it induces Ras activity. Together with constitutive RAF kinase activity, one might suggest this putative dual oncogenic role of the FAM131B:BRAF fusion protein. Interestingly, both the N-myristoylation motif of FAM131B and the transmembrane domains of KIAA1549 are thus retained in their fusion with BRAF, which suggests a preference of BRAF for membrane-localized fusion partners.

The *KIAA1549* gene is expressed in a variety of tissues, including the brain, with variety in expression in all tissues (Nagase et al., 2012). A putative role of *KIAA1549* in the human brain might be hypothesized from the localization of exon 1, containing two start codons, within a CpG island (Jones et al., 2008). CpG islands are relatively rare and their occurrence in or near transcription start sites might be explained by evolutionary selection for regulation of conserved gene expression, such as housekeeping gene expression (Krinner et al., 2014). Also, the *KIAA1549* gene has orthologs in different animal species, which show non-tissue specific expression pattern of the *KIAA1549* gene similar to in human. Taken together, these findings strongly suggest a housekeeping function of the KIAA1549 gene in the human brain. However, the unidentified KIAA1549 protein has not been associated with putative cellular processes involved in cell maintenance, survival or growth.

In sum, it appears that the fusion partners of BRAF do not only provide passive elements of the resulting protein, but are rather proactive in regulating the intracellular localization of the protein towards the cell membrane. However, it remains to be

determined which function(s) these proteins execute and how these are associated with PA biology.

2.2.5. BRAF mutation

Besides oncogenic fusion events involving BRAF, other mutations in this gene have been reported to induce MAPK activity. An activating point mutation in the BRAF gene at codon 600, where a thymidine to adenine change at nucleotide 1799 results in an amino acid change from valine to glutamic acid (BRAF:V600E), is the most common oncogenic genetic mutation in this gene (Wan et al., 2004).

Within the BRAF activation segment, residue 600 and adjacent residues comprise a hydrophobic domain, which blocks hydrophilic amides in the ATP-binding pocket and thereby preventing ATP to kinase binding (Davies et al., 2002). These residues stabilize the inactive conformation of the BRAF kinase domain. Mutation in any of these residues destabilizes this inactive conformation and promotes the active state of the BRAF kinase domain (Wang et al., 2004). The switch from a hydrophobic to a hydrophilic molecule (valine and glutamic acid respectively) within this hydrophobic domain might therefore mimic the presence of an ATP molecule. This gain-of-function mutation constitutively activates the kinase domain, leading to hyper phosphorylation of MEK1/2.

In vivo experiments have shown that ectopic expression of the activated BRAF:V600E kinase domain in mice carrying xenografts of neuro progenitor cells is sufficient to induce PA (Gronych et al., 2011). It has been suggested that the intrinsic stability of the BRAF:V600E is lower than wild type BRAF. This could explain the absence of tumors in mice with melanoma xenografts overexpressing BRAF:V600E. Support of the heat-shock-protein 90 (hsp90) system of activated BRAF protein in this melanoma mouse model is likely required for tumor survival (Grbovic et al., 2006). This is in line with the observation that multiple hsp's are upregulated in melanoma and correlate with clinical parameters (Shipp et al., 2013). Therefore, hsp90 inhibition might be an interesting treatment strategy in not only melanoma, but other tumor types too.

Additionally, insertion of 3 base pairs coding for threonine in the region of codon 600 (BRAF:598insT) results in a similar mutation (Jones et al., 2009). The BRAF:598insT mutation constitutively activates BRAF kinase activity similar to the BRAF:V600E mutation and causes a more-than-sixfold increase in kinase activity in vitro (Eisenhardt et al., 2010; reviewed by Rodriguez et al., 2013). This mutation is only sparsely found in PA.

2.3. NF1

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder with a worldwide incidence of 1 per 2500 to 3000 individuals. This tumor-predisposition syndrome is caused by a mutation in the NF1 gene, located on chromosome 17q11.2 (Viskochil et al., 1990). The NF1 gene codes for neurofibromin, a cytoplasmatic, 2818 amino acids containing protein. Neurofibrimin is widely expressed throughout different tissues of the central nervous system, including neurons and astrocytes. It is believed to be involved in cortical development and astrocyte growth, through influencing signaling pathways (Andersen et al., 1993; Gutmann, 2002; Gutmann et al., 1991; Zhu et al., 2001).

Neurofibromin inhibits RAS by catalyzing the conversion of GTP-bound RAS to its GDP-bound form (Andersen et al., 1993; Basu et al., 1992). Loss of neurofibromin increases RAS activity and induces downstream activity of both the MAPK and the mTOR pathway (Johannessen et al., 2005; Sandsmark et al., 2007). The oncogenic mechanism of NF1-related PA reflects a 'second hit model', in which germline inactivation of a NF1 allele predisposed patients to tumor development. Somatic inactivation of the still functioning remaining NF1 allele is the second hit which leads to tumor development (Gutmann et al., 2000; Kluwe et al., 2001; Knudson, 1985). Different mouse models of PA have confirmed the central role NF1 plays in a major subset of PA (Jones et al., 2012; Rodriguez et al., 2013).

The spatial distribution of NF1-associated PAs might be explained by the relatively high proportion of stromal cells in these tumors (Gutmann et al., 2013). These cells promote tumor formation by paracrine signaling, such as CXCL12-mediated cAMP release. CXCL12 acts as a suppressor of cAMP signaling and is highly expressed in the optic pathway in young children. Increased expression of CXCL12 reduces cAMP which was shown to cause OPG growth in mice heterozygous for NF1. Increased cAMP levels inhibited this process (Warrington et al., 2010).

In the context of NF1, the importance of input derived from the tumor microenvironment is increasingly accepted. However, the role of the tumor micro-environment in sporadic PAs remains to be described.

2.5. Angiogenesis in PA

Angiogenesis is a strongly regulated process in which novel vasculature is formed from existing vessels in the brain. In the development of brain tumors, angiogenesis is a key mechanism in the highly vascular phenotype of PA. Typically, the tumor microvasculature displays vessel immaturity, instability, impaired perfusion and increased permeability (Welsh and Welsh, 2013). Reversal of these vascular abnormalities ('normalization') by treatment targeting angiogenesis restores vascular function but inhibits tumor growth (Jain, 2005; Welsh and Welsh, 2013). Highly vascular tumors such as gliomas, show overexpression of pro-angiogenic factors, including growth factor receptors and angiopoietins (Dasgupta and Haas-Kogan, 2013). Aberrant activity of MAPK and PI3K/mTOR pathways is associated with mutation in these genes and is regarded as a major deregulated mechanism underlying abnormal tumor vascularity.

PAs are highly vascular tumors and display remarkable angiogenic features. PA vasculature contains less vessels compared to its high-grade counterpart, glioblastoma multiforme (GBM), but these vessels are wider (Mustafa et al., 2013; Sie et al., 2010). In contrast to this difference in vascular architecture, a remarkable overlap in vessel immaturity and instability in these tumors has been reported (Sie et al., 2010). Aberrant angiogenic activity is a central oncogenic mechanism in GBM and therefore a better understanding of angiogenesis in PA is necessary for the search of future treatment strategies.

