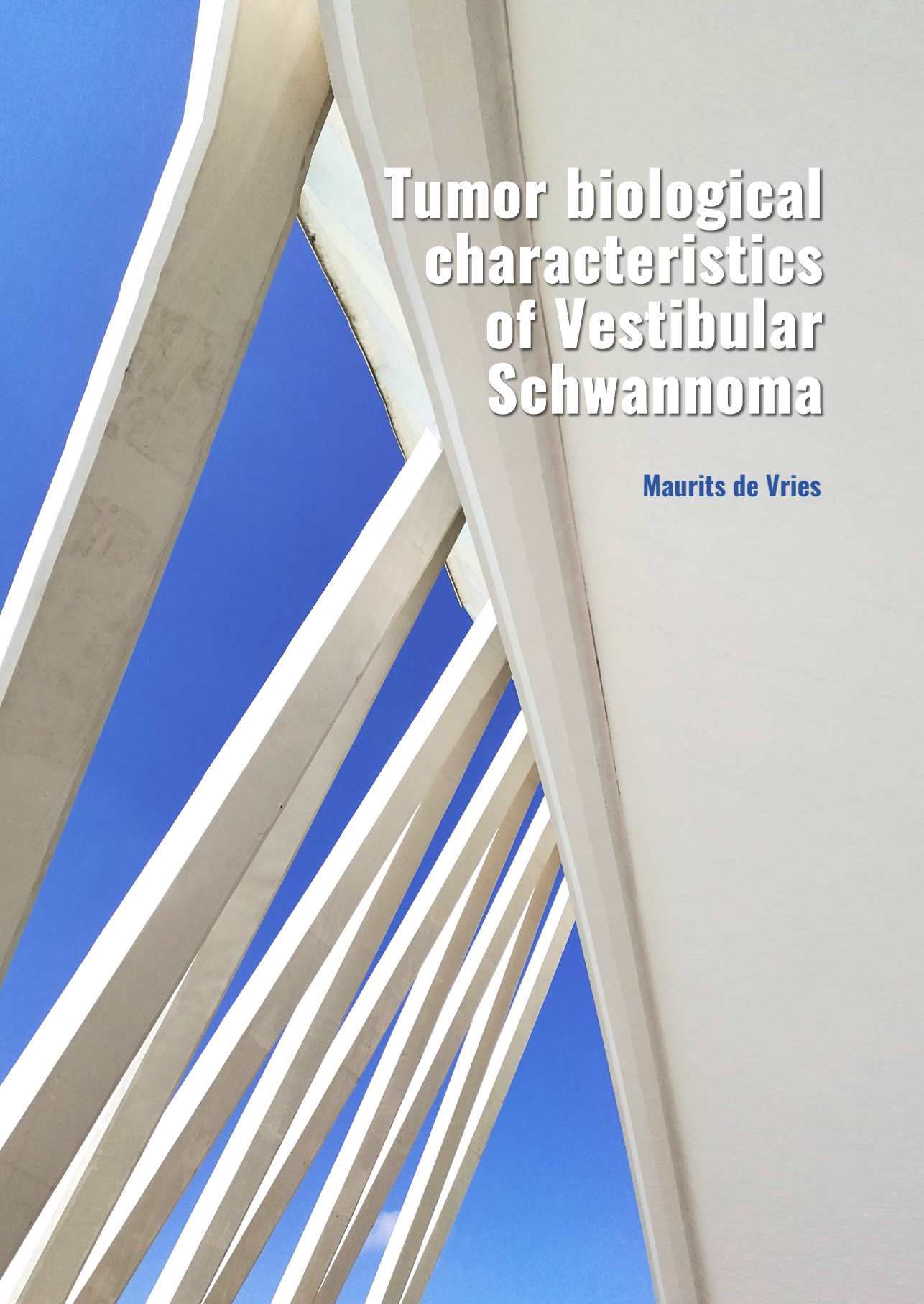
Tumor biological characteristics of Vestibular Schwannoma

Vries, M. de



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Maurits de Vries

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Aan mijn beide ouders

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CHAPTER 1

Introduction and outline of the thesis

Introduction

Vestibular schwannoma (VS) is a benign neoplasm recapitulating the differentiation repertoire of the myelin-forming Schwann cells forming the nerve sheath that insulates the vestibular portion of the eighth cranial nerve. Other more archaic names used to designate this tumor are acoustic neuroma, acoustic neurinoma or vestibular neuroma. Given its origin and biological composition, vestibular schwannoma is the most appropriate terminology[1,2]. Though benign these tumors are not harmless, their location in the internal auditory meatus and cerebellopontine angle can cause severe morbidity and in rare cases may even lead to death by brainstem compression when they are left untreated.

VS account for approximately 80% of all neoplastic lesions found in the cerebellopontine angle (CPA) [3]. Approximately 90% of all VS are unilateral sporadic tumors [4]. In rare cases these tumors occur bilateral which is a pathognomonic symptom for the hereditary disease neurofibromatosis type 2 (NF2). Recent years the number of diagnosed VS is rising. This rise is largely a result of the increased application of magnetic resonance imaging (MRI) scanning which for example leads to the detection of more asymptomatic lesions. Next to its widespread use the continually improving sensitivity of MRI is an important contributor to this phenomenon as well. The current incidence rate of VS lies around 20 per million people per year and this number is still increasing[5-8].

The biological background of schwannomas is still not fully understood. An important step in unravelling the genetic basis of VS formation was made by Rouleau, Seizinger and Trofatter who isolated the neurofibromatosis type-2 gene (*NF2*) during the late 80's of the twentieth century [9-12]. The *NF2* gene is located on chromosome 22q11 and it encodes for the tumor suppressor protein merlin [13]. Heterozygous germline inactivating mutations affecting *NF2* cause neurofibromatosis type-2 while biallelic somatic mutations of *NF2* are found in sporadic, unilateral vestibular schwannomas. [14]

This thesis mainly comprises research that is performed on sporadic vestibular schwannomas. NF2-related and sporadic vestibular schwannomas show considerable differences in their clinical presentation and they often require different and more complex therapeutic approaches. At the same time there are quite some similarities between both tumors and biological or clinical knowledge about one type can be beneficial to the understanding of tumorgenesis of the other type as well.

Unilateral vestibular schwannomas usually occur in adult patients with a peak incidence ranging from forty to sixty years [4,15] Main clinical symptoms are unilateral sensorineural hearing loss, tinnitus and balance disorders. In rare cases larger tumors can cause unsteadiness, trigeminus neuralgia, facial nerve paralysis and long tracks symptoms. If untreated, progressive tumors can finally lead to brain stem compression, obstructive hydrocephalus and increased intracranial pressure.

During the 20th century the tools and techniques to detect vestibular schwannomas have greatly improved from the tunic fork to audiometry and vestibular testing, auditory brain stem evoked response testing, computed tomography and finally magnetic resonance imaging (MRI)[16]. In 1987 gadolinium enhanced MRI was introduced, this technique has become the gold standard for the diagnosis of VS [17]. Tumors as small as 3 mm can now be detected [18]. The ability to detect VS in an ever-earlier phase has led to an increasingly complex decision-making regarding the best way to deal with these tumors once they are diagnosed.

Therapeutic management

Current therapeutic management of sporadic VS comprises three main options: conservative management by observation with scheduled MRI follow-up (wait-and-scan), microsurgery or radiotherapy. Although scarcely applied and not part of standard therapy yet, pharmacotherapeutic options are emerging as well. Choosing the most suitable therapy is mainly based on symptoms, radiological assessment and patient's preference.

Observation

Because of the increase in use and quality of MRI more and more VS are diagnosed in early tumor stages, often without even causing symptoms. Given the fact that VS are relatively slow growing tumors with an average growth rate of approximately 1-3 mm/year [19-21] the initial treatment strategy in these cases will be observation. This strategy was first suggested for the elderly or otherwise frail patients. Because natural history studies have indicated that up to 50% of VS show no significant growth in the first five years after diagnosis [19] this strategy nowadays is applied to younger patients as well. The goal of this strategy is to delay or even avert treatment related morbidity. The main drawback of this policy is that in case of rapid tumor progression the complexity of active treatment will have increased compared to the conditions at initial diagnosis. One of the hallmarks of VS is their variable growth pattern. This makes prediction of future tumor behavior difficult and even a strict wait and scan protocol with systematic radiological follow-up can sometimes result in unexpected and unfavorable situations. Better understanding of the biological processes behind VS growth can help to improve prediction of tumor behavior in order to single out potentially aggressive tumors at an early stage.

Microsurgery

First successful attempts to surgically remove VS were performed over a century ago. Unfortunately, these pioneering surgeons accomplished success in a minority of cases. Shortly before the start of World War One mortality rates were approximately 80% and almost all patients suffered major complications [22]. Over the twentieth century surgical outcomes have greatly improved. A large contribution to this growing success rate was the application of the translabyrinth microsurgical approach introduced by the otologist House

in 1963. Current reviews indicate that mortality associated with VS surgery is rare and occurs in less than 1% of cases [23]. Microsurgical techniques provide good tumor control but there is still an undeniable risk of iatrogenic complications such as damage to adjacent cranial nerves, cerebrospinal fluid leak, intracranial hemorrhage and meningitis. So far, microsurgery is the only treatment strategy to achieve (near total) tumor removal while radiotherapeutic approaches merely prevent additional tumor growth. Surgery has a high efficacy and less than 1% of patients require additional treatment after incomplete tumor resection[23]. Because of the significant risks associated with microsurgery it is important that patients and physicians make a balanced decision in which benefits have to be weighed against potential complications. Main indications to apply microsurgery should be tumors that progress rapidly or large tumors (>30mm) showing radiological or clinical signs of brainstem compression.

Radiotherapy

The Swedish neurosurgeon Lars Leksell introduced the concept of stereotactic radiosurgery (SRS) for brain tumors in 1951. The first time this technique, also known as the Gamma Knife, was applied to a VS was in 1969 [24]. Initially high-dose treatment protocols were used. This led to good tumor control but also had a significant effect on hearing loss and rates of facial and trigeminal neuropathy were high. Over the years the dose threshold has been lowered to 12 or even 11 Gy. At these levels permanent facial palsy is less than 1% and over 90% of patients show durable tumor control[25]. Recent years another type of particle therapy, proton therapy, is increasingly used. This technique uses protons instead of photons and has the advantage of further reducing the radiation dose to surrounding tissues, hypothetically leading to less iatrogenic damage. It used to be a scarce treatment modality but the availability has increased tremendously during the past decades. A recent article reviewing proton therapy indicated that data on its application in VS treatment is scarce and the few reports that are present do not show a favorable outcome in comparison to conventional radiotherapy[26,27]. The role of proton therapy in the treatment of VS remains unclear and additional research is needed to properly verify its effect before making it part of standard treatment options.

Pharmacotherapy

Despite the improvements of surgical and radiotherapeutical techniques these modalities will always carry a certain risk of inflicting iatrogenic damage to surrounding structures. Pharmacotherapeutic treatment might be an additional treatment strategy with less iatrogenic effects or complications. It could also benefit patients that are not eligible to undergo current forms of therapy.

Treating sporadic VS with drugs is not part of standard clinical practice yet. Nevertheless there are promising reports on the effect of antiangiogenic treatments for patients suffering

from NF2 related vestibular schwannomas. The most extensively tested drug in the context of VS is bevacizumab. This is an U.S. Food and Drug Administration approved anti-VEGF antibody used for the treatment of several types of cancer. A number of reports demonstrated stabilization and even tumor shrinkage in a significant amount of patients [28] [29,30]. Other forms of targeted therapy showed encouraging results as well [31] but there are also reports on drug resistant tumors[32]. **Chapter two** provides a more extensive overview of the current knowledge on-, and the future prospectives of pharmacologic therapies and the tumor biological processes behind them. The genetic- and micro environmental factors that determine vestibular schwannoma genesis and development will be discussed in **chapter two** as well.

Choice of therapy

Choosing the most suitable therapy for individual vestibular schwannoma patients can be a complex matter. In the obvious cases, such as fragile patients with small and stable intracanalicular tumors or young patients with progressing large brainstem compressing tumors, the choice can be quite clear. However, most patients find themselves between these extreme ends of the spectrum. For this category of patients there is the possibility to choose between different treatment modalities. This choice has to be made by the patient together with his or her physician. The fact that even among experts there remains controversy when it comes to selecting the correct therapy makes shared-decision-making even more difficult. During the past decades a vast amount of research into this topic has been performed but so far a balances consensus has yet to be reached.

The fact that all modalities have different goals makes a correct comparison between them difficult. For microsurgery an important goal is total tumor removal, for radiosurgery stopping tumor progression is the main outcome to measure success while the desired result of the wait and scan policy is avoiding disproportional treatment related morbidity. Before reaching consensus about treatment there should be more consensus about the goals and criteria by which success can be defined. A more general method to assess the effect of treatment is to determine quality of life (QoL). The PhD theses of Godefroy in 2010 and van Leeuwen in 2016, both at the Leiden University, indicated the importance of this measuring instrument. QoL assessments can help to increase the comparability of the therapeutic modalities, this will benefit the research that is performed to refine the decision-making with regard to the correct timing and method of treatment. Another factor that complicates the controversy regarding the choice of therapy is the unpredictable behavior of VS. As mentioned earlier, for the cases at both ends of the phenotypic spectrum the required treatment strategy can be obvious. Unfortunately in a large proportion of patients, often the ones with medium sized tumors, it is difficult to make a good prognosis of future tumor growth. The controversy over the choice of therapy is most evident in this category of patients.

Increased accuracy of predicting tumor growth will help to narrow down the group of patients for whom it is unclear which therapy will benefit them the most. So far the only proven

prognostic factors of tumor growth are observed growth during follow-up and cystic degeneration [21,33]. More insight into the tumor biology of VS improves the understanding of its growth pattern, which in turn benefits the search for better prognostic factors of tumor growth. A recent example of research with a similar purpose was the identification of loss of H3K27 tri-methylation as a prognostic marker for malignant peripheral nerve sheath tumors[34]. Next to improving the accuracy of predicting tumor progression, tumor biological research has the goal of identifying potential targets for pharmacotherapy. The current thesis can be seen in this context.

Aims and outline of the thesis

In this thesis a number of tumor biological characteristics of sporadic VS are analyzed. We mainly focus on prognostic markers which could benefit individual decision making and processes that can form a potential target for therapy. Prior tumor biological research on VS has predominantly been performed on neurofibromatosis type 2 related tumors, sporadic tumors have been studied to a much lesser extent. **Chapter 2** provides an overview of the tumor biological research that has been performed on both types of VS but it focusses on the sporadic tumors. Current knowledge about the genetic profile of VS is described and the roles of angiogenesis and the microenvironment on tumor progression are discussed. It also comprises a summary of targeted therapy that has been applied so far.

Chapter 3 presents an immunohistochemical analysis of the presence of markers for cell proliferation (Histone H3 and Ki-67), vascularization (CD31), inflammation (CD45 and CD68) and intratumoral bleeding (hemosiderin). The expression of these markers was correlated to clinical parameters such as tumor size, patient age at time of surgery and duration of symptoms.

Chapter 4 describes an allele specific quantitative real-time PCR assay that was performed on tumor specimens of forty-eight patients in order to detect the presence of the thirteen most frequent mutations affecting *BRAF, EGFR, PIK3CA,* and *KRAS*. These genes encode for proteins that are members of the MAPK/ERK cell signaling pathway which is associated with uncontrolled cell growth. This pathway is known to be up regulated in VS and *BRAF* mutations have already been found in other sporadic non-head and neck schwannomas. If present, mutated *BRAF* can function as a potential target for therapy.

In **chapter 5** the inflammatory microenvironment of vestibular schwannomas is investigated by analyzing the presence of tumor associated macrophages (TAM). These macrophages are known to support tumor progression by stimulating processes like angiogenesis. The presence of TAM is analyzed by immunofluorescent staining for CD163. The level of CD163 expression is assessed and compared with the degree of angiogenesis and tumor growth in 20 sporadic vestibular schwannomas.

In **chapter 6** the expression of the breast cancer resistance protein BCRP is studied in a selection of peripheral nerve sheath tumors i.e. vestibular schwannomas, plexiform neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). All three tumor subtypes are known to display a certain degree of intrinsic resistance to drug therapy. BCRP is a transmembrane efflux transporter associated with drug resistance in various types of cancer and it is also part of the blood-brain-barrier. Targeting BCRP can enhance drug susceptibility of neoplastic tissues. In order to investigate the role of this protein in the biology of peripheral nerve sheath tumors an immunohistochemical staining for BCRP is performed on a tissue microarray. This array comprises 22 sporadic vestibular schwannomas, 10 plexiform neurofibromas and 18 MPNSTs.

Chapter 7 elaborates on the inflammatory microenvironment by investigating the macrophage colony stimulating factor (M-CSF) and interleukin-34 (IL-34). These two cytokines play a key role in the recruitment of TAMs and thereby form a potential therapeutic target. An immunohistochemical analysis of these proteins is performed in the same patient cohort as described in chapter 4. The presence of M-CSF and IL-34 will be related to the presence of TAMs, the degree of angiogenesis and volumetric tumor growth. Finally the results of the thesis are summarized and discussed in **chapter 8**, and future perspectives for research are indicated.