Expression of genes involved in vascular proliferation in PA has been identified, although data is not consistent and expression levels differ between studies (Marko and Weil, 2012). Amongst the genes reported in PA are those encoding receptor tyrosine kinases (RTKs), including Kit and vascular endothelial growth factor receptor 2 (VEGFR2, also known as KDR) (Puputti et al., 2010; Sikkema et al., 2011). Both receptors are predominantly found in tumor endothelial cells (ECs), although the exact role these proteins play in PA development is still poorly understood.

Relatively little is known about the vasculature of pediatric low-grade gliomas. Therefore, the following will focus on the 'angiogenic profile' of PA (Sie et al., 2010). The most common VEGF family member and its receptor, VEGFA and VEGFR2 respectively, will be introduced. Its downstream components and their functional association with the distinct angiogenic properties of PA will be discussed.

2.5.1. VEGFR2 in tumor development

In line with other RTKs, such as epidermal growth-factor receptors (EGFRs) or the platelet-derived growth-factor receptors (PDGFRs), VEGFRs display receptor dimerization upon binding to its ligand and activation of the intracellular tyrosine kinase (Olsson et al., 2006). Induced tyrosine phosphorylation recruits signaling protein complexes which transduce the VEGF signal *via* cascades involved in multiple growth-factor associated cellular processes including cell migration, survival and proliferation. The co-receptors of VEGFRs are neuropilins (referred to as NRP1 and NRP2) and increase ligand-receptor interaction between VEGFs and VEGFRs (Klagsbrun et al., 2002). Despite the absence of an intracellular signaling complex associated with these receptors, NRPs are known to be important mediators of tumor induction and growth independent from VEGFRs (Goel and Mercurio, 2013; Prud'homme and Glinka, 2012). However, it is not known whether these mechanisms involve VEGF.

VEGFR2 binds VEGFA, the most common member of a large family of growth factors that also includes VEGFB,-C,-D and placental growth factor. These ligands differ in expression pattern, receptor binding and functional significance (Ellis and Hicklin, 2008; Goel and Mercurio, 2013). VEGFR2 expression is restricted to the vasculature and is devoted to induce an angiogenic response in endothelial cells *via* multiple routes. This response includes proliferation and survival, cell migration and vascular permeability as the most prominent angiogenic mechanisms (Welsh and Welsh, 2013).

VEGFR2-dependent proliferation and survival of ECs is mediated by the Raf/Mek/Erk and PI3K/mTOR pathways. Indirect Erk activation is initiated by VEGFR2-mediated activation of phospholipase Cγ (PLCγ), which in turn promotes Raf/Mek/Erk phosphorylation and activation (Takahashi et al., 2001). Alternatively, conflicting evidence exists for binding of growth receptor bound 2 (Grb2) and the adaptor Shc directly to VEGFR2, which might activate Raf *via* Ras and subsequently further downstream Erk1/2. Being the end-point in a large number of different signaling cascades, Erk1/2 serves a key role in increased EC proliferation. Akt, another key downstream component of VEGFR2, not only mediates migratory and survival-

associated cellular responses in ECs (Fujio and Walsh, 1999; Jiang and Liu, 2009), but it also regulates vascular permeability in angiogenesis via different routes. VEGFA-independent Akt activation leads to increased mTOR-mediated NO expression, a critical component in regulating vessel diameter. It has been shown that sustained Akt signaling in ECs lead to formation of widened, hyperpermeable vessels (Phung et al., 2006). VEGFA-dependent activation of eNOS, which catalyzes NO production, occurs via different adaptor and intermediate proteins conveying the VEGFR2-induced signal, including via direct Akt phosphorylation (Fulton et al., 1999).

However, VEGFA not only affects functioning of endothelial and tumor cells, but in addition targets other cells in the tumor microenvironment (Goel and Mercurio, 2013). NRP1 receptors have been identified on immune cells, including stromal fibroblasts, possibly indicating a mediating role of VEGFA in host-tumor interactions (Hansen et al., 2012; Yacoob et al., 2012). Increased expression of VEGFA in these cells might therefore pose a putative novel mechanism of tumor growth and maintenance, facilitated by non-tumor cells in the microenvironment. Indeed, tumor angiogenesis has been associated with a pro-angiogenic immunophenotype, which features increases VEGFA expression in stromal cells (Allavena et al., 2008).

An emerging trend in literature promotes VEGF not only as a key regulator of angiogenesis and endothelial vascular function, but merely as a crucial factor in tumorigenesis and tumor survival. As Goel and Mercurio (2013) noted, autocrine VEGF signalling in tumor cells can be a crucial feature of tumor initiation and is an important mediator of cancer stem cell functioning and self-renewal. This trend forces us to reconsider the roles of VEGF in tumor biology. A well-acknowledged, important mechanism in regulation of signaling pathways of different RTKs and their co-receptors is endocytosis and associated membrane trafficking (Goel and Mercurio, 2013).

Upon ligand binding, RTKs undergo clathrin-dependent endocytosis, followed by being transported to the endosomal compartment ('trafficking') from which further signaling is initiated. In RTK signaling, the endosomal system is crucial for achieving signaling specificity and specifically early endosomes are likely to comprise a major signal relay center for RTKs (Sigismund et al., 2012; Goel and Mercurio, 2013). This aspect of RTK signaling might provide novel insights in the role of VEGF in PA. We might be able to translate the current knowledge about VEGF signaling in different types of cancer into hypotheses on how VEGF-mediated signaling in PA influences tumor vasculature and behavior.

2.5.2. VEGFR2 in PA

Studies have shown that VEGF signaling could be regulated at the point of vesicle trafficking (Goel and Mercurio, 2013; Welsh and Welsh, 2013). NRPs may play a crucial role in this process, as has been shown in glioma stem cells (Hamerlik et al., 2012). In cancer stem cells, studies done *in vivo* and *in vitro*, emphasize the significance of autocrine VEGFA signalling mediated by NRP1 and VEGFR2. Also, paracrine VEGFA

signaling from tumor cells to adjacent endothelial cells has been suggested to be a driving mechanism behind angiogenesis (Goel and Mercurio, 2013).