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CHAPTER 2

Tumor Biology of Vestibular Schwannoma: A Review of Experimental Data on the Determinants of Tumor Genesis and Growth Characteristics

Maurits de Vries, Andel G. L. van der Mey and Pancras C. W. Hogendoorn

Otology & Neurotology (2015) 36: 1128-1136

Abstract

Objective: provide an overview of the literature on vestibular schwannoma biology with special attention to tumor behavior and targeted therapy.

Background: vestibular schwannomas are benign tumors originating from the eighth cranial nerve and arise due to inactivation of the *NF2* gene and its product merlin. Unraveling the biology of these tumors helps to clarify their growth pattern and is essential in identifying therapeutic targets.

Methods: PubMed search for English language articles on vestibular schwannoma biology from 1994 till 2014.

Results: activation of merlin and its role in cell signaling seem key aspects of vestibular schwannoma biology. Merlin is regulated by proteins like CD44, Rac and myosin phosphatase targeting subunit 1 (MYPT1). The tumor suppressive functions of merlin are related to receptor tyrosine kinases, such as the platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGF). Merlin mediates the Hippo pathway and acts within the nucleus by binding E3 ubiquiting ligase $CRL4^{DCAF1}$. Angiogenesis is an important mechanism responsible for the progression of these tumors and is affected by processes like hypoxia and inflammation. Inhibiting angiogenesis by targeting VEGF seems to be the most successful pharmacological strategy but additional therapeutic options are emerging.

Conclusion: over the years the knowledge on vestibular schwannoma biology has significantly increased. Future research should focus on identifying new therapeutic targets by investigating vestibular schwannoma (epi)genetics, merlin function and tumor behavior. Besides identifying novel targets, testing new combinations of existing treatment strategies can further improve vestibular schwannoma therapy.

Introduction

Sporadic vestibular schwannomas (VS) are benign tumors recapitulating the differentiation repertoire of the myelin-forming Schwann cells of the vestibular branch of the eighth cranial nerve. Vestibular schwannomas derive within the internal auditory canal, often extending into the cerebellopontine angle. Associated symptoms are hearing loss, tinnitus and vertigo. Large tumors can cause paralysis of adjacent cranial nerves and brainstem compression. The majority of vestibular schwannomas occur as unilateral sporadic tumors(>90%)[1]. Bilateral tumors are pathognomonic for the hereditary disorder neurofibrosis type 2 (NF2). In this review we discuss both but mainly focus on the sporadic tumors. Recent years the incidence of vestibular schwannomas has increased to approximately 20 per million people per year [2-4]. This is probably a consequence of the increased application of magnetic resonance imaging (MRI) scanning resulting in the identification of more subclinical cases. Therapeutic management of vestibular schwannomas comprises three strategies i.e. microsurgery, radiotherapy or serial radiological observation. So far pharmacological treatment options are scarce [5-7]. An important aspect determining the most suitable therapy is growth rate. Some tumors remain stable for years while others grow relatively fast (Figure 1.) The biological background of this phenotypical heterogeneity is largely unknown. This review provides an overview of the literature on vestibular schwannoma biology with special attention to tumor behaviour and targeted therapy.

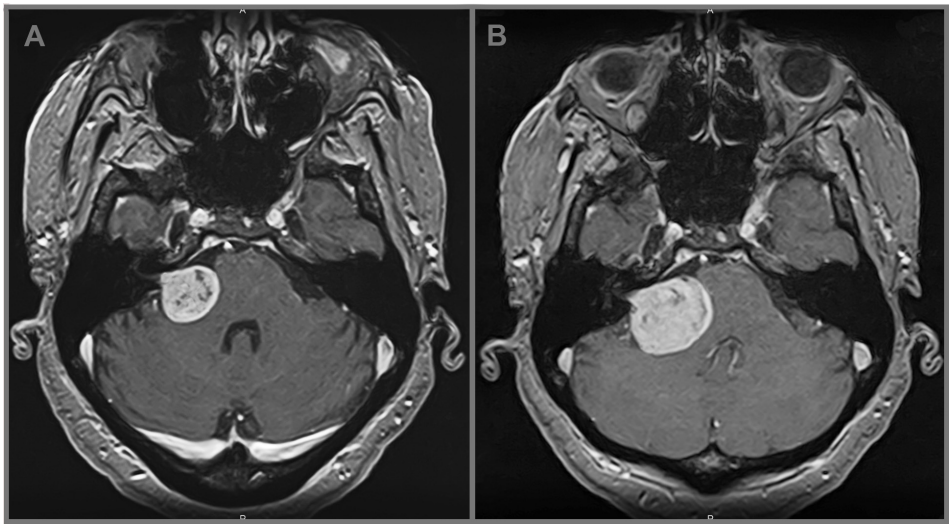

Figure 1. Sequential T1-weighted gadolinium enhanced magnetic resonance imaging scans of a fast-growing sporadic VS. This tumor more than doubled in volume from 4.25 ml (A) to 11.75 ml (B) in less than 10 months causing compression of the brainstem.

NF2 gene

An essential contribution to the understanding of vestibular schwannoma biology was the isolation of the neurofibromatosis type-2 gene (*NF2)* [8,9]. *NF2* encodes for the tumor suppressor protein merlin. This gene is located on chromosome 22q12 and contains 17 exons. Loss of functional merlin is essential in schwannoma pathogenesis [10]. Heterozygous germline inactivating mutations affecting *NF2* cause the autosomal dominant disorder neurofibrosis type-2 and biallelic somatic mutations of *NF2* are found in sporadic vestibular schwannomas [11].

Merlin Structure and Activation

Merlin is a cytoskeletal protein encoded by the *NF2* gene. It shows similarity to the ERM proteins, ezrin, radixin and moesin. These proteins play an important role in linking the actin cytoskeleton with plasma membranes [12]. “Merlin” is an acronym for “Moesin-Ezrin-Radixin-like Protein”. Merlin consists of an relatively conserved N-terminal FERM (Four-point-one, ezrin, radixin, moesin) domain followed by a coil-coil domain and a Carboxyl-terminal domain [13] (Figure 2).

The FERM domain is a membrane-binding module resembling the domain of the ERM proteins except for the C-terminal domain which lacks an actin-binding motif [14-16]. Instead, merlin directly binds actin with residues in the glutathione S-transferase N-terminal domain [17] or indirectly in association with II-spectrin or fodrin [18].
Merlin exists in an open and closed state. Dephosphorylation of merlin causes the protein to close. The closed conformation of merlin is the active tumor suppressor [19-21]. Promitogenic signals initiated by membrane-bound integrins and receptor tyrosine kinases are transduced by the signalling protein Rac which in turn activates p21-activated kinase (PAK) [22,23]. Activated PAK phosphorylates merlin at amino acid serine 518 [19,24,25]. This phosphorylation induces an open conformation of merlin, thereby inhibiting its tumor suppressor function. In addition to PAK merlin is also phosphorylated by protein kinase A (PKA)[26]. PKA mediated phosphorylation not only takes place at serine 518 but serine 10 as well [27]. The effect of phosphorylation at serine 10 is not entirely clear but it is suggested to induce changes of the actin cytoskeleton.
Conversely to Rac, engagement of cadherins or loss of mitogenic signalling inactivates PAK, leading to increased levels of closed, activated merlin [22]. In accordance with these observations, inactivation of merlin leads to loss of contact inhibition [28,29] and accelerated progression of the cell cycle [30]. By combining signals from cadherins and integrins merlin mediates cell cycle progression.
Another regulator of merlin is CD44, a transmembrane hyaluronic acid receptor involved in cell adhesion, matrix adhesion and cell migration [31]. Merlin mediates contact inhibition dependent cell growth by its interaction with CD44. Through these interactions merlin and

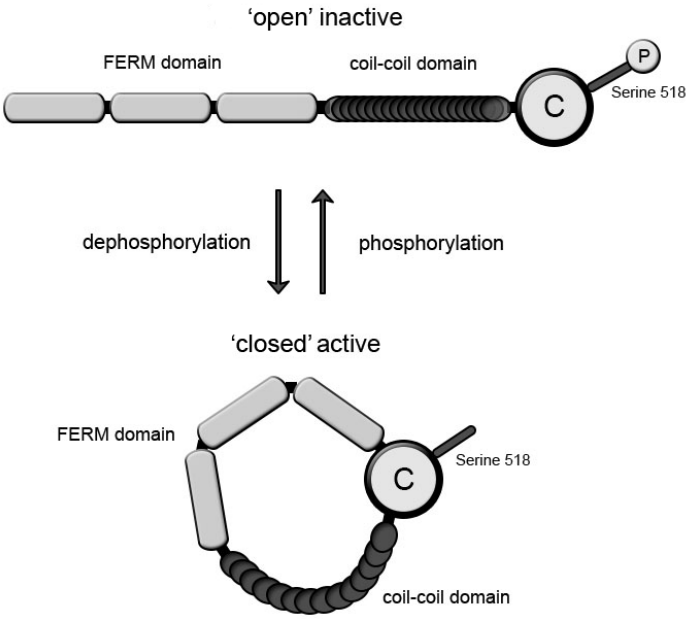

Figure 2. Merlin structure. Merlin has three structural sections: the N-terminal FERM domain followed by a coil-coil domain and a Carboxyl-terminal domain. Dephosphorylation of merlin at amino acid Serine 518 causes the protein to fold and become active

CD44 may function as a switch controlling cell growth arrest or proliferation [32,33]. Merlin can also be activated by myosin phosphatase targeting subunit 1 (MYPT1). This protein dephosphorylates merlin at amino acid serine 518 [20,34]. The concept of MYPT1 mediated activation of merlin is supported by the observation that CPI-17 (protein kinase C-potentiated phosphatase inhibitor of 17 kDa), a cellular inhibitor of MYPT1, causes loss of function of merlin [34]. Figure 3 provides an overview of various interactions involved in merlin regulation.

Merlin's role in cell signalling

Contact mediated inhibition is an important mechanism regulating cell growth. The tumor suppressive role of merlin seems largely affect by contact inhibition [23,32,35,36]. Identifying cellular pathways in which merlin participates may provide targets for treatment. Examples of targeted therapy are tyrosine kinase inhibitors, which have been successfully employed for solid neoplasms like gastrointestinal stromal tumors as well as leukemia's[37,38]. Tyrosine kinases are enzymes involved in the activation of numerous cell signalling cascades, when inhibited they can slow down or arrest tumor progression. It is because of developments like these that a significant proportion of schwannoma related research is now devoted to clarifying the function of merlin and the cell signalling pathways it affects. To maintain comprehensibility only the most well-established interactions will be discussed.

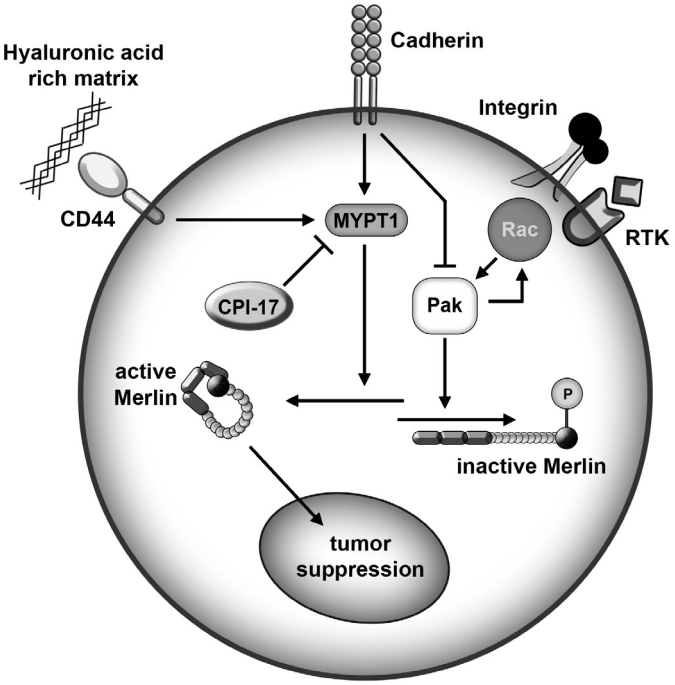

Figure. 3. Merlin activation. Cell-to-cell adhesions and CD44 activate MYPT1, which dephosphorylates merlin resulting in a closed and active protein conformation. Conversely, integrins and receptor tyrosine kinases activate Pak, which phosphorylates merlin, inducing an open and inactivated confirmation.

Merlin's tumor suppressor function is linked to the integrin mediated Rac pathway, which is involved in actin remodelling, cell cycle control, transcription and apoptosis. Shaw et al. [19] were the first to describe this association. They demonstrated merlin's ability to negatively regulate Rac, this was confirmed by additional studies [23,39]. A downstream target of Rac is PAK, the kinase responsible for the activation of merlin. The interaction between merlin, Rac and PAK suggests a positive feedback loop between merlin and PAK.

Merlin has been proposed to suppress proliferation by inhibiting receptor tyrosine kinases (RTKs) including the ErbB receptors, the platelet-derived growth factor receptor (PDGFR), the insulin-like growth factor 1 receptor (IGF1R) and the vascular endothelial growth factor (VEGF) receptor [28,35,40-42]. This is confirmed by the fact that proteins of the oncogenic Ras/Raf/MEK/ERK and PI3K/AKT pathways, which are downstream of these RTKs, are strongly activated in merlin deficient schwannoma cell models [43,44].
Merlin seems to act as a negative regulator of the mammalian target of rapamycin complex 1 (mTORC1), a kinase complex that regulates cell growth, cell proliferation, cell motility and cell survival [45]. mTORC1 seems activated in merlin deficient meningioma cells [46]. This is

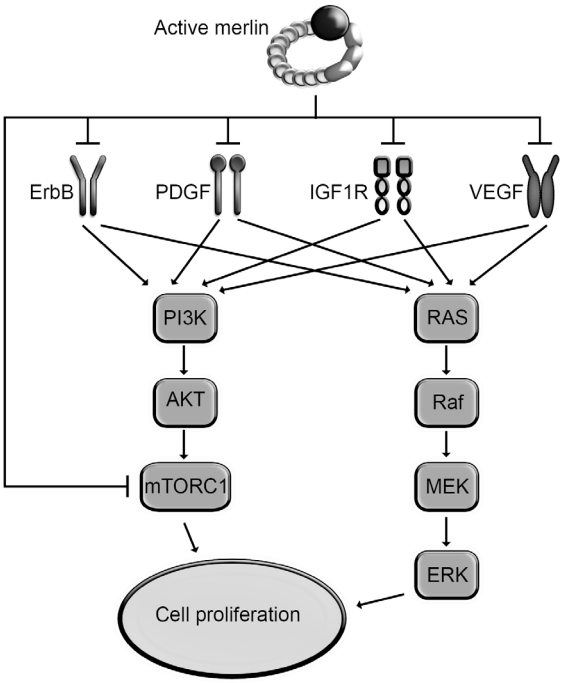

Figure 4. Merlin signalling. Merlin is proposed to suppress proliferation by inhibiting several receptor tyrosine kinases and their downstream signalling pathways.

supported by the correlation between loss of merlin and mTORC1 activation observed in mesothelioma cell lines [30]. The significance of mTORC1 as an effector of merlin is emphasized by the fact that the mTOR kinase inhibitor, Torin 1, could successfully block mTORC1 and AKT in merlin deficient meningioma cells leading to inhibited cell proliferation[47]. See figure 4 for an overview of kinases affected by merlin.

Merlin also mediates the Hippo pathway. This pathway controls organ size through regulation of cell proliferation and apoptosis. Mutations affecting this pathway are associated with different types of cancer [48]. A study investigating Hippo signalling in Drosophila showed that merlin is required for cell proliferation arrest and apoptosis [49]. This observation is supported by studies on meningioma cell lines and primary meningioma tumors which demonstrated that merlin suppresses the oncoprotein YAP (Yes-associated protein), a member of the Hippo pathway capable of stimulating cell proliferation [50,51].

Most of merlin's interactions take place around the plasma membrane. A recent study showed that merlin also accumulates in the nucleus where it binds the E3 ubiquitin ligase $CRL4^{DCAF1}$ blocking its activity [52-54]. $CRL4^{DCAF1}$ has been implicated to induce an elaborate oncogenic program of gene expression [55]. Interactions between merlin and $CRL4^{DCAF1}$ seem to be essential for tumor suppression by merlin.

NF2 mutations in sporadic VS

To date, the genetic profile of vestibular schwannomas has not been fully characterized. The only consistent genetic alteration is inactivation of the *NF2* gene. Multiple mutation analyses screening for *NF2* mutations in sporadic vestibular schwannomas have been described. Reports on the number of tumors containing a proven *NF2* mutation range from 15 to 84% (table 1). Most mutations are small deletions and point mutations [56-61], resulting in truncated proteins. A significant proportion of vestibular schwannomas did not harbour a proven *NF2* mutation. Studies investigating the *NF2* gene product, both at RNA- as well as protein level, demonstrated decreased expression of *NF2* gene products in a much higher percentage of tumors then expected with regard to the percentage of tumors containing an *NF2* mutation [10,62-65]. This difference could be explained by the involvement of other (epi)genetic changes that cause down-regulation of *NF2* expression. Another reason could be the fact that the utilized mutation detection methods were not sensitive enough. A possible factor impairing the sensitivity of these analyses is contamination of tumor tissue with for instance tumor invading cells of the intratumoral infiltrate. This theory is supported by our findings regarding the presence of tumor infiltrating macrophages in vestibular schwannomas [66,67].

Epigenetic alterations are involved in the development of many tumors [71,72]. Hypermethylation of CpG Islands in the promoter region leading to gene silencing is an important epigenetic mechanism causing tumor suppressor inactivation [73]. Aberrant methylation of *NF2* has been investigated in several studies. Kino et. al.[74] analysed 23 vestibular schwannomas and demonstrated aberrant methylation of *NF2* in 14 tumors, suggesting it as an alternative pathway of *NF2* inactivation. Gonzalez-Gomez [75] et. al. reported hypermethylation of *NF2* in just 6 out of 31 sporadic schwannomas. An even lower percentage was reported by Kullar et al.[68]. They found aberrant methylation of *NF2* in 4 out of 40 sporadic vestibular schwannomas. Finally Lee et. al.[69] investigated 30 vestibular schwannomas and found no aberrant methylation at all. The results of these studies vary considerably and do not provide a sufficient explanation for the subpopulation of vestibular schwannomas lacking a proven *NF2* mutation.

Global gene expression profiling of sporadic vestibular schwannomas

Global gene expression profiling experiments provide powerful methods to analyse the expression pattern of a large panel of genes. Welling et al. [76] were one of the first to perform a cDNA microarray analysis on vestibular schwannomas. They studied 7 tumors and identified several deregulated genes. Among the up-regulated genes were osteonectin (SPARC), an angiogenesis mediator, and RhoB GTPase, which is important in cell signaling. Among the down-regulated genes was LUCA-15 which is related to apoptosis. Ezrin, a relative of merlin, was also down-regulated in a majority of tumors. This studies' main limitation was the small number of samples, making statistical analysis difficult.

Table 1. NF2 mutations in sporadic vestibular schwannomas

Author (ref.nr.)	Year	NF2 mutation rate
Irving et al. [58]	1994	13 out of 85 (15%)
Sainz et al.[10]	1994	17 out of 26 (65%)
Welling et al. [56]	1996	19 out of 29 (66%)
Jacoby et al. [57]	1996	41 out of 49 (84%)
Hadfield et al. [61]	2010	65 out of 98 (66%)
Aarhus et al. [59]	2010	19 out of 25 (76%)
Kullar et al. [68]	2010	12 out of 40 (30%)
Lee et al. [69]	2012	16 out of 30 (53%)
Lassaletta et al. [60]	2013	25 out of 51 (49%)
Zhang et al.[65]	2013	50 out of 145 (35%)
Torres-Martin [70]	2013	23 out of 31 (74%)

Epigenetic alterations of *NF2*

A microarray analysis by Cayé-Thomasen et al. [77] investigated 16 vestibular schwannomas and compared their gene expression pattern with 3 vestibular nerves. An interesting up-regulated gene was platelet-derived growth factor D which is involved in cell cycle regulation. PTEN (phosphatase and tensin homolog deleted on chromosome 10), a tumor suppressor gene and major regulator of the PI3K/AKT pathway, was also up-regulated. The authors suggested PTEN up-regulation as compensatory for the lack of merlin inhibition. Comparison of the results of Cayé-Thomasen et al. and Welling et al. revealed an association related to SPARC. Welling et al found this gene to be up-regulated while the scavenger receptor stabilin-1, involved in SPARC degradation, was up-regulated in the analysis by Cayé-Thomasen et al. Subsequently Aarhus et al. [59] demonstrated up-regulation of SPARC as well, emphasizing the role of this gene in vestibular schwannoma biology.

Another finding of Aarhus et al. was the down-regulation of tumor suppressor gene CAV1, suggesting that loss of CAV1 participates in vestibular schwannoma formation. Additionally they performed a network and pathway analysis which indicated the ERK pathway as the central core linking the differentially expressed genes.

Coinciding results were reported in a microarray analysis by Torres-Martin et al. [70] They postulated that down regulation of CAV1 in schwannomas leads to deregulation of MET, a tyrosine kinase receptor involved in cellular mechanisms like proliferation, motility and migration. Table 2 provides an overview of these data.

An important consideration when interpreting the results of these expression profiling studies is the issue of the control tissue. Most studies used peripheral nerve tissue. Peripheral nerves predominantly contain axons surrounded by Schwann cells whereas tumor tissue mainly consists of schwannoma cells. This proportional discrepancy in tissue type can cause non-tumor-related differential cDNA expression which may obscure the actual results.

Table 2. global gene expression

Series (refnr.)	Gene	Function	Regulation status
Welling et.al. [76]	SPARC	angiogenesis	up-regulated
	RhoB GTPase	promotion of cellular functions related to cancerous cells	up-regulated
	LUCA-15	apoptosis	down-regulated
Cayé-Thomassen et.al [77]	PDGFD	cell growth and division	up-regulated
	PTEN	tumor supressor	up-regulated
	Stabilin-1	degradation of SPARC	up-regulated
Aarhus et.al. [59]	SPARC	angiongenesis	up-regulated
	CAV1	tumor supressor	down-regulated
Torres-Martin et.al. [70]	CAV1	tumor supressor	down-regulated

Angiogenesis and the role of the tumor microenvironment

Although vestibular schwannomas are relatively slow-growing neoplasms they still require angiogenesis to progress beyond a certain size [78]. Multiple angiogenesis stimulating factors have been identified; the best established is vascular endothelial growth factor (VEGF). VEGF is expressed by vestibular schwannoma cells [79-82] and several studies have correlated the degree of VEGF expression with clinical parameters such as tumor growth [79,81], tumor volume [83] and microvessel-density [83]. VEGF expression can be induced by hypoxia in response to the production of HIF-1alpha (Hypoxia inducible factor 1alpha) [84,85]. Diensthuber et al. [86] studied HIF-1alpha in sporadic vestibular schwannomas and demonstrated a relation between HIF-1alpha expression and cell proliferation. Next to hypoxia there are other microenvironmental factors regulating angiogenesis and tumor progression. Moller et. al. investigated matrix metalloproteinase-9 (MMP-9), an enzyme involved in migration and invasion of endothelial cells during angiogenesis. They studied 37 sporadic vestibular schwannomas and demonstrated a correlation between MMP-9 expression and tumor growth [87]. Inflammation is also capable of influencing tumor behavior. Macrophages form the major determinants of intratumoral inflammation. These so called tumor associated macrophages are associated with angiogenesis, cell growth and down-regulation of the immune response [88]. We performed a study on 68 sporadic [89]vestibular schwannomas and found a correlation between the expression of CD68 positive macrophages, tumor size and angiogenesis [66]. In a subsequent study we were able to support the concept of inflammation mediated tumor progression by linking macrophage expression to tumor growth [67]. The active role of inflammation in vestibular schwannomas is denoted by the presence of the enzyme COX-2. [90] COX-2 is expressed at sites of inflammation and effects angiogenesis [91]. Vestibular schwannomas with higher proliferation rates show higher COX-2 expression[90]. Vestibular schwannoma angiogenesis also seems to be stimulated by the down-regulation of the antiangiogenic factor semaphoring 3F (SEMA3F)[7]. A study by Wong et al.[92]

demonstrated the ability of merlin to up-regulate SEMA3F through Rac1 thereby decreasing angiogenesis. All together these studies support the importance of angiogenesis in vestibular schwannoma development, making it an interesting target for pharmacotherapeutic treatment.

Targeted therapy

As mentioned in previous paragraphs the increasing biological knowledge on vestibular schwannomas helps to identify targets for therapy. Next to angiogenesis other targets are emerging. Various components of the cell signalling pathways affected by merlin, like the receptor tyrosine kinases IGF1R, EGFR and PDGF, might also form targets for therapy. These growth factors are normally supressed by merlin but can be inhibited pharmacologically as well.

Current research on targeted therapy for vestibular schwannomas primarily focuses on the neurofibrosis type 2 related tumors. This paragraph discusses the latest developments regarding these pharmacotherapeutic options.

The angiogenesis inhibiting drug bevacizumab is an anti-VEGF antibody approved by the U.S. Food and Drug Administration for the treatment of several types of cancer. Plotkin et al. [7] were the first to investigate the effect of bevacizumab in NF2 patients. They demonstrated tumor shrinkage and mild hearing improvement in 9 out of 10 subjects. Mautner et al. [5] reported similar results. The effect of anti-VEGF therapy was also confirmed by Wong et al. [6]. They showed that angiogenesis inhibitors bevacizumab or vandetanib decreased vascularisation and growth rate of schwannoma xenografts in mice. Finally, a retrospective study on 31 NF2 patients demonstrated hearing improvement and tumor shrinkage with bevacizumab in more than 50% of the patients [93]. It should be noted that sustainable tumor control requires long term treatment with bevacizumab [94]. Because side effects of bevacizumab include hypertension, disrupted blood coagulation, embolism and kidney complications great caution should be exercised before starting therapy.

Another way to inhibit angiogenesis is by blocking the platelet-derived growth factor (PDGF) pathway. Besides having a proangiogenic function PDGF serves as a mitogen for Schwann and schwannoma cells. Therefore PDGF inhibitors have a direct effect on the schwannoma cells themselves as well. A drug capable of inactivating this pathway is imatinib mesylate (Glivec) [37,95]. The possibility that PDGF serves as a target for vestibular schwannoma treatment was first suggested by Altuna et al. [37,96]. They demonstrated that vestibular schwannomas express the PDGF Receptor-β and showed the ability of imatinib to alter cell cycle distribution and induce apoptosis in the vestibular schwannoma cell line HEI193. They additionally demonstrated that imatinib inhibited cell proliferation in HEI193 and in primary vestibular schwannomas cells. Yener et al. [97] confirmed the growth inhibitory effect of imatinib. They conducted angiogenesis assays on vestibular schwannomas. Imatinib proved to be effective in reducing the angiogenic activity. Ammoun et al. [98] compared imatinib with the

more potent PDGFR receptor inhibitor nilotinib (Tasigna). They found nilotinib to effectively inhibit proliferation of vestibular schwannoma cells at concentrations 6-10 times lower than imatinib. Additionally they demonstrated that a combination of nilotinib with selumetinib (AZD6244), a MAPK kinase inhibitor, even further inhibited cell proliferation.
Apart from antiangiogenic therapies other therapeutic agents have been tested. Lapatinib is a dual EGFR/ErbB2 inhibitor. In vitro studies have demonstrated that this drug achieves inhibition of cell proliferation in vestibular schwannoma cell cultures and it induces apoptosis in the HEI193 cell line [100,101]. This effect was endorsed by a phase II trial testing volume and hearing responses in NF2 patients. Results showed ≥15% tumor volume decrease in 4 out of 17 patients. Hearing was monitored in 13 patients, 4 of which experienced an improvement in pure tone average of at least 10 dB [107].
The recently developed histone deacetylase inhibitor AR42 capable of blocking the PI3K/AKT pathway proved to be a potent growth inhibitor of schwannoma- and meningeoma cells [103]. Targeting PDK1, which is a crucial activator of this pathway, can also inhibit AKT signalling in schwannoma cells [108] Yet another tyrosine kinase inhibitor tested for vestibular schwannoma therapy is erlotinib. It acts through HER-1/EGFR inhibition. Erlotinib showed to inhibit growth of vestibular schwannoma xenografts in nude mice [109]. Subsequent tests on the efficacy of erlotinib in 11 vestibular schwannoma patients [99] showed no radiographic or hearing responses but some patients did experience prolonged stable disease.
FRAX597 is an inhibitor of PAK, the kinase responsible for the phosphorylation of merlin (Figure 3). Licciulli et al. [104] found it to reduce proliferation in schwannoma cell lines and impair tumor development in an NF2 mouse model. Giovannini et al. showed that targeting the mTORC1 pathway with rapamycin (Sirolimus) leads to antagonization of tumorgenesis. This observation was made in in vitro as well as in vivo schwannoma models. They even seemed to induce tumor growth arrest in an NF2 patient [105] It should be noted that these observations are opposed by results of a trial describing no tumor resonse in 9 patients treated with mTOR inhibitor everolimus [102]
Finally there is the remarkable observation that plain aspirin is also associated with halted growth of vestibular schwannomas[106]. It is suggested that the COX2 inhibiting effect of aspirin dampens the pathologic immune response and its tumour promoting stimuli resulting in halted tumor progression. Table 3 provides an overview of target therapy tested for vestibular schwannomas.

Future prospects

To date a wide range of potential therapeutic targets for vestibular schwannoma treatment has been studied. Most drugs seem to induce an antiangiogenic or cytostatic response. An actual cytotoxic effect resulting in apoptosis has also been observed, for example with drugs

Table 3. Summary of research on targeted therapy

Drug	Target	Author (ref. nr.)	Main results
Bevacizumab	VEGF	Plotkin et al. [7]	Tumor shrinkage and mild hearing improvement in 9 out of 10 patients.
		Plotkin et al.[93]	Tumor shrinkage in >50% of 31 analysed patients
		Mautner et al. [5]	>40% tumor shrinkage in 2 out of 2 patients.
		Wong et al. [6]	Mouse model showing decrease of tumor vasculature after bevacizumab. Tumor growth decreased and the survival of treated mice extended by 50%.
Erlotinib	EGFR	Plotkin et al.[99]	No radiographic or hearing response in 11 patients.
Imatinib	PDGF	Altuna et al. [96]	In vitro study demonstrating apoptosis and inhibition of cell proliferation
		Yener et al. [97]	In vitro study demonstrating reduction of angiogenesis in tissue specimens of NF-2 related as well as sporadic tumors.
Lapatinib	EGFR/ErbB2	Ahmad et al. [100]	In vitro study demonstrating decrease of cell growth and proliferation in vestibular schwannoma cell cultures.
		Ammoun et al. [101]	In vitro study demonstrating decreased proliferation in a human schwannoma model
		Karajannis et al. [102]	Phase II trial demonstrating hearing an volume responses in lapatinib treated NF2 patients
AR42	PI3K/AKT	Bush et.al.[103]	Growth inhibition of schwannoma cells
FRAX597	PAK	Licciulli et al.[104]	In vitro study displaying reduced schwannoma cell proliferation and an in vivo experiment indicating impairment of tumor development.
Rapamycin	mTORC1	Giovannini[105]	Tumor growth arrest in one NF2 patient
Aspirin	COX-2	Kandatil et. al.[106]	Inverse association between aspirin use and tumor growth

targeting EGFR and PDGF. By simultaneously targeting EGFR and ErbB2 actual tumour shrinkage could be accomplished in a number of NF2 patients. This outcome emphasizes the potential effect of combining different therapies. The ideal would be a therapeutic regimen of drugs tailored to the gene- or protein expression pattern within each individual tumour. Such an approach would require analysis of tumour tissue, which is virtually impossible in non-surgically treated patients, but could be applied in a subgroup of patients suffering from tumour recurrence after surgery. The knowledge of having a good treatment alternative after surgery may also lessen the need for radical tumour extirpation, allowing more limited surgery with less morbidity.

Combining drugs with radiotherapy, a treatment approach already applied for different types of cancer, is another strategy worth considering. A recent study combing a c-Jun N-terminal kinase (JNK) inhibitor with gamma radiation[110] shows that the combination of these therapeutic strategies can be successful in vestibular schwannomas as well. Altogether these emerging therapeutic targets will help to further reduce the need for surgical intervention.

Conclusion

During the past years there has been a tremendous increase in knowledge of vestibular schwannoma biology. The mechanisms through which merlin carries out its functions are gradually elucidated. This process goes hand in hand with the identification of novel therapeutic targets. Up till now targeting angiogenesis seems the most successful pharmacological strategy but additional therapeutic options are emerging. Other tumor biological issues that deserve to be part of future research are the processes responsible for the variable growth pattern of these tumors, the discrepancies regarding the occurrence of (epi) genetic changes to *NF2,* and the potential involvement of additional genes and signalling pathways.

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CHAPTER 3

Intratumoral hemorrhage, vessel density, and the inflammatory reaction contribute to volume increase of sporadic vestibular schwannomas

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Abstract

Objective: identification of clinicopathologic factors related to vestibular schwannoma progression.

Background: vestibular schwannomas show a large variation in growth rate, making prediction and anticipation of tumor growth difficult. More accurate prediction of clinical behaviour requires better understanding of tumor biological factors influencing tumor progression.

Methods: biological processes like intratumoral haemorrhage, cell proliferation, microvessel density, and inflammation were analysed in order to determine their role in vestibular schwannoma development. Tumor specimens of 67 patients surgically treated for a histologically proven unilateral vestibular schwannoma were studied. Preoperative magnetic resonance imaging (MRI) scans were used to determine tumor size and to classify tumors as homogeneous, inhomogeneous, and cystic. Immunohistochemical studies evaluated cell proliferation (histone H3 and Ki-67), microvessel density (CD31), and inflammation (CD45 and CD68). Intratumoral haemorrhage was assessed by hemosiderin deposition. The expression patterns of these markers were compared with tumor size, tumor growth index, MRI appearance, patients' age, and duration of symptoms.

Results: no relation between cell proliferation and clinical signs of tumor volume increase or MRI appearance was found. Intratumoral hemosiderin, microvessel density, and inflammation were significantly positively correlated with tumor size and the tumor growth index. Cystic and inhomogeneous tumors showed significantly more hemosiderin deposition than homogeneous tumors. The microvessel density was significantly higher in tumors with a high number of CD68-positive cells.

Conclusion: the volume increase of vestibular schwannomas is not based on cell proliferation alone. Factors like intratumoral bleeding, (neo)vascularization, and intensity of the inflammatory reaction also influence tumor volume.