Activation of VEGFR2 by VEGFA induces auto-phosphorylation of tyrosine residues located within the intracellular domain of the receptor. This leads to clathrin-dependent endocytotic internalization of the entire receptor, which is mediated at least partially by ephrin-B2 (Lampugnani et al., 2006; Sawamiphak et al., 2010). VEGFR2 endocytosis might be a crucial step in the activation of Erk1/2 in vascular endothelial cells (Gourlaouen et al., 2013). In this study, inhibitors of VEGFR2 endocytosis blocked activation of MEK1/2 and Erk1/2, but had no effect on VEGFR2 and Raf activity. This suggests that internalization is not required for activation of the receptor and its direct downstream components, but it affects more distal components. Indeed, absence of proteins mediating intracellular trafficking of VEGFR2 compromises downstream activation of Erk1/2 and Akt (Lanahan et al., 2010). High rates of VEGF uptake and VEGF receptor endocytosis have been shown *in vivo* in mouse retinal endothelial cells at the 'angiogenic front', i.e. where relatively immature endothelial cells show increased migratory and mitogenic behavior. Both processes are opposed in more stable and mature vessels (Nakayama et al., 2013). The vasculature of PA is markedly unstable and immature, which might imply VEGFR2 endocytosis as a central mechanism in PA growth.

Suppression of Ras and its downstream components, including BRAF, PI3K and Akt has been shown to attenuate expression of pro-angiogenic factors (Goel et al., 2011). These components comprise a major part of the downstream signaling network of VEGFA. Reversely, knock-in of the BRAF:V600E allele in human epithelial cells promoted their angiogenic response (Bottos et al., 2012). It is likely that constitutive kinase activity in RAF fusion proteins has similar implications in PA vasculature.

As mentioned earlier, transmembrane components of both KIAA1549 and FAM131B proteins are retained in the fusion protein with BRAF, which suggests localization of the fusion protein to the cell membrane. Also, the FCH domain in the SRGAP3 protein is retained in the fusion protein with RAF1, which enables this protein to localize towards the cell membrane. These findings suggest a functional role of the fusion proteins in PA which might be associated with oncogenic mechanisms exerted by proteins localized at the cell membrane, such as RTK internalization leading to pro-angiogenic and mitogenic signaling.

The fusion protein might associate with proteins localized at the cell membrane which are important in RTK-induced intracellular signaling. For example, Grb2, in addition to its key function in signaling through Ras, has a major regulatory role at the initial steps of EGFR internalization, where it mediates the binding of the ubiquitin ligase Cbl, which subsequently induces the endocytotic process (Jiang et al., 2003). Given the overlap in attributions of endocytosis between EGFR and VEGFR2 internalization, Grb2 might play a similar role in VEGFR2-mediated signaling.

To elaborate on RTK internalization, one might suggest a functional role of BRAF fusion proteins in direct Ras activation by RTKs. Internalization of an RTK after binding

its ligand might be mediated by direct binding of the Grb2 adaptor complex. Subsequent association with the membrane-bound KIAA1549:BRAF protein might induce constitutive activity of the BRAF kinase domain, which leads to increased Ras-independent MEK1/2 phosphorylation. Activated Erk1/2 then stimulates mTOR activation which leads to increased VEGFA transcription. Another hypothetical model features membrane-bound KIAA1549:BRAF which constitutively phosphorylates autophosphorylation site Y1175 in the intracellular RTK tyrosine domain. This leads to sustained PLC-γ activation by phosphorylated Tyr1175 and constitutive Ras/Raf activation.

Conclusions

- The MAPK and PI3K/mTOR pathways are involved in PA pathogenesis.
- The KIAA1549:BRAF fusion gene causes constitutive MAPK activation and is the major oncogenic driver of sporadic PA.
- BRAF fusion proteins retain elements that are associated with localization towards the cell membrane.

3. Supra- versus infratentorial PA: exploiting molecular differences in diagnosis and prognosis

Mounting data provide us with more and more knowledge about the underlying molecular background of PA. Although current knowledge does not allow us yet to stratify the differential of PAs in molecularly distinct subgroups, it is possible to distinct PAs based on their localization. Furthermore, studies have attempted to associate PA location with molecular features. The next section compared supra- and infratentorial PAs at levels of localization, gene expression and most common BRAF alteration featured in PA. Finally, a brief overview is given about the methods involved in detection of BRAF alterations and how they might aid in stratifying PAs.

3.1. General gene expression profiles

In contrast to emerging evidence of differential gene expression in PA compared to other low-grade gliomas, other studies report conflicting data on different gene expression profiles of differently located PAs. Homozygous p16 deletion was found to be more common in supratentorial PAs (Jacob et al., 2009). Loss of this OIS- checkpoint protein might attribute to the more aggressive nature of non-cerebellar PA. However, other studies did not report similar findings (Belirgen et al., 2012; Marko and Weil, 2012).

The only gene showing consistent increased expression in supratentorial PAs is *LHX2*, which is involved in cellular proliferation and forebrain development (Mascelli et al., 2013). This gene has been proposed to play a key role in platelet-derived growth factor (PDGF-) mediated signaling to support blood vessel functionality in breast cancer (Kuzmanov et al., 2014). Expression of this gene might affect vascularity in differently localized PA, and might thereby influence their clinical behavior. However, more data is needed to confirm at least the differential expression of LHX2 in PA.

Although differences in molecular background and biological behavior between supra- and infratentorial PAs are reported, there is still debate in the clinical practice on the complicated diagnosis of PA and the limited options in clinical prognosis. Different biological features of PA have been proposed, each with their pros and cons.

3.2. Tumor location

It was shown that location of the tumor correlates with progression-free survival (PFS). Patients with cerebellar PA had better prognosis than patients with chiasmatic/hypothalamic tumors (Belirgen et al., 2012). This might be attributed to the fact that these tumors are difficult to completely resect, which leads to recurrence with worse prognosis than before. However, even among tumors that were completely resected, PFS differed between supra- and infratentorial locations, so it is possible that other factors, such as BRAF alterations play a role in the clinical outcome.

3.3. Tumor behavior

The role of the BRAF oncogene is well-acknowledged in PA. Altered regulation of BRAF activity is associated with multiple cancers, including most pediatric low-grade gliomas. Given the notion of BRAF being a rather powerful oncogene together with the relative benign nature of PA, this association raises an interesting question as to how BRAF activation leads to such indolent tumors such as PA. However, aberrant MAPK activity is known to initially render cells oncogenic, followed by 'burning out' the tumor, a process termed oncogene-induced senescence (OIS). This phenomenon may play a role in indolent PAs (Jacob et al., 2011). These authors described a general *in vitro* mRNA expression profile for OIS in two PA cohorts. Using human neurospheres overexpressing BRAF, Raabe et al. (2011) found in mice initial tumor proliferation, followed by growth arrest. These hallmark studies further appointed the p16 protein as being a crucial checkpoint for OIS. Loss of p16 saves cancer cells from OIS and is associated with clinically worse outcome in PA (Jacob et al., 2011; Raabe et al., 2011; Rodriguez et al., 2013). Although the details of OIS as putative mechanism in indolent PA remain to be fully determined, the model of oncogene-induced senescence is gaining evidence and is more and more recognized (Dimauro and David, 2010; Horbinski et al., 2013).

Alternatively, PAs might behave more aggressively in rare cases, which is accompanied by expression of multiple anaplastic features. Horbinski and colleagues reported that PAs with BRAF:V600E mutations showed an increased risk of progression, compared with BRAF fusion cases (2012). This is in line with a study in malignant pediatric astrocytomas, which had the BRAF:V600E mutation but not BRAF rearrangement (Nicolaides et al., 2011). Additionally, NF1-patients with PA show overall lower progression-free survival (PFS) than sporadic PA patients (Sadighi and Slopis, 2013), which again might be attributed to the extent of resection possible.