Introduction

Vestibular schwannomas (acoustic neuromas) are benign tumors originating from the myelin-forming Schwann cells of the vestibular branch of the eighth cranial nerve in the internal auditory canal or cerebellopontine angle. Clinically, these tumors grow slowly and progressively, eventually causing brainstem compression.

One of the problems in the treatment of these tumors is the large variation in the evolution of increase in volume in time. Why this is so variable remains largely unknown, but clinically, this makes prediction and anticipation of the evolution of symptoms difficult. Current therapeutic management is mainly based on symptoms and radiological assessment. Unfortunately, these indicators do not always correlate with actual tumor volume increase. More accurate prediction of tumor expansion rate could lead to more balanced decision-making regarding therapeutic actions when a vestibular schwannoma is diagnosed.

Accurate assessment of potential tumor volume increase requires better understanding of tumor biological (growth) factors and concurrent pathological events like cyst formation. To gain more insight in the role of these factors, we assessed the relationships between (immuno)histopathological markers, radiological observations on tumor appearance, and clinical growth of vestibular schwannomas.

One way for tumors to expand is by cell proliferation. A well-known proliferation marker is the Ki-67 antigen [1,2] , which is present during all phases of the cell cycle but is absent in noncycling cells. A less known indicator for proliferation is the phosphorylated histone H3 protein, which is expressed during mitosis [3-5]. Both these markers were included in this study. In addition to proliferation, we investigated to what extent intratumoral haemorrhage, microvessel density, and the degree of inflammation are involved in tumor expansion. To quantify intratumoral haemorrhage, hemosiderin (i.e., iron) deposition was evaluated. The endothelial marker CD31 was used to measure microvessel density, and the degree of inflammation was determined by quantifying the number of leukocytes and macrophages through the expression of the markers CD45 and CD68, respectively.

Material and methods

Patients

From the vestibular schwannoma database at the Leiden University Medical Center, a total of 67 patients (26 males and 41 females) were identified. They involved a group of patients surgically treated for a histologically proven vestibular schwannoma from January 2000 to November 2005. The main criteria for surgical treatment were clinical symptoms (e.g., progressive or debilitating vertigo and hearing loss) and tumor size, both initial as well as in terms of progression over time. Patients' personal preference was also of great importance

in deciding if and when to apply treatment. Only cases of unilateral sporadic schwannomas were selected, patients diagnosed with NF2 were excluded.

Tumor measurement

Information on radiological data was obtained from radiology records which are all composed according to specific clinical and scientific incentives. These standardized reports include measurement of the greatest tumor diameter according to the guidelines of the American Academy of Otolaryngology Head and Neck Surgery [6]. Additionally, the reports comprise evaluation of tumor density based on gadolinium-enhanced T1-weighted as well as T2-weighted scans. Depending on the presence of microcystic and or macrocystic components, tumors were classified as homogeneous, inhomogeneous, or cystic. All patient data were prospectively discussed at a multidisciplinary conference attended by representatives of the neurosurgery, radiology, radiotherapy, pathology, and otolaryngology department. Clinical and radiological data were cross checked before being entered in the vestibular schwannoma database (for the exact patient characteristics, including figures illustrating cystic, inhomogeneous, and homogeneous tumors, see Electronic supplementary material Figures 1, 2, and 3).

Immunohistochemistry

The immunohistochemical tests were conducted at the Department of Pathology of the Leiden University Medical Center. The tests were conducted on specimens preserved in formalin and stored in paraffin. All assessments were performed on tissue sections obtained from one single tumor block per tumor sample. Immunohistochemical reactions were performed according to standard laboratory methods [7]. In brief, heat-induced antigen retrieval was performed using microwave treatment of all sections after dewaxing and rehydration, followed by blocking of endogenous peroxidase with 3 % H2O2 in methanol. Incubation with primary antibodies was overnight (for sources, working dilutions, and positive controls used, see Table 1). Subsequently, sections were incubated with poly-HRP-antimouse/rabbit/rat conjugate. Visualization was carried out with a H2O2–diaminobenzidine solution. All washing procedures were performed in phosphate-buffered saline. Slides were counterstained with hematoxylin. In addition to the immunohistochemical stains, hemosiderin staining was performed.

Microscopic analysis

In order to obtain an overall impression of the staining pattern of each marker, all sections were first evaluated using a × 10 objective lens. Hemosiderin, CD45, and CD68 displayed an irregular/patchy staining pattern, making computer-assisted analysis less reliable. For this reason, further analysis of these markers was performed manually in a semiquantitative score. Per specimen, 10 randomly chosen fields of view (FOV) were evaluated at a × 40

Table 1. Antibody concentrations and positive control tissues used

Antibody	Supplier	Concentration	Positive control
Ki-67	DAKO	1:800	Tonsil
Histone H3	Comproscientific	1:3,200	Tonsil
CD31	DAKO	1:150	Tonsil
CD45	DAKO	1:4,000	Tonsil
CD68	DAKO	1:20,000	Tonsil

objective lens. Scoring was independently assessed by two authors. The degree of staining was scored as follows: 0, absent; 1, mild; and 2, strong.

Staining for Ki67 and histone H3 showed a more homogeneous distribution, permitting the application of a computer-based image analysis method in order to determine the index of proliferation. In brief, of each specimen, five randomly chosen digital snapshots were taken. Positively stained nuclei were identified through spectrometry using a Leica DM4000B microscope fitted with a CRI Nuance spectral analyser (Cambridge Research and Instrumentation, Inc., Woburn, MA, USA). Using Mirax software (Zeiss, Germany) and ImageJ (National Institutes of Health, Bethesda, MD, USA), the number of pixels representing positively stained nuclei was determined, as well as the number of pixels representing the total nuclear area in one FOV. The index of proliferation was calculated by dividing the number of immunopositive pixels by the number of pixels representing the total nuclear area. The mean value of the five snapshots was used for statistical analysis.

Microvessel density was determined with the Chalkley point overlap morphometric technique, which allows for rapid analysis with a relatively low interobserver variability [8]. This method has been described in detail [9,10]. In brief, CD31 stained sections were scanned for hot spots of high vascular density. Using an ocular grid with 25 random points, microvessel density was scored in these hot spots with a × 20 objective lens. The grid was oriented to permit the maximum number of points to hit the stained microvessels (Figure 1). The Chalkley count was the mean of the maximum number of points hitting a microvessel in three hot spots per tumor specimen.

Tumor growth index

Accurate measurement of tumor growth depends on serial magnetic resonance imaging (MRI) scanning over a longer period of time. Because only six patients in our selection underwent serial MRI scanning prior to surgery, this method of growth assessment could not be used in our study. We used the tumor growth index as a surrogate parameter. This index has been described earlier regarding vestibular schwannoma growth [11,12]. Its basic assumption is that the age of onset of the tumor varies randomly. The tumor growth index is calculated by dividing the maximal tumor diameter by the age of the patient.

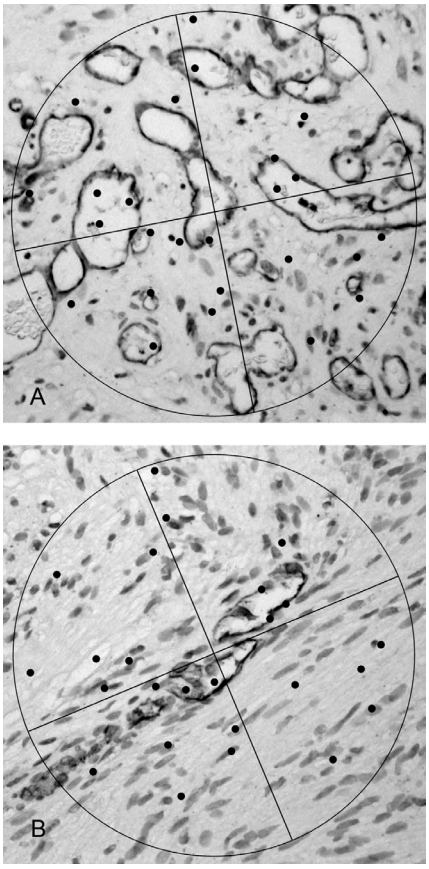

Figure 1. Examples of Chalkley counts in vascular hotspots, specimen **a** and **b** score 10 and 6, respectively (original magnification × 200)

Statistical analysis

For evaluation of the relationships between the tumor biological markers and parameters of clinical growth, the Spearman rank correlation test was used. The difference in hemosiderin deposition between cystic and inhomogeneous tumors versus homogeneous tumors was determined by the chi-square test. Tumor size of cystic and inhomogeneous tumors versus homogeneous tumors was compared with the unpaired t test. The relation between microvessel density and CD68 expression was evaluated using the one-way analysis of variance (ANOVA) and Scheffe test. For all statistical tests, $p < 0.05$ was considered as significant. All calculations were performed using SPSS Inc. software, version 17.0.

Results

A total of 67 patients (age: range, 15–72 years; mean, 49.04 ± 14.06 years) was studied. Maximum tumor diameter varied between 5 and 50 mm (mean, 24.03 ± 11.52 mm) and the

tumor growth index (which does not represent actual increase in size but a parameter for tumor growth rate) varied between 0.07 and 2.11 mm/year (mean, 0.56 ± 0.40 mm/year)
The mean value of the Ki-67 index in this studied group was 0.6 % (range, 0.1–1.8 %). For the histone H3 index, the mean value was 3.9 % (range, 0.4–15.5 %). No significant correlation was found between the Ki-67 or histone H3 index and maximal tumor diameter, tumor growth index, and radiologically measured tumor growth.
The number and distribution of hemosiderin-, CD45-, and CD68-positive cells in the tissue sections were very heterogeneous. Some specimens showed no staining at all, yet other specimens showed a substantial number of positive cells (Figure 2). The mean Chalkley count was 9.59 (range, 3.3–23.0; SD, 3.70). The Spearman rank correlation test demonstrated several significant positive correlations between these markers (Table 2, for the corresponding scattergrams, see Electronic supplementary material Figures 4, 5, and 6).
The number of hemosiderin-positive cells correlated with tumor size ($p < 0.001$; $r = 0.39$) and tumor growth index ($p < 0.006$; $r = 0.33$). CD45 expression correlated with tumor size ($p < 0.006$; $r = 0.33$) and tumor growth index ($p < 0.002$; $r = 0.38$), and CD68 expression also correlated with tumor size ($p < 0.0001$; $r = 0.46$) and tumor growth index ($p < 0.0001$, $r = 0.48$).

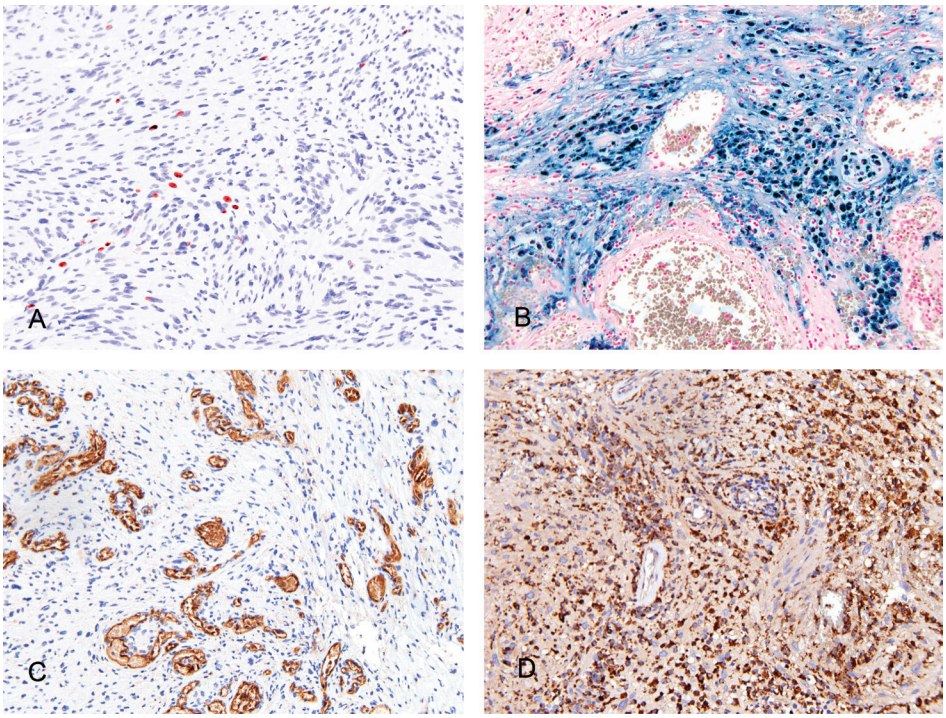

Figure 2. a Proliferating cells (Ki-67/histone H3), **b** intratumoral bleeding (hemosiderin), **c** high microvessel density (CD31), and d intratumoral inflammation (CD45/CD68) (original magnification × 400)

The Chalkley count demonstrated a positive correlation with tumor size ($p < 0.001$; $r = 0.40$) and tumor growth index ($p < 0{,}002$; $r = 0.37$). Furthermore, the Chalkley count showed a significant correlation with the number of hemosiderin-positive cells ($p < 0.003$; $r = 0.36$), CD45-positive cells ($p < 0.021$ $r = 0.28$), and CD68-positive cells ($p < 0.0001$; $r = 0.47$). A one-way ANOVA and a Scheffe test confirmed the positive relation between the number of CD68-positive cells and microvessel density. Tumors with a high number of CD68-positive cells displayed a significantly higher microvascular density than tumors with low or no CD68-positive cells (Figure 3).

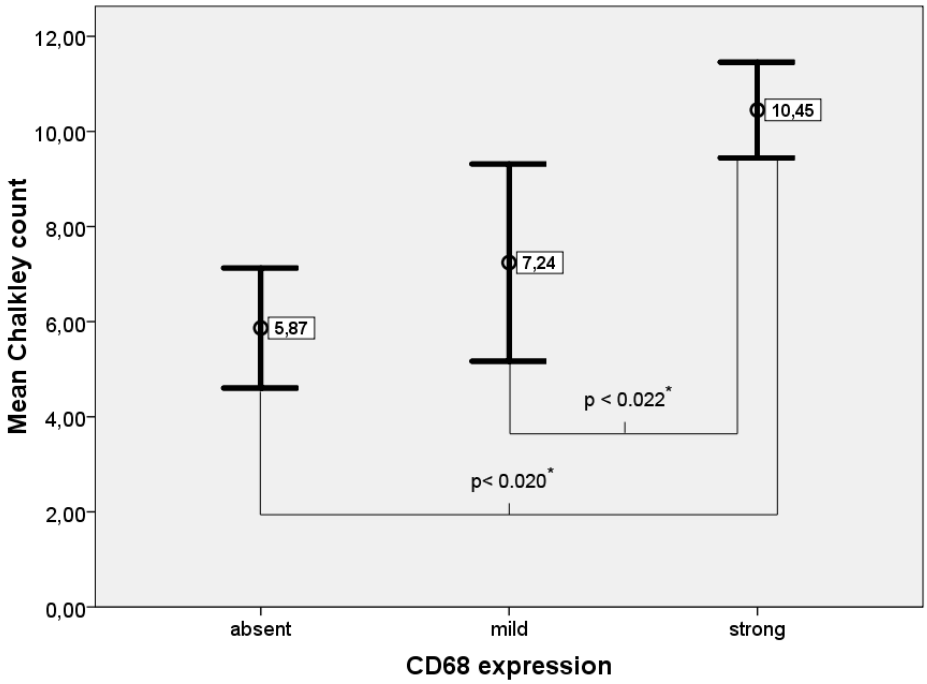

Figure 3. Relation between CD68 expression and microvessel density. The mean Chalkley count is significantly higher in tumors with strong CD68 expression. Asterisk denotes statistical differences calculated with the Scheffe test.

In 61 patients, the tumors were morphologically classified based on their MRI appearance. Twenty-four tumors were classified as homogeneous, 8 as inhomogeneous, and 29 as cystic. Cystic and inhomogeneous tumors were significantly larger than homogeneous tumors (Table 3). Cystic and inhomogeneous tumors also displayed a significantly higher number of hemosiderin-positive cells than homogeneous tumors (Table 3). No statistically significant correlations or differences were observed when patient age or duration of symptoms was taken into account.

Table 2. Spearman's correlation test for pathological markers and clinical characteristics

		Ki-67	Histone H3	CD45	CD68	Siderin[a]	MVD
Size	Correlation coefficient	0.129	0.326	0.333**	0.457**	0.392**	0.397**
	Significance (two-tailed)	0.301	0.120	0.006	0.000	0.001	0.001
TGI	Correlation coefficient	0.091	0.275	0.375**	0.482**	0.330**	0.373**
	Significance (two-tailed)	0.466	0.243	0.002	0.000	0.006	0.002
MVD	Correlation coefficient	0.140	0.240	0.281*	0.472**	0.363**	-
	Significance (two-tailed)	0.261	0.102	0.021	0.000	0.003	-

TGI tumor growth index, *MVD* microvessel density, [a]Hemosiderin

Table 3. MRI appearance compared by hemosiderin deposition and size

	Hemosiderin deposition [a]						Size [b]	
MRI [c]	absent	mild	strong	x^2	df	p	mean ± SD	p
Homogeneous	15	7	2	8.67	2	<0.013	13.46 ± 8.44	< 0.0001
Inhomogeneous/cystic	10	15	12				30.46 ± 8.43	

[a] chi square test; [b] independent t-test ; [c] magnetic resonance imaging.

Discussion

To gain more insight into the mechanisms responsible for volume increase of vestibular schwannomas, possible correlations between histopathological markers and radiological and clinical characteristics of vestibular schwannomas were investigated. For studying the growth rate of a tumor, serial radiological observation is the preferred method. As most patients in this study were operated on shortly after diagnosis, in the majority of cases, only one preoperative MRI scan was obtained, which excluded this approach. As a surrogate, we used the growth index, which is only a rough estimate of the rate of tumor volume increase but allowed us to include a larger number of patients in the study.

To evaluate the role of proliferative activity in the volume increase of vestibular schwannomas, the cell cycle markers Ki-67 and histone H3 were used. Ki-67 as a parameter of growth of vestibular schwannomas has been studied by Niemczyk et al. [2], who compared clinically stable vestibular schwannomas with clinically growing cases. They found a significant difference in Ki-67 labeling index between the two groups. The mean labeling index in the growing tumors was 3.17 % compared to 1.11 % in the stable tumors. Overall, the Ki-67 index ranged from 0.22 to 5 %, with an average of 1.86 %. Gomez et al. [13] also investigated cell proliferation in vestibular schwannomas, but did not find a significant correlation between tumor growth and Ki-67 labeling index, which ranged from 0.2 to 2.2 %. We conclude that the labeling index of Ki-67 in our study (ranging from 0.1 to 1.8 %, with a mean of 0.6 %) is comparable with earlier published data.

Histone H3 as a proliferation marker has not been studied in vestibular schwannomas before. We did not find a correlation between the histone H3 labeling index and the tumor growth index. Taken together, our data and those from the literature indicate that cell proliferation is not a decisive factor in the expansion of vestibular schwannomas.

Degenerative changes such as cysts may contribute to tumor volume increase. Reports on the incidence of cyst formation in vestibular schwannomas vary between 5.7 and 48 %, with more recent studies indicating incidences of approximately 10 % [14-17]. Cystic tumors can display a relatively rapid increase in volume and generally become larger than noncystic tumors [18]. This also applies to our case series, the cystic and inhomogeneous tumors being significantly larger than the homogeneous tumors. The mechanisms responsible for cyst formation in vestibular schwannomas remain unclear. Gomez et al [13] . demonstrated a significant correlation between hemosiderin deposition, tumor size, and tumor heterogeneity and suggested that hemosiderin resorption might induce cyst formation. The same suggestion is made by Park et al. [19]. Our results are in line with these studies. We found significantly more hemosiderin deposition in cystic and inhomogeneous tumors compared to homogeneous tumors. The significant correlations between hemosiderin deposition and tumor size and tumor growth index also support this notion.

These results may offer a clue in the search for markers of tumor volume increase. Both cysts and iron deposition are radiologically demonstrable and might, therefore, constitute clinically applicable markers. A radiological study on pituitary macroadenomas by Stadlbauer et al. [20] described a significant difference in spectral signals measured in hemorrhagic versus nonhemorrhagic tumors. The authors suggested that the paramagnetic effect of hemosiderin deposition in hemorrhagic tumors on the MR images might provide an explanation. Using this approach to study iron deposition and cyst formation in vestibular schwannomas might establish a causal relationship between intratumoral hemorrhage and cyst formation.

Although vestibular schwannomas are relatively slow-growing neoplasms, they depend for further growth on a functional vascular system, as any other tumor [21]. The positive correlation between microvessel density and tumor size and tumor growth index we found is consistent with other published data [22,23]. These findings suggest that angiogenesis may be important for the volume increase of vestibular schwannomas. Determining the degree of vascularization of vestibular schwannomas might also be possible using MRI diffusion or perfusion techniques [24-26]. Further research into this hypothesis could lead to another clinically applicable marker of tumor volume increase. These results also suggest that antiangiogenesis therapy might contribute to controlling tumor growth.

The degree of inflammation measured by the expression of CD68 and CD45 showed a positive significant correlation with tumor size and tumor growth index. Similar correlations have not been reported in the literature yet. In a study performed by Brieger el al. [27] on angiogenic growth factors in vestibular schwannomas, tumor-infiltrating CD68-positive lymphocytes were not detected. Labit et al. [28] only found a correlation between the number

of CD45-positive cells and the duration of symptoms. The mechanism responsible for or the significance of an inflammatory reaction in vestibular schwannomas has not been elucidated as yet. Studies on breast cancer have addressed the role of the inflammatory microenvironment in tumor progression [29-35]. It has been proposed that inflammation which triggers angiogenesis might contribute to tumor progression. Macrophage activity is a major determinant of the intratumoral inflammatory milieu but other components of the inflammatory infiltrate also seem to modulate tumor behaviour [36]. The significant associations between the degree of inflammation (i.e., CD45 and CD68) and tumor size and tumor growth index and, in addition, the significant increase of microvessel density in tumors with a higher number of CD68-positive cells (Figure 3) suggest that similar processes take place in vestibular schwannomas. Several approaches targeting macrophage activity are currently under investigation [37,38], making additional research of the inflammatory process in vestibular schwannomas even more interesting. The first step would be further typing of inflammatory cells present in vestibular schwannomas. Furthermore, their activation state and relationship with angiogenic growth factors should be examined.

The results of this study indicate that tumor volume increase of vestibular schwannomas is not based on cell proliferation alone. Contributing factors are intratumoral hemorrhage, vascularization, and degree of inflammation.

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CHAPTER 4

Mutations affecting *BRAF*, *EGFR*, *PIK3CA* and *KRAS* are not associated with sporadic vestibular schwannomas

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Abstract

Objective: determine whether the most frequent mutations affecting the MAPK/ERK pathway contribute to vestibular schwannoma progression

Background: sporadic vestibular schwannomas are benign tumors originating from the Schwann cells of the vestibular portion of the eigth cranial nerve. An important clinical hallmark of these tumors is their variable growth rate. Investigating vestibular schwannoma biology can help to clarify this variable growth rate and may offer targets for therapeutic treatment. A recent mutation analysis on sporadic non head and neck schwannomas detected *BRAF* mutations in around 20 % of tumors. *BRAF* is part of the MAPK/ERK pathway. MAPK/ERK activation is associated with uncontrolled cell growth. Mutated *BRAF* can function as a target to inhibit this pathway. Mutations in *BRAF* and other members of the MAPK/ERK pathway have not been investigated in sporadic vestibular schwannomas before.

Methods: Tumor specimens of 48 patients surgically treated for a sporadic vestibular schwannoma were analyzed. An allele specific quantitative real-time PCR assay was performed to detect the thirteen most frequent mutations affecting *BRAF, EGFR, PIK3CA,* and *KRAS*. Radiologically measured tumor growth was included in the analysis to identify potential relationships between these mutations and tumor progression.

Results: No activating hotspot mutations in *BRAF, EGFR, PIK3CA* or *KRAS* were detected in the investigated vestibular schwannomas.

Conclusion: the thirteen most frequent mutations affecting *BRAF, EGFR, PIK3CA* and *KRAS* are not involved in sporadic vestibular schwannoma development. These results are in contrast to the recent detection of these *BRAF* mutations in non-head and neck schwannomas and are as such no explanation of the clinically observed variable growth pattern.

Introduction

Sporadic vestibular schwannomas are benign tumors recapitulating the differentiation repertoire of the myelin-forming Schwann cells of the vestibular branch of the eighth cranial nerve in the internal auditory canal or the cerebellopontine angle. These tumors grow clinically slowly and progressively, extending into the cerebellopontine angle ultimately causing brainstem compression.

Therapeutic management of these tumors can be divided into three different strategies i.e. surgical removal, stereotactic radiotherapy or serial radiological observation, also known as the wait & scan policy. So far, unlike neurofibromatosis type 2-related tumors[1], sporadic vestibular schwannomas are not pharmacotherapeutically treated. One of the clinical problems regarding vestibular schwannoma therapy is the large variability in growth rate these tumors can display. More understanding of this variable growth rate would be of great benefit when determining the most suitable therapeutic approach. This requires more insight into tumor biological factors affecting vestibular schwannoma progression. Studying the biology of the vestibular schwannoma not only contributes to a better understanding of its growth pattern, it may also help to identify potential therapeutic targets.

To date the tumor biology of sporadic vestibular schwannomas is poorly understood. An important factor in the development of schwannomas in general is loss of function of *NF2* (neurofibromin 2), which acts as a tumor suppressor gene[2-5]. Inactivation of the *NF2* gene has been described in both neurofibromatosis type 2-related as well as sporadic vestibular schwannomas[6-9]. Reports regarding the sporadic tumors described *NF2* mutations in a majority of cases [6-11]. Nevertheless a significant proportion of sporadic vestibular schwannomas do not seem to harbour a proven *NF2* mutation. Studies investigating the *NF2* gene product in schwannomas, both at RNA- as well as protein level, demonstrated absent or decreased expression of *NF2* gene products in a higher percentage of tumors then expected with regard to the percentage of tumors containing a proven *NF2* mutation [9,12-14]. These findings suggest that in addition to mutational changes of *NF2* other mechanisms are implicated in deregulating *NF2* gene products. A study by Kino et. al.[15] demonstrated aberrant methylation of *NF2* in 14 out of 23 schwannomas, both NF2-related(n=3) as well as sporadic(n=20). A more recent study by Kullar et. al. [16] also investigated the role of methylation in vestibular schwannomas. They reported aberrant methylation of *NF2* in only 10% of the investigated samples. In summary, quite some discrepancies between reports on the incidence of *NF2* mutations, loss of *NF2* gene product and epigenetic aberrations of *NF2* exist. The combination of these discrepancies and the fact that no associations between aberrant *NF2* expression and tumor growth have been demonstrated, leads to the impression that next to loss of function of *NF2* other mechanisms are implicated in vestibular schwannoma development.

A recent finding supporting this suggestion was reported by Serrano et al. [17] They identified

the presence of *BRAF*^{v600E} mutations in a number of sporadic non head and neck schwannomas. The presence of *BRAF* mutations in schwannomas has been investigated before. Schindler et al. [18] investigated *BRAF*^{v600E} mutations in 1320 nervous system tumors, including 14 schwannomas. None of these schwannomas contained the *BRAF*^{v600E} mutation. Among the tumors that did harbour *BRAF* mutations were WHO grade II pleomorphic xanthoastrocytomas (42/64; 66%), pleomorphic xanthoastrocytomas with anaplasia (15/23;65%), WHO grade I gangliogliomas (14/77; 18%), WHO grade III anaplastic gangliogliomas (3/6) and pilocytic astrocytomas (9/97;9%). Alterations of *BRAF* in pilocytic astrocytomas have been described in other reports as well [19,20]; Additioinal tumors of glial origin associated with *BRAF* mutations are glioblastomas [21] and oligodendroglial tumors [22], in both cases mutations occurred at low frequency. A relatively recent study on malignant peripheral nerve sheath tumors (MPNST) screened for multiple gene mutations including *BRAF, PIK3CA* and *RAS* [23]. No *BRAF* or *PIK3CA* mutations could be identified but 2 out of 11 sporadic MPNSTs contained mutations to the *RAS* gene.

Both *NF2* [24-28] as well as *BRAF* [29] are known to be involved in the regulation of the MAPK/ERK pathway (Figure 1). The MAPK/ERK pathway consists of a cascade of tyrosine kinase proteins that mediate cellular responses like cell division, differentiation and survival[30,31]. An estimated 30% of all human cancers harbour mutations related to this pathway [32] with mutations of the *BRAF* gene being the most frequent[29]. A relatively recent global gene expression profile analysis performed by Aarhus et. al. [6] subscribes the role of this pathway in the pathogenesis of sporadic vestibular schwannomas. The fact that mutated *BRAF* and other members of the MAPK/ERK pathway form potential targets for pharmacological treatment[29,33] emphasizes the relevance of investigating their involvement in vestibular schwannoma development.

The goal of this study was to investigate the hypothesis that mutations affecting the MAPK/ERK pathway, *BRAF*^{v600E} in particular, play a role in the development of sporadic vestibular schwannomas and may even account for the subgroup of tumors exhibiting rapid tumor growth.

In order to test this hypothesis we have conducted an allele specific quantitative real-time PCR assay with hydrolysis probes for the most frequent activating mutations related to MAPK/ERK pathway activation. The results of this mutation analysis were compared with clinical parameters such as tumor size and tumor growth rate.

Materials and Methods

Patient selection

From the vestibular schwannoma database at the Leiden University Medical Center material of a total of 48 out of 315 patients, operated between January 2005 and July 2011, was

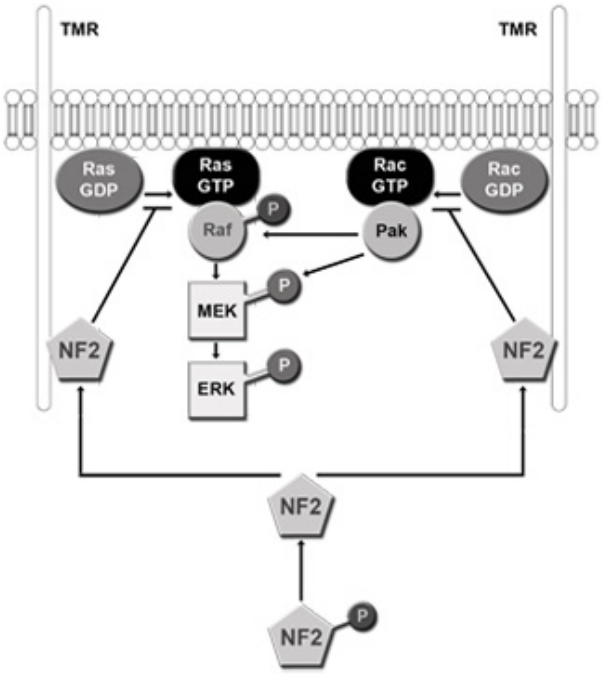

Figure 1. NF2 involvement in the MAPK/ERK pathway. NF2 becomes active through dephosphorylation. Dephosphorylated NF2 binds to a transmembrane receptor (TMR). NF2 then blocks the activation of Ras and Rac thereby inhibiting phosphorylation of Raf and MEK by PAK. NF2 also inhibits signaling from constitutively active RAS. (Cell signaling model as adopted from Morrison H. et al Cancer Res 2007;67:520-527)

selected. The selection consisted of patients surgically treated for a histologically proven primary sporadic vestibular schwannoma. Patients with a tumor exceeding 20 mm in diameter were operated in cooperation with the Department of Neurosurgery; no patients diagnosed with neurofibromatosis type 2 were included. Decision for surgical treatment was based on clinical symptoms (e.g. tinnitus, vertigo, hearing loss, increased cerebrospinal pressure and tumor size), radiologically observed tumor growth and patients' personal preference.

We selected two different patient groups based on first clinical presentation. Group 1 consisted of 30 consecutive patients with tumors smaller than 20 mm in diameter at first diagnosis. These patients initially enrolled into the wait and scan protocol. Surgery was performed only after tumor growth was observed during radiological follow up. Tumor growth rate was determined by comparing the maximal tumor diameter measured on two sequential MRI scans and is expressed in millimetres per year[34]. Because only patients with tumors of small- to moderate size were monitored over time, few patients with larger tumors were present in this first group. In order to study a patient cohort representing the entire spectrum of tumor sizes we added a second group of patients to our selection. Group 2 consisted of

18 consecutive patients with large tumors, exceeding 30 mm in size at initial diagnoses. Because patients with large tumors are operated shortly after diagnosis, no radiological follow up on tumor growth rate is available for these patients.
All tumor samples were handled in a coded fashion and all procedures were performed according to the ethical guidelines of the Code for Proper Secondary Use of Human Tissue in The Netherlands (Dutch Federation of Medical Scientific Societies).

DNA isolation

DNA was extracted from formalin fixed paraffin embedded tumor blocks. To ensure the tumor samples contained sufficient amounts of tumor cells (> 70%) H&E-stained slides of all samples were evaluated on percentage of tumor cells in relation to non-neoplastic cells. In one case microdissection was required in order to obtain an adequate percentage of tumor cells. DNA was extracted from 10 µm sections of paraffin-embedded tissue and subsequently purified using a NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany). For DNA quantification a UV/VIS spectrometry analysis with a NanoDrop ND-1000 spectrometer (Thermo scientific®, New York, New York) was performed.

***BRAF, EGFR, PIK3CA* and *KRAS* mutation analysis**

Exact details regarding the allele specific quantitative real-time PCR (qPCR) with hydrolysis probes (Applied Biosystems, Nieuwerkerk a/d IJsel, NL) that was conducted have been described before[35]. In short, the assay contained mutation specific hydrolysis probes for the detection of one *BRAF*, two *EGFR*, three *PIK3CA* and seven *KRAS* mutations (table 1.). The $BRAF^{v600E}$ mutation included in this assay accounts for more than 90% of all *BRAF* mutations described in human cancer [36]. The deletion of exon 19 and the point mutation in exon 21 at nucleotide 2573 of chromosome 7 account for approximately 90% of all mutations affecting *EGFR* [37]. For *KRAS* 95% of all activating mutations are located in exon 1 (codons 12 and 13) [38]. The hotspot mutations of *PIK3CA* included in this analysis cover approximately 80% of all mutations to this gene [39].
All qPCR reactions were performed on a sealed LightCycler 480 multiwell Plate 384 (Roche Applied Science) in a LightCycler 480 Multiwell system (Roche diagnostics). For quality assessment the quantification cycle (Cq) was taken into account. Samples with Cq values exceeding 35 in the wild-type channel were rejected and excluded from further analysis. The endpoint fluorescence ratio R_m/R_{wt} was calculated to determine the presence or absence of a mutation. In case the R_m/R_{wt} ratio exceeded 0.7 a sample was considered positive for that specific mutation. An R_m/R_{wt} ratio smaller than 0.3 indicated the mutation was absent. Allele specific quantitative real-time PCR is a reliable and sensitive technique for the detection of mutations in *BRAF, EGFR*, *PIK3CA* and *KRAS* and has been validated in several studies investigating different types of tumors [35,40-45]. This technique is also part of the routine mutation detection protocol deployed by the Molecular Diagnostics department in our hospital.