3.4. KIAA1549:BRAF fusion

Infratentorial sporadic PAs occur usually in the cerebellum. These tumors harbor frequent chromosomal aberrations in the region of the BRAF gene which are almost exclusive to sporadic PAs (Lawson et al., 2010; Marco and Weil, 2012). The anatomic location of sporadic PAs appears to correlate with their underlying molecular abnormalities (Rodriguez et al., 2013; Sharma et al., 2007). BRAF gene rearrangements, and in particular KIAA1549:BRAF fusion, are found in about 70% of PAs, which are most commonly infratentorially localized (Horbinski et al., 2013). Also, the sparsely reported SRGAP3:RAF1 and FAM131B:BRAF fusions were found in the cerebellum (Cin et al., 2011; Forshew et al., 2009). It was thought that this fusion was restricted to PA, but recent work suggest otherwise by identifying BRAF fusions in other low-grade gliomas (Hawkins et al., 2011; Jones et al., 2012; Lin et al., 2012). However, these cases might actually represent misdiagnosed PAs, since diagnosis based on histological features is shown to be difficult with these tumors.

Interestingly, KIAA1549:BRAF fusions are expressed in infratentorial PAs at levels that are almost similar as those of the endogenous KIAA1549 gene. This suggests that the fusion protein is transcribed under the KIAA1549 promoter in a region-specific

manner (Lin et al., 2012). It is likely that BRAF rearrangements are oncogenic in specific cell types in the cerebellar area whereas it does not lead to tumor formation in cells residing in the supratentorial regions, which suggests a role of the local cellular environment.

In addition to the ongoing debate about the diagnostic value of the KIAA1549:BRAF fusion, its value in predicting the clinical course of growth and outcome is not better appreciated either. One study included a group of pediatric patients with non-cerebellar, non-NF1 associated and surgically inoperable PA and found that supratentorial PAs with BRAF fusion behave as typical grade I PAs, whereas PAs without BRAF fusion are more likely to behave like grade II diffuse astrocytomas. The fusion positive group had better clinical outcome (Hawkins et al., 2011). A similar trend was seen in PAs in the cerebellum (Horbinski et al., 2010). However, other studies did not find a correlation between KIAA1549:BRAF fusion and increased progression-free survival of PA patients (Lin et al., 2012; Horbinski et al., 2010; Horbinski et al., 2012). Overall, the KIAA1549:BRAF fusion might not be the desired diagnostic and prognostic marker as previously thought.

3.5. BRAF mutation and NF1

3.5.1. NF1

In general, one-third of tumors in the optic pathway are PAs, of which 10% is associated with NF1 (Listernick et al., 2007; Sadighi and Slopis, 2013). Loss of the neurofibromin gene is almost never reported in sporadic PAs and NF1-associated and sporadic PAs show different gene expression patterns (Rodriguez et al., 2013). Anterior localization of these tumors is associated with less severe clinical phenotype compared to posterior PAs with hypothalamic involvement (Louis et al., 2007). These tumors are usually indolent, show low progression rates compared to sporadic optic pathway gliomas (OPGs) and might even exhibit spontaneous regression (Sadighi and Slopis, 2013). Patients with optic pathway PAs should be tested for NF1 and children with NF1 should conversely be examined for presence of optic pathway gliomas (Hernáiz Driever et al., 2010; Sadighi and Slopis, 2013).

3.5.2. BRAF:V600E

In addition of NF1-associated PAs, there has been described a significant association between BRAF:V600E expression and supratentorial localization in PA (Schindler et al., 2011). However, the frequency of BRAF mutations in PA is much lower than BRAF fusion and therefore, data on BRAF:V600E mutations and outcome are very sparse and it remains difficult to confirm its supratentorial distribution (Rodriguez et al., 2013; Schindler et al., 2011). Although no histological differences between BRAF fusion and mutation exist in PA and no significant association between these alterations and PA localization is consistently reported, the data to date indicate that there is likely a stratification in localization of BRAF alterations in PA.

The BRAF:V600E mutation is known to be non-specific to any low-grade glioma (Dougherty et al., 2010; Schindler et al., 2011). However, it appears to be more associated with other tumors in the PA differential and also with higher-grade gliomas (Hawkins et al., 2011; Horbinski, 2012; Schiffmann et al., 2010). Therefore, the presence of a BRAF:V600E mutation suggests the tumor diagnosed being less likely a PA.

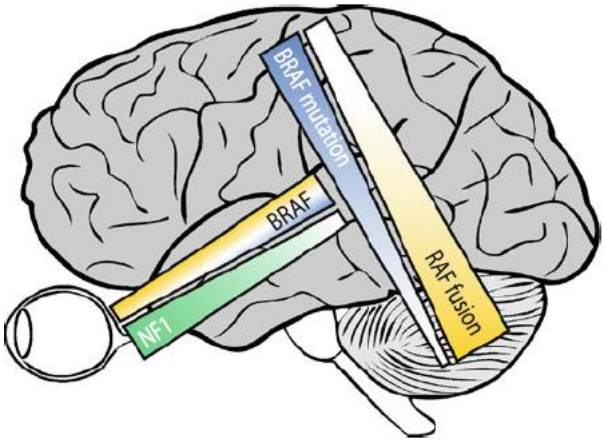

Fig 2. MAPK pathway alterations occur at different places in the brain. NF1 loss is mostly seen in optic pathway gliomas, but also in other areas. RAF activation occurs at different locations, with fusions mostly found in cerebellar PA and mutation occurring in supratentorial PA. *Adapted from: Jones et al. (2012) Cell Mol Life Sci (69)*

Presence of a BRAF:V600E mutation suggests a shorter PFS than BRAF fusion, which is likely due to the cerebellar localization of BRAF fusion, where surgical resection of tumors is less difficult than in non-cerebellar areas (Horbinski et al., 2012). There is no conclusive evidence for appointing either KIAA1549:BRAF fusion of BRAF:V600E mutation as prognostic marker of favorable or unfavorable clinical outcome respectively. Instead, the literature suggests that tumor location is by far the most accurate predictor of clinical outcome in PA (Horbinski, 2013).

3.6. Detection of BRAF fusion and mutation

Several strategies enable detection of BRAF fusion genes and transcripts, including FISH, array-based comparative genomic hybridization (array-CGH) and specialized RT-PCR for analysis of both fusion breakpoints and transcripts. A major advantage shared by all strategies is that they allow the use of formalin-fixed paraffin-embedded (FFPE) tissue in absence of fresh tissue, which is of the greatest practical usefulness.