Table 1. analyzed mutations

Gene	DNA mutation	Protein modification
KRAS	c.34G>A	p.G12S
	c.34G>C	p.G12R
	c.34G>T	p.G12C
	c.35G>A	p.G12D
	c.35G>C	p.G12A
	c.35G>T	p.G12V
	c.38G>A	p.G13D
EGFR	c.2573T>G	P.L858R
	deletion exon 19	deletion
BRAF	c.1799T>A	p.V600E
PIK3CA	c.1624G>A	p.E542K
	c,1633G>A	p.E545K
	c.3140A>G	p.H1047R

Results

A total of 48 patients, 21 (44%) male and 27 (56%) female were studied. Patient age ranged from 21 to 81 years with an average of 53.2 (SD ± 11.9) years. In patient group 1 tumor size varied from 7 to 49 mm (mean 16.4 ± 9.8) and tumor growth rate varied from -1.3 to 33.9 millimetres per year (mean 4.1 ± 6.1). Tumor size in patient group 2 varied from 30 to 46 millimetres (mean 36.3 ± 5.3) Tumor size in the total patient selection varied from 7 to 49 millimetres (mean 23.8 ± 12.8). As expected, tumor size in group 2 was significantly larger than tumor size in group 1. No other significant differences existed between the two groups. Exact details on statistical analysis and patient characteristics of clinical data are listed in table 2 and table 3 respectively.

BRAF, EGFR, PIK3CA and *KRAS* mutation analysis

In none of the 48 investigated patients mutations affecting *BRAF, EGFR, PIK3CA* or *KRAS* were detected (see Figure 2 for an example of the qPCR results). All assays for *BRAF, PIK3CA* and *KRAS* gave Cq values <35. In four cases the Cq values of the *EGFR* assay exceeded 35 making interpretation of the endpoint fluorescence ratio for these samples less reliable, however indications for the presence of a mutation in these cases were unlikely.

Table 2. statistical analysis of clinical data

Clinical parameter	Total	Group 1	Group 2	P
age, year	53,2 ± 11,9	55,4 ± 11,2	49,3 ± 12,2	0,85†
female, %	56	56	56	0,94‡
size	23,8 ± 12,8	16,4 ± 9,8	36,3 ± 5,3	<0.00001†
growth, mm/year	-	4,1 ± 6,1	-	-

P: † t-test and ‡ chi-squared; ± indicate SD

Table 3a. patient characteristics and qPCR results of smaller tumors

Sample	Sex	Age (yr)	Size (mm)	Growth mm/yr	KRAS	EGFR	BRAF	PIK3CA
Group 1								
L3716	M	69	17,6	-1,3	WT	WT	WT	WT
L3717	F	67	12,9	2,7	WT	WT	WT	WT
L3718	M	46	9,9	3,5	WT	WT	WT	WT
L3719	F	60	11,6	2,1	WT	WT	WT	WT
L3720	M	48	17,3	0,6	WT	WT	WT	WT
L3721	M	60	6,8	0,5	WT	WT	WT	WT
L3722	M	53	12,7	3,4	WT	WT	WT	WT
L3723	M	53	19,7	0	WT	LS	WT	WT
L3724	F	34	10,7	4,5	WT	WT	WT	WT
L3725	F	62	15,1	2,6	WT	WT	WT	WT
L3727	F	46	9	1,5	WT	LS	WT	WT
L3728	M	56	13,7	3	WT	WT	WT	WT
L3729	M	52	16,8	4,4	WT	LS	WT	WT
L3730	F	65	11,3	2,8	WT	WT	WT	WT
L3731	F	81	45,9	5,8	WT	WT	WT	WT
L3732	F	69	13,2	8,4	WT	WT	WT	WT
L3734	M	35	24,8	5,1	WT	WT	WT	WT
L3735	F	41	8,3	0	WT	WT	WT	WT
L3736	F	69	24,3	4,2	WT	WT	WT	WT
L3737	M	59	14,2	3,1	WT	WT	WT	WT
L3738	F	60	10,8	2,6	WT	WT	WT	WT
L3739	M	61	9,2	1,8	WT	WT	WT	WT
L3740	F	52	14,6	9,4	WT	WT	WT	WT
L3741	F	71	24,8	2,9	WT	WT	WT	WT
L3742	F	54	12	4,3	WT	WT	WT	WT
L3743	F	38	11,1	3,5	WT	WT	WT	WT
L3744	M	46	7,9	0	WT	WT	WT	WT
L3745	F	56	5,6	5,6	WT	WT	WT	WT
L3746	M	46	49,3	33,9	WT	WT	WT	WT
L3747	F	53	15,4	2,7	WT	WT	WT	WT

WT: wild type signal , LS : Low signal.

Table 3b. patient characteristics and qPCR results of larger tumors

Sample	Sex	Age (yr)	Size (mm)	Growth mm/yr	KRAS	EGFR	BRAF	PIK3CA
L3611	F	39	43	-	WT	WT	WT	WT
L3749	F	59	32	-	WT	WT	WT	WT
L3613	M	49	36	-	WT	WT	WT	WT
L3618	F	49	46	-	WT	WT	WT	WT
L3752	M	30	35	-	WT	WT	WT	WT
L3753	F	48	3	-	WT	WT	WT	WT
L3754	M	51	39	-	WT	WT	WT	WT
L3755	F	61	34	-	WT	WT	WT	WT
L3756	M	58	43	-	WT	WT	WT	WT
L3757	M	65	37	-	WT	WT	WT	WT
L3758	F	58	38	-	WT	WT	WT	WT
L3759	M	47	30	-	WT	WT	WT	WT
L3760	F	21	38	-	WT	WT	WT	WT
L3761	F	30	45	-	WT	LS	WT	WT
L3762	M	60	30	-	WT	WT	WT	WT
L3751	M	60	30	-	WT	WT	WT	WT
L3750	F	53	33	-	WT	WT	WT	WT
L3748	F	50	35	-	WT	WT	WT	WT

WT: wild type signal , LS : Low signal.

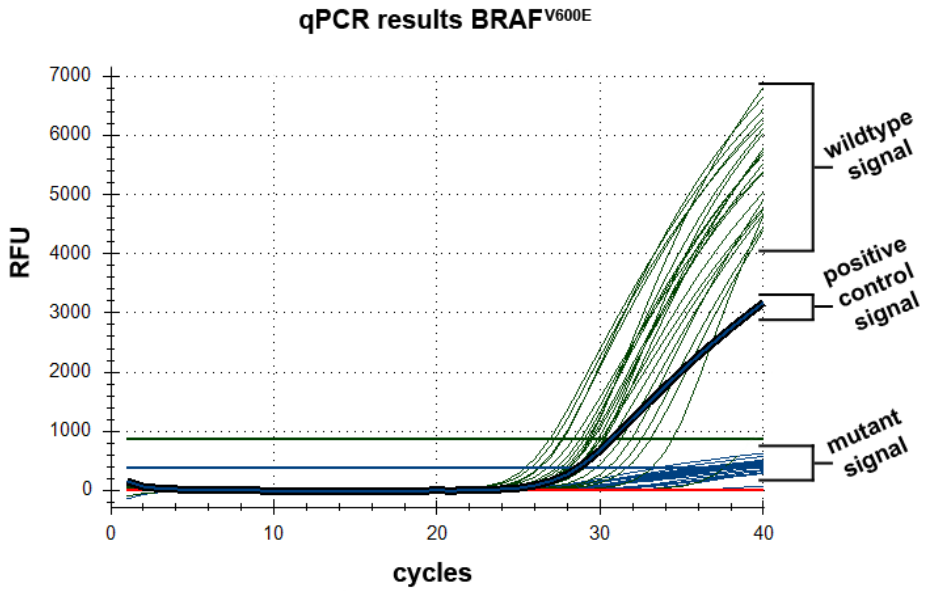

Figure 2. Example of qPCR results for the BRAF assay.

Discussion

In this study we report on the results of an allele specific quantitative real-time PCR assay for the most frequent activating mutations of *BRAF, EGFR, PIK3CA* and *KRAS* in 48 sporadic vestibular schwannomas. The allele specific quantitative real-time hydrolysis probe PCR assay that was conducted is a reliable and highly sensitive technique for the detection of mutational hotspots in the *BRAF, EGFR, PIK3CA* and *KRAS* genes.

Using this technique no mutations could be demonstrated. The fact that no mutations were found in this cohort of 48 tumors suggests that hotspot activating mutations of *BRAF, EGFR, PIK3CA* and *KRAS* do not play a significant role in sporadic vestibular schwannoma pathogenesis. This outcome supports the results by Shindler et al. but remains in contrast with the findings of Serrano et. al. [17]. They detected *BRAF* mutations in 3 out of 16 investigated sporadic non head and neck schwannomas. The origin of the schwannomas investigated by Schindler et al was not specified but maybe analogous to the situation in the uveal melanomas, which in contrast to cutaneous melanomas very rarely contain *BRAF* mutations [46,47], there is a location dependency for *BRAF* mutations in sporadic schwannomas as well. So far the biological mechanisms responsible for this apparent location dependent incidence of *BRAF* mutations remain unclear.

Next to *BRAF, EGFR* has been analysed in earlier studies on schwannoma pathogenesis as well. An immunohistochemical study on 22 vestibular schwannomas by Sturgis et al. [48] demonstrated positivity for *EGFR* in three fourths of their samples. Prayson et al. investigated *EGFR* via immunohistochemistry and fluorescent in situ hybridization but was not able to detect any *EGFR* expression or amplification [49]. The later study combined with our results indicate that if *EGFR* is upregulated in schwannomas this probably is not caused by changes to the gene itself but more likely a result of diminished inhibition by its upstream regulator *NF2* [50].

To date the genetic profile of vestibular schwannomas has not been fully characterized. As mentioned before, the major genetic alteration involved in sporadic vestibular schwannoma genesis is inactivation of the *NF2* gene. However, there seems to be a subpopulation of tumors without a proven *NF2* mutation[6-8,11]. One explanation for this subpopulation might be the involvement of genes other then *NF2* but so far no clear evidence proving the presence of such genes has been provided. Another explanation could be that the mutation detection techniques that have been used were simply not sensitive enough. Even the most extensive mutation assays did not cover the entire *NF2* gene [51]. Other factors that may have reduced the sensitivity of these tests are contamination of tumor tissue or epigenetic processes like aberrant methylation [15,52].

Recent developments in DNA sequencing technologies like “next-generation sequencing”[53] make analysis of the entire *NF2* gene, including epigenenomic assays [54], possible and might offer solutions to clarify this matter. If indeed all sporadic vestibular schwannomas

arise by inactivation of *NF2* and no other genetic alterations play a major role in vestibular schwannoma biology the question remains which other mechanisms are responsible for these tumors' phenotypical variability.

In this context we have recently performed a pilot study which focussed on the intratumoral microenvironment of the vestibular schwannoma and its role in tumor growth[55]. In this study we detected CD68 positive macrophages in a majority of tumors; the expression rate of these macrophages correlated with the degree of tumor vascularisation and with clinical markers of tumor growth. The importance of the intratumoral inflammatory microenvironment has been established in several types of cancer [56] and might be an important biological mechanism affecting tumor growth of the vestibular schwannoma as well.

In summary we conclude that the most frequent mutations affecting *BRAF, EGFR, PIK3CA* and *KRAS* do not play a major role in sporadic vestibular schwannoma biology. So far no genes other than *NF2* have been proven to be associated with this type of tumor. Whether the variable growth pattern of sporadic vestibular schwannomas is based on a specific genetic background or other biological mechanisms such as the intratumoral microenvironment remains to be established.

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CHAPTER 5

Tumor-associated macrophages are related to volumetric growth of vestibular schwannomas

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Abstract

Objective: assess whether the expression of CD163 positive macrophages in sporadic vestibular schwannomas is associated with angiogenesis and tumor growth.

Background: an important clinical problem regarding vestibular schwannoma treatment is their variable growth rate. Tumor biological research can help to clarify this growth rate and may offer targets for therapy. Inflammation is an important biological process involved in the development of many solid tumors. Macrophages are major determinants of intratumoral inflammation. Macrophages can be divided into two groups; the M1- and M2-type macrophages. M2-type macrophages are associated with tumor promoting processes like angiogenesis, tumor cell growth and downregulation of the antitumor immune response. Both macrophages and angiogenesis can serve as targets for therapy. CD163 is a specific marker for M2-type macrophages.

Methods: CD163 expression in 10 fast growing vestibular schwannomas was compared with CD163 expression in 10 slow growing vestibular schwannomas. Tumor growth was determined by comparing preoperative tumor volume measurements on MRI. The relation between macrophage expression and angiogenesis was evaluated by assessing microvessel density (CD31).

Results: CD163 expression and microvessel density were significantly higher in fast growing vestibular schwannomas ($p<0,001$ and $p=0,019$ respectively). Tumors with higher CD163 expression contained significantly more microvessels ($p=0.014$).

Conclusion: this study demonstrates that M2-type macrophages in vestibular schwannomas relate to angiogenesis and volumetric tumor growth. These results imply that the M2-type macrophage infiltrate contributes to progressive tumor growth making it a potential target for pharmacological therapy.

Introduction

Vestibular schwannomas are benign tumors recapitulating the differentiation repertoire of the myelin-forming Schwann cells of the vestibular branch of the eighth cranial nerve in the internal auditory canal or the cerebellopontine angle. These tumors grow slowly and progressively, ultimately causing brainstem compression. Therapeutic management of vestibular schwannomas can be divided into three main strategies i.e. microsurgery, stereotactic radiotherapy or serial radiological observation, also known as the wait & scan policy. So far, unlike neurofibrosis type 2-related tumors [1,2], sporadic vestibular schwannomas are not pharmacotherapeutically treated One of the main problems in determining the most suitable strategy is the large variability in growth rate these tumors can display. More understanding of this variable growth rate would be of great benefit when determining the most suitable therapeutic approach. This requires more insight into tumor biological factors affecting vestibular schwannoma growth. Studying vestibular schwannoma biology not only contributes to a better understanding of its growth pattern, it may also help to identify potential therapeutic targets.

Recently we have performed a pilot study on histopathological characteristics of vestibular schwannoma growth which indicated a significant correlation between CD68 positive macrophages and clinical tumor growth [3]. So far little is known about the mechanism and biological value of the inflammatory process in vestibular schwannomas. A possible explanation may come from research on other types of tumors [4-9]. It is supposed that the inflammatory microenvironment has many tumor promoting effects [10]. One of the major determinants of the inflammatory infiltrate are macrophages [11]. These so called tumor associated macrophages (TAMs) can, based on their immunological functions, roughly be divided into two different categories [12]. The first category comprises the classically activated or M1 type macrophages. M1 macrophages have a defensive purpose; they are inflammatory and initiate cytotoxic responses against tumor cells and intracellular pathogens [13,14]. Monocytes differentiate into M1 type macrophages in response to signals produced by bacterial products like lipopolysaccharides or by cytokines such as IFNγ [15]. M1 macrophages are characterized by the expression of proinflammatory cytokines such as IL-12, IL-1 and IL6[16]. Upon activation M1 macrophages start to produce nitric oxide and reactive oxygen species, they stimulate cytokine-induced cytotoxicity and promote natural killer and T-cell activity. Altogether these processes result in a strong antitumor effect. The alternatively activated M2 macrophages on the other hand possess different functions. Monocytes differentiate into M2 type macrophages in response to cytokines like IL-4 and IL-13 [17]. M2 macrophages express scavenger receptors such as CD163 [4,15,18-22] and are associated with production of for instance IL-10, IL-1b and vascular endothelial growth factor (VEGF). M2 macrophages dampen anti-tumor inflammatory responses, participate in remodelling and repair of damaged tissues and stimulate angiogenesis [11,12,23]. The combination of these

functions results in a tumor promoting effect. It should be noted that the classification of macrophages into M1 and M2 subtypes is an oversimplification of the actual macrophage population in order to increase the comprehensibility of macrophage differentiation.
The aim of this study was to investigate if the expression of M2 type macrophages in sporadic vestibular schwannomas is associated with angiogenesis and tumor growth.

Materials and methods

Multiple studies on different types of tumors indicate that the majority of tumor associated macrophages are of the M2 type and highly express CD163 [19-22], making it an excellent marker to study M2 macrophages. We performed immunofluorescent stains against CD163 on formalin fixed paraffin embedded vestibular schwannoma tissue. To establish the relation between macrophage activity and vestibular schwannoma growth we analysed and compared the expression pattern of this marker in tumor samples of 10 radiologically observed fast growing and 10 radiologically observed slow growing tumors. In order to study the association between M2 type macrophage expression and angiogenesis the degree of CD31 positive microvessels was evaluated.

Patient selection

Patients were retrospectively selected from the vestibular schwannoma database at the Leiden University Medical Center. The selection involved a group of consecutive patients surgically treated for a histologically proven vestibular schwannoma from October 2006 to December 2011. Two separate patient cohorts were composed. The first cohort comprised 10 cases of radiologically observed evident slow growing tumors. The second cohort comprised 10 cases of radiologically observed evident fast growing tumors. Decision for surgical treatment was based on clinical symptoms (e.g. tinnitus, vertigo and hearing loss), the presence of increase of tumor size on sequential MRI scanning and patients personal preference. Patients diagnosed with NF2 were excluded from this study.
All patient samples were handled in a coded fashion and all procedures were performed according to the ethical guidelines of the Code for Proper Secondary Use of Human Tissue in The Netherlands (Dutch Federation of Medical Scientific Societies).

Tumor measurement

All tumor measurements were conducted on T1-weighted gadolinium enhanced MRI examinations and carried out by one and the same author. Volume measurements were performed with a contour measurement method using Vitrea View software (Vital Imaging ™, Minnetonka, Minnesota, U.S.A.) (Figure 1). For validation purposes tumor volume was measured twice for each MRI scan. The mean of these two measurements was used for

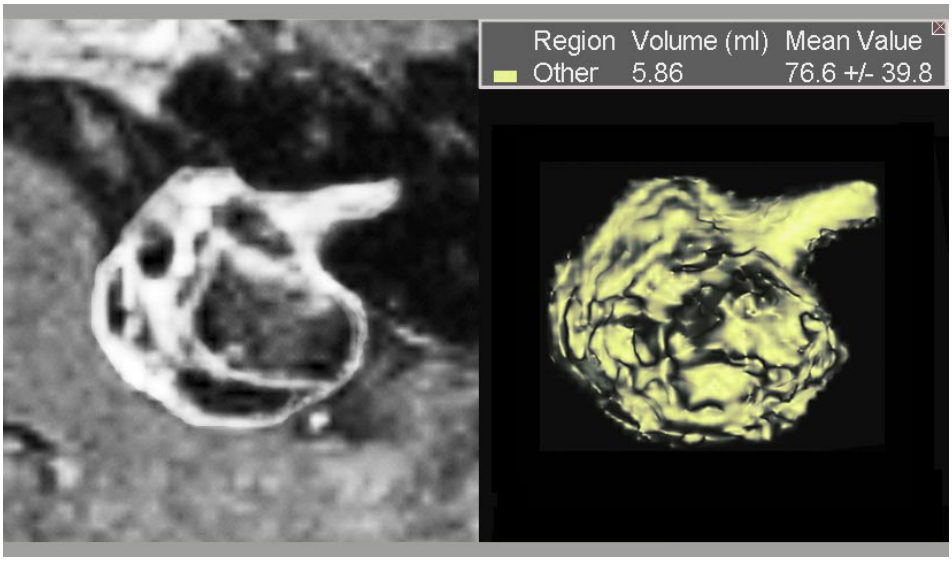

Figure 1. Example of volume measurement of left sided vestibular schwannoma performed with the contour measurement method on a T1-weighted gadolinium enhanced MRI scan.

further evaluation of tumor growth. Tumor growth rate was determined by comparing tumor volume on two sequential MRI scans and expressed in millilitres per year.

Enzymatic and fluorescent immunostainings

Immunohistochemical stainings were conducted on 4-µm thick slides obtained from formalin fixed and paraffin embedded vestibular schwannoma tissue. All stainings were performed on one single tumor block per tumor sample. For CD31 (DAKO) enzymatic immunohistochemical reactions were carried out according to standard laboratory methods [24]. In brief, heat-induced citrate buffer antigen retrieval was performed using microwave treatment of all slides after dewaxing and rehydration, followed by blocking of endogenous peroxidase with 3% H2O2/methanol. Primary antibodies were incubated over night at room temperature. Slides were incubated with Poly-HRP-Anti mouse/rabbit/rat I 69 (immunologic, DVPO 110HRP). Visualisation was carried out in a 3.3'-diamino-benzidine-tetrahydrochloride (DAB) solution after the addition of hydrogen peroxide 30% shortly before application. All washing procedures were performed in phosphate buffered saline (PBS). Slides were counterstained with haematoxylin.

For CD163 immunofluorescent (Novocastra, Newcastle Upon Tyne, UK) stains were performed. After tris EDTA (pH 9.0) heat induced-antigen retrieval the primary antibody was incubated over night, followed by incubation with a secondary fluorescent antibody (Alexa goat-anti-mouse IgG1 AL-647 (Invitrogen) .

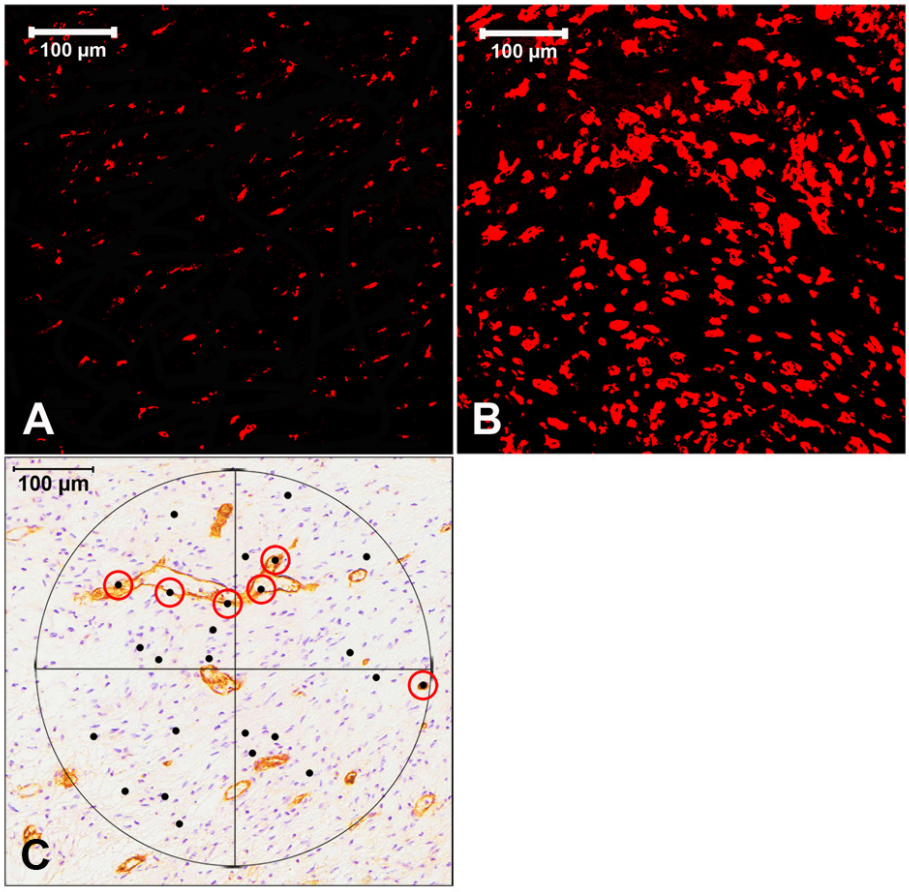

Figure 2. CD163 immunofluorescent stain for macrophages in vestibular schwannoma (A) low expression; (B) high expression. Original magnification X250. (C) example of the Chalkley count for microvessel density. The circled grid points hit CD31 stained micro vessels. Original magnification X 200

Microscopic analysis

CD163 stained slides were analysed using a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany). This analysis method has been described with regard to macrophage expression analysis before [22]. Slides were scanned using a fixed laser-filter. Alexafluor 647 (red) was exited at 633 nm and detected with a 650 nm long-pass filter. On the digitalized scans the fluorescent signal was represented by an artificial colour (red). Per scan the microscope was focussed to pick up the maximum amount of fluorescent signal. All images were 1,024 x 1,024 pixels, stack size 521.2 µm x 521.2 µm and a 25x objective was used (Figure 2a ,2b).

All assessments were carried out blinded with regard to clinical tumor characteristics. Per tumor slide 5 randomly chosen scans were obtained. These scans were saved in a JPEG

format. Using ImageJ (National Institutes of Health, Bethesda, MD) images were converted to a binary configuration. All images were uniformly thresholded and 'despeckeled' in order to minimize overexposure effects and to reduce background noise. The remaining number of positive, red pixels was calculated and expressed as the area fraction which served as the score for macrophage expression. The average count of the five scans per tissue slide was used for statistical analysis.

Microvessel density was determined using the Chalkley point overlap technique. This technique allows for rapid analysis with a relatively low interobserver variability and has been described in detail before [25,26] . In brief, CD31 stained slides were scanned for vascular hot spots. Using an ocular grid with 25 random points these hot spots were scored at a 200x magnification. The grid was orientated to permit the maximum number of points hitting the stained microvessels (Figure 2c). The Chalkley count was the average of the maximum number of points hitting a microvessel in the three most prominent vascular hotspots per tumor slide.

Statistics

The difference in CD163 expression and microvessel density in the slow vs. fast growing tumors was determined with the Mann-Whitney U test. Two tests were performed to evaluate the relationship between CD163 expression and microvessel density. For the first test the total patient cohort (n=20) was divided into two groups with regard to the degree of CD163 expression. The Mann-Whitney U test was used to compare the degree of microvessel density in tumors displaying lower CD163 expression with the degree of microvessel density in tumors displaying higher CD163 expression. The second test was a Spearman correlation test comparing CD163 expression and microvessel density. For all statistical tests a level of significance of $p < 0.05$ was taken into account. All calculations were performed using SPSS inc. software, version 16.0.

Results

Details on patient characteristics are listed in table 1. No significant differences in distribution of age, gender and duration of preoperative radiological follow up were observed in the two patient cohorts. As expected, there was a significant difference in tumor growth rate and tumor volume between the two groups.

Immunofluorescent staining for CD163 was performed on tissue sections obtained from 10 fast growing and 10 slow growing sporadic vestibular schwannomas. All tumors displayed a certain degree of immunopositivity for CD163. CD163 expression was significantly higher ($p<0.001$) in fast growing tumors compared to slow growing tumors (Figure 3a). The degree of microvessel density was also significantly higher in fast growing tumors ($p=0.019$) compared to slow growing tumors (Figure 3b). The degree of microvessel density was

Table 1. patient characteristics

Case	Sex	Age (yrs)	Tumor volume (ml)	Growth rate (ml/yr)	Preoperative mri follow up (months)
Slow growing group					
L3721	M	60	0.35	0.06	13
L3742	F	54	0.27	0.21	7
L3773	F	52	0.22	0.07	6
L3774	F	50	0.88	0.19	27
L3775	M	53	0.69	0.17	10
L3779	F	51	1.46	0.18	32
L3780	F	56	0.74	0.25	11
L3781	F	44	0.53	0.19	12
L3787	M	58	0.52	0.18	12
L3797	F	60	2.13	0.23	12
mean (±SD)		53.8 (±4.96)	0.77 (±0.59)	0.17(±0.06)	14.2 (±8.46)
Fast growing group					
L3725	F	62	3.26	1.33	11
L3731	F	81	14.41	4.41	19
L3733	F	76	11.66	8.84	10
L3740	F	52	2.27	2.50	7
L3741	F	71	7.25	1.83	21
L3745	F	56	5.62	2.18	6
L3746	M	46	30.73	43.98	4
L3792	F	75	6.92	3.43	21
L3793	F	39	7.32	5.52	10
L3805	F	39	23.05	2.90	9
mean (±SD)		59.7 (±15.65)	11.24 (±9.16)	7.69 (±12.94)	11.8(±6.26)
difference fast vs. slow (p)	0.26a	0.41b	*<0.0001b*	*<0.0001b*	0.31b

[a] Chi-square test, [b] Mann-Whitney U test, results at p ≤ 0,05 are shown in italic

significantly higher (p=0.014) in tumors displaying high CD163 expression (Figure 3c), and the Spearman correlation test showed a significant positive relation (p=0.024; r=0.50) between CD163 expression and microvessel density.

Discussion

Many, if not all solid tumors contain a certain degree of inflammation[27]. Macrophage activity is a major determinant of the intratumoral inflammatory microenvironment [11]. As mentioned earlier, these tumor infiltrating macrophages, especially the alternatively activated M2 type macrophages, have been associated with tumor progression by stimulating angiogenesis,

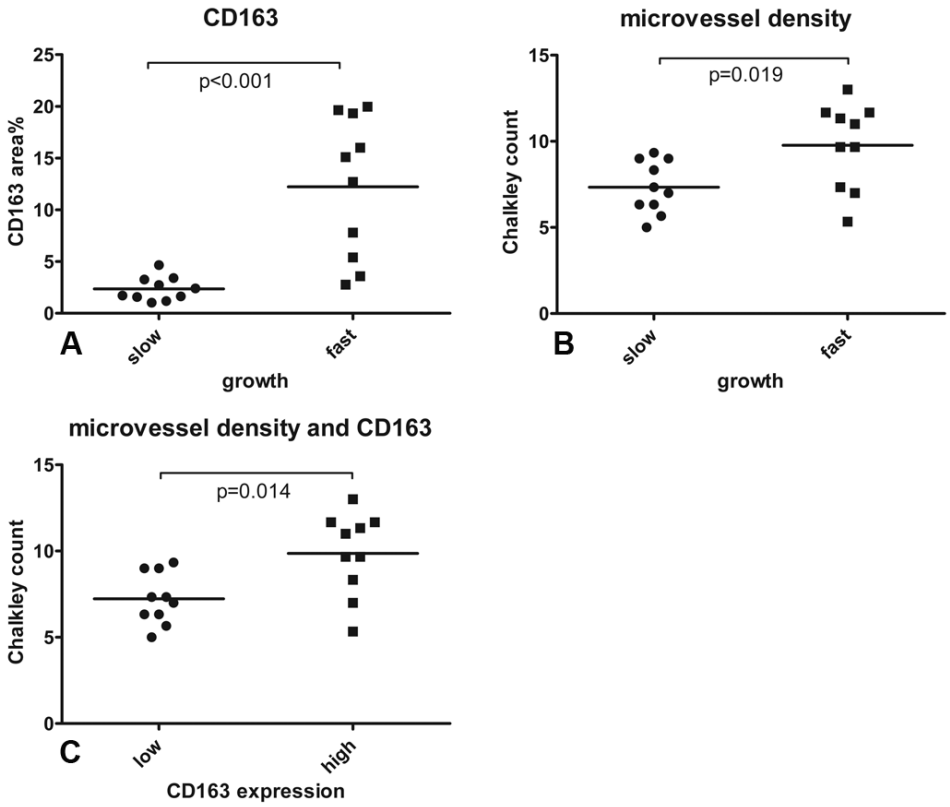

Figure 3. (A) CD163 expression in slow versus fast growing tumors (Mann-Whitney U test), (B) microvessel density in slow versus fast growing tumors(Mann-Whitney U test) and (C) microvessel density in tumors with low versus high CD163 expression (Mann-Whitney U test).

tumor cell growth and down regulation of the antitumor immune response. Although the majority of studies on macrophages in human tumors support this tumor promoting model it should be noted that a number of studies reported contradicting results in which high macrophage counts were associated with better prognosis [28-30]. Presumably different types of tumors give rise to different types of inflammatory infiltrates resulting in different effects on tumor development.

In this study we have compared the expression of the M2 macrophages in a group of 10 slow growing tumors with the expression of this marker in a group of 10 fast growing tumors. We also compared the degree of microvessel density between these two groups and finally we investigated the relationship between macrophage expression and microvessel density. It should be taken into account that the outcomes of these comparisons remain observations of association. There is always a possibility that these findings are epiphenomena of a larger biological growth dynamic and therefore not directly linked to one another.

We used volumetric measurements to assess tumor size because this technique is more precise than the conventional 2 dimensional tumor measurements [31,32] Tumor growth rate was measured by comparing tumor volume on serial preoperative MRI scans and expressed in millitres per year. As demonstrated in table 1. the group of fast growing vestibular schwannomas contained significantly larger tumors than the group of slow growing vestibular schwannomas. For this reason our findings might not only be based on tumor growth rate alone, they may be a related to overall tumor size as well.

In this study we only included evidently slow growing tumors and evidently fast growing tumors. In reality a significant proportion of vestibular schwannomas display a more intermediate growth rate. However, because of the explorative nature of this study we chose to leave out this portion of intermediate growing tumors in order to maximize the chances of demonstrating or ruling out significant differences between the patient groups.

The degree of CD163 positive macrophages was significantly higher in the group of fast growing tumors. These results indicate that M2 macrophages are associated with fast growing vestibular schwannomas. The fact that the fast growing tumors also displayed a higher degree of microvessel density combined with the fact that there was a significant relationship between macrophage expression and microvessel density supports the concept that M2 macrophages play a substantial role in vestibular schwannoma biology.

Several therapeutic strategies targeting macrophage activity at different levels are currently under investigation. There are studies aiming to inhibit the attraction and induction of M2 macrophages [23,33,34] while others investigate the possibility of redirecting macrophages with a tumor promoting M2 phenotype towards macrophages with a antitumor M1 phenotype[35]. If these strategies prove to be effective, tumor associated macrophages might become novel therapeutic targets for the treatment of vestibular schwannomas. However, before the inflammatory process can be considered a realistic target in vestibular schwannoma therapy more extensive research on tumor related effects of macrophages and their interactions with other elements of the immune system is required.