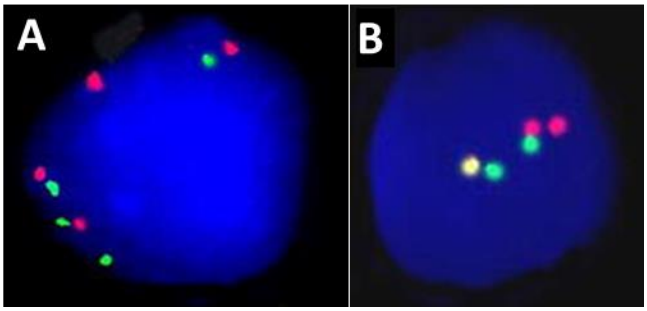

Fig 3. Examples of a FISH analysis for the BRAF:KIAA1549 fusion probe. A) Astrocytoma grade II with polysomic gain of 7q34 exhibits signals for KIAA1549 (in green) and BRAF (in red) without evidence for fusion. **B**) PA exhibiting tandem duplication of BRAF and KIAA1549 resulting in fusion of these loci (yellow signal). *Adapted from: Korshunov et al. (2009) Acta Neuropathol (118)*

In a FISH assay using two-colored probes, a normal cell should show two signals, e.g. green and red (representing BRAF and KIAA1549 respectively), while a cell with KIAA1549:BRAF fusion should show a major yellow signal, corresponding to fusion of green and red signals

(Horbinski, 2012). BRAF FISH can detect all BRAF fusion variants, including FAM131B:BRAF, but it cannot distinguish between those variants. Naturally, this method will not suffice in diagnosing patients when future mounting experimental and clinical data might suggest a differential in MAPK activation caused by different BRAF fusion variants.

Detection of BRAF alterations occurs naturally in pre-determined genomic areas. Consequently, additional genetic events playing significant roles in PA pathology, such as loss of p16, is neglected (Rodriguez et al., 2013). Array-CGH overcomes this limitation since it covers the entire genomic sequence. In this assay, fluorescently-labeled DNA from both tumor and normal tissue is co-hybridized with mapped DNA sequences. A dominant color associated with tumor or normal DNA indicates respectively a gain or loss in tumor tissue. Using this approach, the 7q34 amplification was identified. Despite its whole-genome coverage, array-CGH is not suitable for direct identification of fusion transcripts.

Analysis of BRAF fusion breakpoints and transcripts using RT-PCR is highly specific by using probes flanking breakpoint sites. Due to different sizes of fusion transcripts, it is rather easy to detect them in a gel. However, despite high specificity for detecting fusion breakpoints, high-quality cDNA is required for PCR, which is not provided by FFPE samples. Interestingly, pyrosequencing, an alternative form of RT-PCR, was shown to reach over 90% sensitivity in detecting BRAF CNV's in FFPE tissue (Setty et al., 2011). This technique might be proven very useful in molecular diagnosis of PA.

To summarize, there is no standard approach to detect BRAF fusion in PA. Although there is good correlation between BRAF FISH and RT-PCR breakpoint analysis (Hawkins et al., 2011), the amount of tissue available might be the most important factor. With sufficient (FFPE) tissue available, the optimal method is likely RT-PCR aimed at detecting breakpoints or fusion transcripts, since specificity and sensitivity obtained could be over 90% (Horbinski, 2013; Jones et al., 2008; Tian et al., 2011). However, spatial resolution is lacking, in contrast to FISH which presents correlating subregions with a matched hematoxylin and eosin section (Horbinski, 2013). Also, very few tissue is required for FISH analysis, although manual labor required is rather intensive.

In contrast to BRAF fusions, detection of BRAF mutations, including V600E is rather simple. Sequencing and hybridization assays, including pyrosequencing, on frozen and FFPE tissue have been standardized and show high specificity and sensitivity (Ihle et al,. 2014; Rodriguez et al., 2013). Additionally, a novel monoclonal antibody specific for the BRAF:V600E protein has been developed and showed almost perfect correlation with BRAF direct sequencing in papillary thyroid carcinoma and melanoma (Capper et al., 2011). Despite inter-study variation in staining intensity and frequently reported false-negatives in frozen or damaged tissue (Horbinski, 2013), this approach might be a valuable tool in future diagnostics.

3.7. VEGFA and VEGFR2

Although gene and protein expression of VEGFA and VEGFR2 has been described in PA, the spatial distribution of VEGFA expression in differently localized PA is still largely unknown, in contrast to GBM or other high-grade astrocytomas (Sikkema et al., 2011). In general, no significant relation between different PA localizations and VEGFA expression pattern has yet been found (Belirgen et al., 2012). However, in optic pathway gliomas, Bartels et al. (2006) stressed the importance of angiogenesis in low grade gliomas. Microvessel density was suggested to be the best prognostic marker of progression-free survival in low grade glioma.

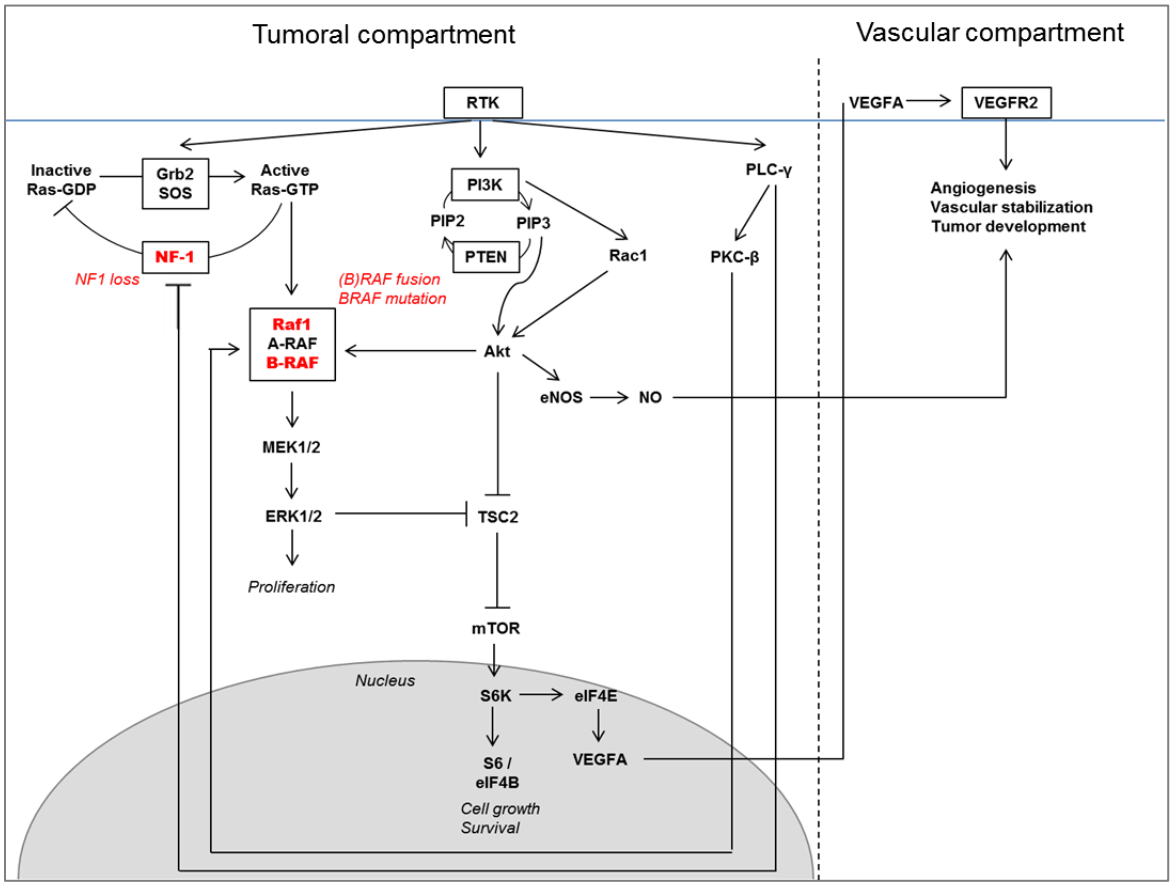

Fig 4. Overview of signaling pathways involved in PA. In the tumoral compartment, MAPK and mTOR signaling components are downstream of different RTKs. Mutations in the MAPK pathway, i.e. loss of NF1 and fusion or mutation of the (B)RAF gene causes up regulation of Erk and increased mTOR activity. Increased VEGFA transcription directly affects the vascular compartment by binding VEGFR2, which subsequently induces an angiogenic response.

In PA, VEGFA expression was found to be mainly restrained to the tumor endothelial area, which is in contrast with high-grade gliomas which show increased expression in the tumor cells (Sikkema et al., 2011). This implies a two-compartment model, in which the tumor compartment is distinct from the vascular compartment. It might be that up regulation of oncogenic signaling pathways in the former compartment leads to increased activity in the latter compartment. It has been found that cerebellar

PAs show higher vessel immaturity than supratentorial PAs, as reflected by increased VEGFA expression. This finding might be supported by the different molecular alterations underlying supra- and infratentoral PAs.

Conclusions

- There is no consistent evidence for differential gene expression profiles in differently localized PA.
- BRAF fusion and mutation are not exclusive to PA and do not predict clinical course.
- Data of VEGFA and VEGFR2 in PA are sparse and do not show a significant relation with PA localization.

4. Treatment

The preferred treatment in pediatric PA patients is surgical resection of PAs in surgically accessible areas such as the posterior fossa, localized in the cerebellar area. However, tumors residing in brain areas that are rather inaccessible are subtotally resected, which often leads to tumor recurrence and sometimes progression to a higher-grade tumor. Radiotherapy or chemotherapy is the first line of treatment for these tumors. Patients undergoing these treatments often experience severe side effects. Novel treatment strategies targeting components of the MAPK pathway and its related pathways, including PI3K/Akt/mTOR and VEGFA/VEGFR2, using small molecular kinase inhibitors are under current investigation. This section provides an overview of the most recent developments in the therapeutic field.

4.1. Inhibiting the MAPK pathway: from Ras to Erk

A study done *in vitro* showed that both silencing BRAF and inhibition of MEK1/2 blocked proliferation of cultured low-grade astrocytoma cells (Pfister et al., 2008). In PA, the KIAA1549:BRAF protein is an evident drug target. However, no study has reported successful inhibition of this protein and it remains unknown whether targeting BRAF fusions is feasible. However, BRAF fusion and mutation are generally not present in NF1-associated PA and therefore targeting Ras activation, essential in both sporadic and syndromic PA, might be proven useful. However, clinical trials with farnestyltransferase inhibitors, which interfere with the interaction between Ras and the cell membrane, have not shown therapeutic response in patients with NF1-associated plexiform neurofibromas (Widemann et al., 2006). It appears that targeting components downstream of this 'hub' might be proven more efficient.

The prevalence of the BRAF:V600E mutation in different human cancers necessitated the design of specific inhibitors that down regulate MAPK activity. The development of agents targeting BRAF:V600E and the downstream components MEK1/2 and ERK1/2 in cancers other than pediatric low-grade gliomas, has provided promising results, which might be relevant in PA (Dasgupta and Haas-Kogan, 2013).

Specific BRAF:V600E inhibitors include vemurafenib and dabrafenib, which show great efficacy in phase III clinical trials with BRAF:V600E mutated metastatic melanoma and malignant astrocytoma (Chapman et al., 2011; Hauschildt et al., 2012; Patrawala et al., 2012). These drugs have not been investigated in pre-clinical models of low-grade astrocytomas yet. However, their potential use in treatment of pediatric glioma is becoming more likely, as a case report on the therapeutic effect of vemurafenib on childhood ganglioglioma reflects (Rush et al., 2013; Kilday et al., 2014).

Pre-clinical data in *in vivo* murine models of pediatric gliomas suggest a therapeutic role of BRAF:V600E specific inhibitors (Dasgupta and Haas-Kogan, 2013). Treatment with PLX4720 (the pre-clinical research analog of vemurafenib) decreased tumor growth and increased overall survival in mice bearing BRAF:V600E mutant xenografts of high-grade astrocytomas (Nicolaides et al., 2011). Importantly, when translating this work to clinical pediatric low-grade gliomas, it is important to remind

that, with exception of the Rush study (2013), there is very little experience using dabrafenib or vemurafenib in children. Also, the efficacy of specific BRAF:V600E inhibitors in PAs with the KIAA1549:BRAF fusion is unknown (Dasgupta and Haas-Kogan, 2013).

Clinical trials with MEK inhibitors, such as selumetinib, have shown preliminary encouraging results in a variety of cancers and are ongoing (Rusconi et al., 2012). Interestingly, pre-clinical validation of selumetinib drug in PA- cell lines provides the first step towards clinical trials in children with low-grade gliomas (Kolb et al., 2010).

At the bottom of the MAPK signaling cascade poses is Erk a crucial putative therapeutic target, since activity of multiple oncogenic pathways also converge on this component. Pre-clinical models have confirmed its central role in oncogenic mutations. In mouse neural progenitor cells, the pro-proliferative effect of a BRAF:V600E construct is attenuated by sorafenib, a kinase inhibitor targeting Erk (Gronych et al., 2011). Treatment with sorafenib improved survival of pediatric patients with renal cell carcinoma and hepatocellular carcinoma (Kleir et al., 2010). Clinical studies of sorafenib in pediatric recurrent low-grade gliomas are currently ongoing (Sadighi and Slopis, 2013).

4.2. Inhibiting mTOR

The archetypical low-grade glioma associated with mTOR activation is the subependymal giant cell astrocytoma (SEGA) in patients with the tuberous sclerosis complex (TSC) (Kilday et al., 2014). TSC is an autosomal dominant disorder in which germline mutation in two tumor suppressor genes - TSC1 (hamartin) or TSC2 (tuberin) – leads to formation of benign neoplasms in different organs, including the brain (Chan et al., 2004; Kilday et al., 2014). In a study in neural stem cells, Kaul et al. (2012) found that mTOR activation is a feature shared by both sporadic and NF1-associated low-grade gliomas. The KIAA1549:BRAF protein activates mTOR by MEK-dependent tuberin inactivation, which induces tumorigenesis as in SEGAs. NF1 loss leads to TORC2-induced Akt and mTOR activation (Dasgupta and Haas-Kogan, 2013). The PI3K/Akt/mTOR pathway is up regulated in a variety of cancers. Therefore, mTOR might be an interesting drug target in PA.

Rapamycin inhibits mTOR, hence its name, and its analogs temsirolimus and everolimus are currently used in the clinic. Patients with TSC-associated SEGA showed regression upon oral treatment with rapamycin (Franz et al., 2006). The study of Krueger et al. (2010) reported significant long-term therapeutic responses in children with SEGA after treatment with everolimus. This was confirmed in a larger international study (Franz et al., 2013). These results suggest that mTOR inhibition is useful in other low-grade gliomas, including PA.

However, studies done in PA with everolimus yield inconsistent results, with conflicting reports on therapeutic responses in children with low-grade astrocytomas that differ between sporadic and NF1-associated PA (Kilday et al., 2014).

It is clear that identifying novel molecular biomarkers that predict therapeutic response is becoming vitally important. As drugs targeting the MAPK and AKT/mTOR pathways are tested, these markers will be as important as the drugs themselves.

4.3. Upstream targeting: anti-angiogenic agents

The RTK family is comprised of receptors, including VEGFRs, that are upstream drivers of MAPK and PI3k/Akt/mTOR pathways. They are involved in different cellular processes, of which angiogenic signaling is of special interest in PA, as these tumors are highly vascular. Agents inhibiting angiogenic signaling include monoclonal antibodies that bind growth factor ligands and inhibitors that target the tyrosine kinase domain (Kilday et al., 2014). The mostly evaluated anti-angiogenic agents are the anti-VEGFA monoclonal antibody bevacizumab and the RTK inhibitors sorafenib and sunitinib.

The efficacy of bevacizumab has been shown in different types of cancer (as reviewed by Ellis and Hicklin, 2008). In pediatric patients with low-grade gliomas, the mean therapeutic response shows high variation across studies. In general, the effect of bevacizumab on 2-year progression-free survival was not better than conventional treatment strategies (Kilday et al., 2014). Neither sunitinib nor sorafenib had significant therapeutic effect in children with low-grade gliomas (Dubois et al., 2011; Dasgupta and Haas-Kogan, 2013). It is important to note that sorafenib and sunitinib are specific, but not selective small molecule inhibitors, i.e. these 'multi-kinase' inhibitors bind to ATP pockets not only in the VEGFR2, but also other kinases (Ellis and Hicklin, 2008). Other less-specific antiangiogenic agents for treatment of pediatric low-grade astrocytomas are in development and the first reports of the therapeutic value of these drugs are awaiting (Kilday et al., 2014).

4.4. Treatment resistance

Targeting the MAPK or PI3K/Akt/mTOR pathway occurs mostly by inhibiting one component, such as BRAF, MEK or mTOR. Although PA oftenly is regarded as a 'single-pathway' disease, findings in other malignancies suggest that targeting a single component might be proven unsuccessful in the end (Kilday et al., 2014).

Resistance to BRAF: V600E inhibitors has been reported in V600E mutated melanomas and different colon cancers, which showed disease progression after initial therapeutic response (Jang and Atkins, 2013). Additional pathways, such as PI3K/Akt/mTOR and RTK-mediated signaling cascades might provide an escape route from the inhibitor for the tumor cells, thereby contributing to treatment resistance.

Indeed, treatment with sorafenib, a kinase inhibitor targeting BRAF, VEGFR, PDGFR and c-KIT, in recurrent pediatric low-grade gliomas showed significant early progression rates. This might indicate downstream paradoxical Erk1/2 activation. (Karajannis et al., 2011). Also, resistance to vemurafenib analogues and subsequent growth activation in low-grade glioma cell lines expressing the BRAF:KIAA1549 fusion have been observed in vitro (Lang et al., 2012; Sievert et al., 2013). These findings suggest that combined therapy, including agents that simultaneously target multiple components of different pathways might provide a solution to treatment resistance. Indeed, combinations of BRAF, MEK, and PI3K/Akt/mTOR inhibitors have shown to

overcome resistance to the BRAF inhibitors vemurafenib and dabrafenib in melanoma cell lines (Greger et al., 2012). It remains undetermined if such therapies are useful in PA, but these findings are encouraging.

Conclusions

- Targeting BRAF:V600E has therapeutic effect in different types of cancers and *in vitro* models indicate that these might be of clinical use in PA.
- Inhibition of MEK and ERK has therapeutic effect *in vitro* whereas this remains to be determined in the clinic.
- Targeting mTOR appears to have different clinical outcome in sporadic and NF1-associated PA.
- Conventional anti-angiogenic treatment strategies do not significantly improve clinical outcome in PA.
- Combined treatment targeting multiple pathways at once could overcome therapy resistance in PA.
- Identification of novel biomarkers that predict clinical outcome are becoming highly important in the development of more efficient and specialized treatment strategies.

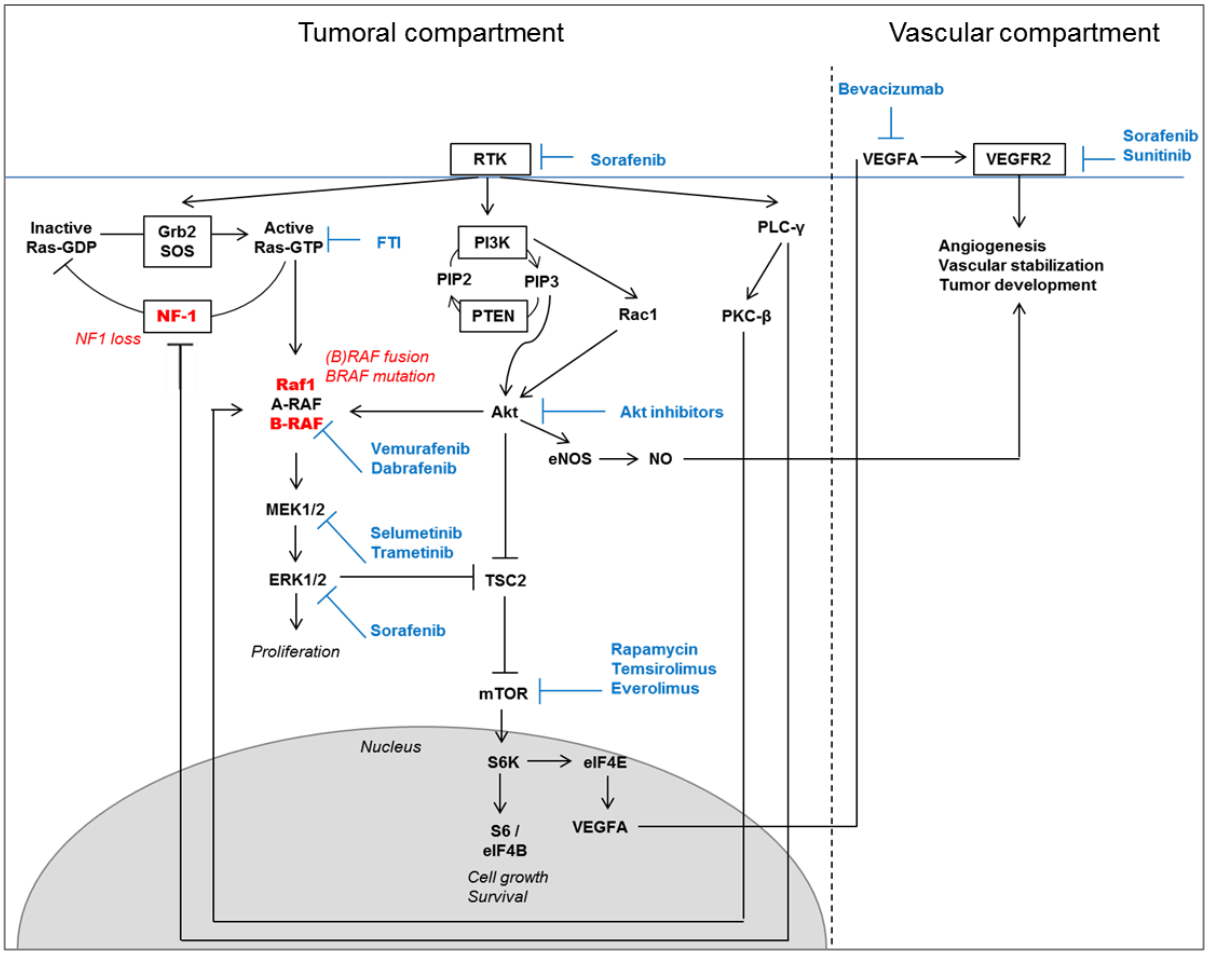

Fig 5. Inhibitors in signaling pathways involved in PA. Components of the MAPK and PI3K/mTOR pathways are known therapeutic targets of kinase inhibitors (depicted in blue). Also in the vasculature, drugs inhibiting VEGFA and VEGFR2 are known.

5. Discussion

- Which pathways are involved in PA and which mutations are associated with them?
- Do supra- and infratentorial pilocytic astrocytomas show different angiogenic profiles?
- Are there differences in behavior and clinical prognosis between supra- and infratentorial pilocytic astrocytomas and could they be correlated with molecular phenotype?
- What are the therapeutic consequences of different molecular phenotypes in PA?

Although PA is one of the most common pediatric tumors in the CNS, our understanding of the biology behind the disease has only recently started to emerge. Up regulation of the MAPK MEK/ERK and PI3K/mTOR pathways is consistently reported and is the major oncogenic driver in PA. Specific mutations in these pathways are associated with the molecular and clinical phenotype of PA.

BRAF fusions and mutations show different spatial expression patterns in sporadic PA, whereas NF1-associated PAs are almost exclusively arising in the optic pathway. The diagnostic value of BRAF fusion, mutation and NF1 loss cannot however decisively distinct PA from other low-grade gliomas. Furthermore, the prognostic value of these molecular alterations pale in comparison to tumor location, although this is most likely due to other factors than a different molecular phenotype. It is remarkable that no significant association between either tumor location or molecular signature and tumor behavior and prognosis exists. This appears to be a vicious cycle; since no clear PA diagnosis could be done, studies investigating the molecular basis of PA most likely include by accident a number of non-PA low-grade gliomas in their sample. Their different biology naturally reduces significance of molecular differences found, and so forth.

It becomes clear that VEGF is more relevant in the biology of virtually all cancers and the highly vascular nature of PA suggests likewise. Differential VEGFA expression is thought to be the cause of the difference in vessel architecture found in PA. However, to what extent BRAF alterations affect VEGFA expression and vessel status is not fully understood yet.

Very little is known about the fusion partners of the BRAF gene. BRAF fusion proteins most likely are localized to the cell membrane and might associate with membrane-bound receptors or other proteins. However, to what extent the functional consequences of KIAA1549, FAM131B and SRGAP3 in fusion protein localization and functioning overlap and differ remains poorly understood. The putative association between KIAA1549:BRAF and RTKs and increased VEGFA expression as a result, might explain the association between the highly vascular nature of PA and up regulated MAPK and PI3K/mTOR signaling.

Besides surgical resection, there is a limited number of therapeutic options available for PA. Drugs blocking components of the MAPK and PI3K/mTOR pathways have been reported to have small therapeutic effect in pediatric gliomas, whereas anti-angiogenic agents used for treatment of pediatric gliomas show little to no clinical effect in PA. The molecular phenotype of different PAs might attribute to this problem.

In summary, the molecular alterations underlying PA are known, but their effects further downstream are not fully understood. There is a discrepancy in molecular phenotype and to a lesser extent in behavior between PAs localized differently, although these differences cannot be used in clinical practice yet.

Future directions

The current means of PA diagnosis are not conclusive and therefore a more specific methodology is required. In contrast to the BRAF:V600E antibody, a selective KIAA1549:BRAF monoclonal antibody could be excluding diffuse astrocytomas in histological diagnosis, thereby being of more value in PA.

In contrast to other childhood brain tumors, there is no standardized *in vitro* model for PA. A cell line representing most features of typical sporadic PA, including expression of KIAA1549:BRAF fusion and high levels of VEGFA, enables research on BRAF fusions, their relationship with putative membrane-bound components and the design of novel treatment strategies. An abstract mentioned the development of a glioma cell line expressing the KIAA1549:BRAF fusion, although it is not known whether this represents the full nature of PA or another type of pediatric low-grade astrocytoma (Lang et al., 2012). In another PA-like cell line, the effects of combined BRAF/mTOR inhibition were evaluated in murine subcutaneous xenografts (Dasgupta et al., 2013).

In line with this, is the development of a conditional transgenic mouse strain in which increased expression of KIAA1549:BRAF resulted in increased proliferation of neural progenitor cells (Kaul et al., 2013). It would be interesting to develop an intracranial xenograft model with either neural progenitor cells or newly developed PA-like cell lines and investigate not only potential therapeutic agents, but also more fundamental issues that are not fully understood. These might include the effect of the cellular microenvironment on tumor development in sporadic PA, as its importance in NF1-associated PA has been emphasized and the apparent crosstalk between BRAF and PI3K/mTOR signaling, which might be the underlying mechanism of treatment resistance.

To conclude, a better understanding of the molecular biology of PA is warranted. As our knowledge grows and changes, our methods of investigation must change with it. Future research must follow both courses for finding better biological markers for diagnosis and prognosis in order to develop more specific novel targeted therapies.

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