In conclusion, this study demonstrates that M2-type macrophage expression in vestibular schwannomas is associated to angiogenesis and volumetric tumor growth. These results imply that the M2-type macrophage infiltrate may contribute to the progression of these tumors making it a potential target for future pharmacotherapeutical therapy.

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CHAPTER 6

BCRP expression in schwannoma, plexiform neurofibroma and MPNST

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Abstract

Objective: to assess if BCRP is expressed in vestibular schwannomas, plexiform neurofibromas and MPNST

Background: peripheral nerve sheath tumors comprise a broad spectrum of neoplasms. Vestibular schwannomas and plexiform neurofibromas are symptomatic albeit benign, but a subset of the latter pre-malignant lesions will transform to malignant peripheral nerve sheath tumors (MPNST). Surgery and radiotherapy are the primary strategies to treat these tumors. Intrinsic resistance to drug therapy characterizes all three tumor subtypes. The breast cancer resistance protein BCRP is a transmembrane efflux transporter considered to play a key role in various biological barriers such as the blood brain barrier. At the same time it is associated with drug resistance in various tumors. Its potential role in drug resistant tumors of the peripheral nervous system is largely unknown..

Methods: immunohistochemical staining for BCRP was performed on a tissue microarray composed out of 22 sporadic vestibular schwannomas, 10 plexiform neurofibromas and 18 MPNSTs.

Results: sixteen out of twenty-two vestibular schwannomas (73%), nine out of ten plexiform neurofibromas (90%) and six out of eighteen MPNST (33%) expressed BCRP.

Conclusion: BCRP is present in the vasculature of vestibular schwannomas, plexiform neurofibromas and MPSNT. Therefore, it may reduce the drug exposure of underlying tumor tissues and potentially cause failure of drug therapy.

Introduction

Peripheral nerve sheath tumors (PNST) are relatively common neoplasm's that comprise a broad spectrum of different subtypes. Most of these tumors are histologically benign such as schwannomas and neurofibromas [1,2]. Next to these benign tumors there is a subset of malignant lesions like the malignant peripheral nerve sheath tumors (MPNST) [3,4]. Neurofibromas, MPNST and schwannomas are examples of PNST that occur either sporadically or as part of hereditary neurocutaneous diseases like neurofibromatosis type I (NF1) and neurofibromatosis type II (NF2) respectively. Both these disorders seem to result from the inactivation of a classic tumor suppressor gene. Neurofibromas and MPNST show loss of *NF1* expression. The *NF1* gene is located on chromosome 17q11.2. and encodes the tumor suppressor protein neurofibromin [5]. NF1 is caused by germline mutations in *NF1* but there are also mosaic forms of this disease[6]. MPNST or plexiform neurofibromas without other symptoms of NF1, i.e. sproradic tumors, are probably caused by somatic mosaicism for an *NF1* mutation. A similar situation is seen in NF2. NF2 is caused by biallilec inactivation of the *NF2* gene, located on chromosome 22q11, which encodes the tumor suppressor protein merlin[7]. Bilateral vestibular schwannomas are pathognomonic for this rare disease. However, most vestibular schwannomas occur as sporadic unilateral tumors[8]. Schwannomas occur in a wide range of anatomical sites, including the subcutaneous tissues of the distal extremities and the head and neck region. Schwannomas in the head and neck region have a predilection to derive from the vestibular portion of the eighth cranial nerve, better known as vestibular schwannomas (VS). Schwannomas are neoplastic proliferations that exclusively comprise Shwann cells while neurofibromas contain multiple cell types such as perineurial cells, fibroblasts and to a lesser extent Schwann cells [1,9-11] There are two types of neurofibromas: dermal and plexiform. Plexiform neurofibromas are strongly related to NF1, affecting 20% to 40% of patients suffering from this condition[12,13]. These tumors often occur in the head, skull base, or neck but also manifest themselves on the trunk and limbs. Plexiform neurofibromas have the potential to transform into MPNST. However, not all MPNST develop from pre-existing neurofibromas, as approximately half of all MPNST arise sporadically without a known precursor[14].

These different types of tumors require different types of therapy. To date surgical excision is the only effective treatment for plexiform neurofibromas, but recent literature demonstrated that targets for pharmacological treatment are emerging[15,16]. Therapeutic management of (vestibular) schwannomas consist of surgery or radiotherapy and pharmacological treatment options were recently tested as well [17]. Although there are promising results in individual NF2 patients treated with targeted therapy there are also reports indicating drug resistance in these tumors[18]. A similar situation exists in MPNST. The triad of surgery, radiotherapy and multi-agent chemotherapy is applied to these tumors as well. Despite promising reports on for instance (neoadjuvant) doxorubicin-ifosfamide treatment regimens, these tumors are

often characterized by a highly aggressive behavior and resistance to multidrug therapy, resulting in poor long-term survival rates [19-21]. In short, despite the different therapies that are applied to these PNST one of their common dominators is the fact that they show a certain degree of drug resistance.

Acquired and/or innate drug resistance of tumor cells is a common phenomenon and a major hurdle to effective chemotherapeutic intervention. An important mechanism contributing to drug resistance concerns the expression of ATP binding cassette (ABC) transporter proteins that are capable of extruding drugs from tumors[22]. These energy-dependent transmembrane proteins transport a wide range of substrates, including many anticancer drugs, across cell membranes [23-27]. So far 49 genes have been identified to encode for members of the ABC transporter family [28], but only a subset of these is involved in drug resistance. Of these drug transporters ABCB1 (P-gp) and ABCG2 (BCRP) are the most extensively studied. They were first discovered in tumor cells[29,30], but are also expressed at the apical membranes of epithelial cells in biological barrier tissues such as in the intestines, kidneys and liver and have an important role in the clearance of xenobiotics from the body [23]. In addition, they are expressed in specialized endothelial cells that form the blood-brain, blood-testis and blood-placenta barriers where they help to limit the exposure of the underlying tissues (brain, testis and fetus) to xenobiotics [31-33]. Besides efflux transporters, these specialized endothelial cells also present other barrier properties, such as tight junctions and lack of fenestrations that limit para-cellular entry of drugs. In the brain, the surrounding glial cells (astrocytes, pericytes) govern the expression of these barrier markers in these endothelial cells. The blood-brain barrier (BBB) may thereby “protect” tumor cells that reside within the central nervous system [34].Similar to the situation in the brain, tumors originating from the peripheral nerve sheath may be protected by the so called blood-nerve-barrier (BNB). Our hypothesis is that the blood-nerve-barrier might hinder drugs from reaching their target cells in peripheral nerve sheath tumors, thereby contributing to drug resistance (Figure 1).

The three tumor types we included in this analysis were selected because all of them originate from the peripheral nerve sheath and, as mentioned earlier, each of them are characterized by some form of drug resistance. The BNB is located in microvasculature of the endoneurium and the inner most layers of the perineurium[35] and there are reports that, analogous to the situation at the BBB, the BNB contains members of the ABC transporter family such as BCRP and P-gb [36,37]. Apart from the concept of protection by the BNB, these tumors may also be drug resistant because the tumor cells themselves express ABC drug transporters. Since its discovery, BCRP expression has been observed in several types of tumors [38-47] and elevated expression levels of this transporter have been correlated with poor prognosis in a number of studies [48-50]. Moreover, the expression of BCRP in tumor cells has been associated with a rare subset of so-called cancer stem cells, similar to the expression of BCRP in normal stem cells [51,52]. Consequently, the expression of BCRP both

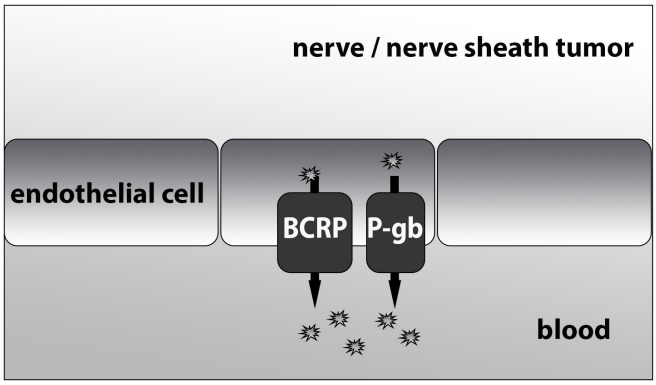

Figure 1. A mechanistic figure of the proposed function of BCRP and P-gb at the blood-nerve-barrier.

in tumor blood vessels or in tumor cells can mediate drug resistance.

The aim of this study was to assess the presence and localization of BCRP in peripheral nerve sheath tumors. We investigated the expression pattern of BCRP in twenty-two sporadic vestibular schwannomas, ten plexiform neurofibromas and eighteen MPNST using an immunohistochemical assay performed on a tissue microarray (TMA) composed of these tumors. We used TMA technology because it provides the advantage of simultaneously analyzing a large panel of tumors with a high degree of experimental standardization[53]. Is has also been shown that the clinico-pathological findings obtained by this technique are highly representative of their donor tissues[54]L.</author><author>Nocito, A.</author><author>Moch, H.</author><author>Sauter, G.</author></authors></contributors><auth-address>Institute of Pathology, University of Basel, 4003 Basel, Switzerland.</auth-address><titles><title>Tissue microarray (TMA. The results of our analysis show that BCRP is expressed in all three of these tumor types. This observation indicates that BCRP might reduce drug accumulation in these peripheral nerve sheath tumors thus creating a hurdle to effective drug treatment.

Results

The results from this immunohistochemical, tissue micro array based study indicate the presence of BCRP in the microvascular endothelium of MPNST, plexiform neurofibromas and vestibular schwannomas (Figure 2). Six out of the eighteen MPNST samples showed vascular BCRP expression. Two of the positive specimens were NF1 related and the other four were sporadic tumors. Tumor cells were negative for BCRP. One of the studied MPNSTs was a recurrent tumor and matched with another specimen in this study. In both cases, the vasculature of these samples was BCRP negative. None of the MPNST patients received

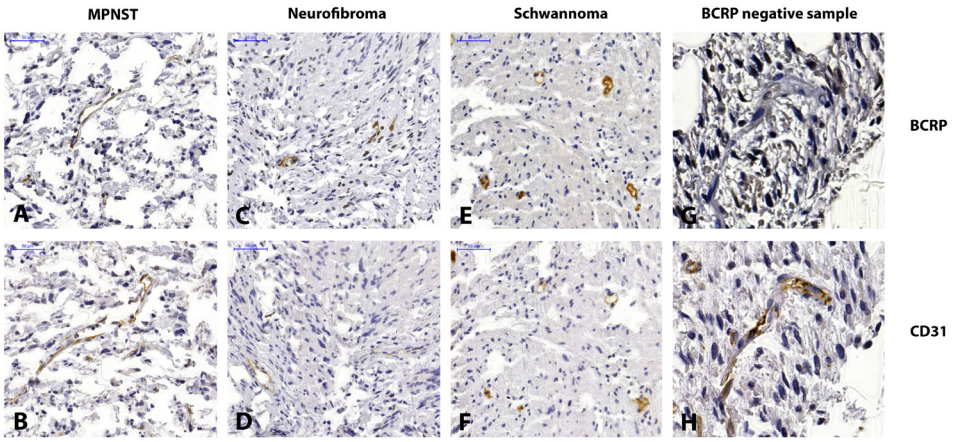

Figure 2. Immunohistochemistry images showing clear BCRP and CD31 positivity in the microvascular epithelium of MPNST(A and B), plexiform neurofibroma (C and D) and vestibular schwannoma (E and F) respectively. Images G and H show a CD31 positive yet BCRP negative sample of a vestibular schwannoma.

chemotherapy prior to resection. The vasculature of nine out of ten plexiform neurofibroma samples was BCRP positive as well as the vasculature of sixteen of the twenty-two schwannomas. Two separate plexiform neurofibroma samples originated from the same NF1 patient and both these tumors had BCRP positive vasculature. Unfortunately, the analyzability of some of the MPNST tumor specimens on the TMA slide was impaired due to necrosis. We found that in four out of eighteen MPNST cases one or two specimens showed intrinsic tumor necrosis making them unsuitable for microscopic analysis. Nevertheless, at least one of the three specimens of these tumors contained representative tumor tissue. Therefore it was still possible to perform adequate microscopic analysis on tissue from all the tumors in the analysis. Of these four MPNST one was scored positive for BCRP and the other three were scored negative.

Discussion

Present treatment of peripheral nerve sheath tumors is mainly a surgical matter. The unraveling of the underlying molecular pathologies and the ongoing development of new therapeutic agents may provide potentially effective drugs as an alternative- or concomitant therapeutic strategy. Unfortunately, however, the occurrence of innate or acquired drug resistance of tumors is a common event. Drug resistance is a frequently encountered problem in MPNST, but it is also observed in benign or precursor lesions. Multidrug resistance is a complex phenomenon and frequently multifactorial. One important reason is impaired drug delivery to the target tissues because of the expression of drug efflux

proteins in the (micro)vasculature of tumor tissues or because of expression of these proteins by tumor cells themselves. In central nervous system (CNS) tissues, the restricted entry is due to drug transporters located at the interface between the blood and the brain (the BBB). A similar situation may be present at the interface between blood and peripheral nerves, but this has not been well established yet. The most extensively studied efflux transporters of the BBB are P-gp (ABCB1) [55] and BCRP (ABCG2) [56]. Together, these two efflux transporters team up to restrict the CNS penetration of a wide range of substrates including many potentially useful drugs [56-59]. In this study, we have investigated the expression of BCRP in tumors of the peripheral nervous system. Based on our results it is not entirely clear if the vascular BCRP expression we observed in a selection of the investigated tumors is a specific characteristic of these tumors, or if it is a remaining part of the blood-nerve-barrier. Dahin et al [60] identified BCRP expression in retinal nerve fibers suggesting that BCRP is part of the blood-nerve-barrier that protects retinal nerve fibers from injury by removing intracellular toxins and xenobiotics. A contradictory observation was made by Huang et al [61] . They investigated BCRP and P-gp in peripheral nerves using a tissue distribution assay on rats but did not find a difference in drug distribution between wild type- or BCRP/P-gp knock out rats. However, in the Huang study the Abcg2 KO had little effect on the brain distribution of known BCRP substrate drugs and these findings are at odds with other ABC KO studies and have not been replicated independently since.[37,62,63]Furthermore, they are in stark contrast with multi drug resistance observed in clinical studies in NF1 patients with known P-gp and/or BCRP substrate drugs.[15,16,64-66]Similarly, prior studies in MPNST confirmed the presence of drug resistant sarcoma stem cells[67,68] and the P-gp and BCRP efflux pumps[47,50,69,70], while drug trials in MPNST patients have not improved outcomes[50,71] Our findings provide a rationale to further study the hypothesis that endothelial BCRP expression may be part of the reason why drug therapy of PNST often fails [47]. If this hypothesis is correct it could mean that inhibition of BCRP may aid in rendering these tumors more susceptible to drug therapy. A potential strategy to achieve this is to co-administer elacridar, a potent, selective inhibitor of both P-gp and BCRP with molecularly targeted drugs to enhance drug levels in diseased neural tissues and improve outcomes as has been observed in animal models of other pump-protected diseases [34,72-82].

In conclusion, our results demonstrate the expression of BCRP in the vascular endothelium in a substantial fraction of MPNST, plexiform neurofibromas and sporadic vestibular schwannomas. Similar to CNS tumors, the presence of BCRP, and perhaps other members of the ABC efflux transporter family, may reduce the drug exposure of underlying tumor tissues and mediate resistance to drug therapy.

Methods

Patients

The cases included in this study were retrospectively selected from the files of the bone- and soft tissue tumor database at the department of Pathology of the Leiden University Medical Center, Leiden the Netherlands. Tumor specimens were obtained from patients surgically treated for their tumors between January 1999 and December 2012. Formalin-fixed paraffin-embedded samples of twenty-two sporadic vestibular schwannomas, ten plexiform neurofibromas and eighteen MPNST were selected. Of these selected tumors two separate plexiform neurofibromas originated from the same patient and one MPNST was a recurrence of a primary tumor included in this analysis as well. Surgery was performed at the departments of Neurosurgery, Otolaryngology, Orthopedic surgery and General surgery of the Leiden University Medical Center. In each case the diagnosis was made according to the WHO classification of soft tissue tumors [83]. All tumor samples were handled in a coded fashion and all procedures were performed according to the ethical guidelines of the Code for Proper Secondary Use of Human Tissue in The Netherlands (Dutch Federation of Medical Scientific Societies). Additional clinicopathological data are shown in table one.

Tissue microarray (TMA) preparation

Preparation of the TMAs was performed at the department of pathology of the Leiden University Medical Center. TMAs were constructed from 1mm cores of all tumor samples using a TMA Master (3DHISTECH Ltd, Budapest, Hungary). Per tumor three randomly selected cores were included in the TMA in order to compensate for intra-tumoral heterogeneity. Normal colon, tonsil, placenta, prostate and spleen tissue together with mamma carcinoma were used to serve as internal controls and points of orientation. In line with data provided by the manufacturer we found high BCRP expression in placenta and low expression in colon tissue.

Immunohistochemistry (IHC)

Immunohistochemical reactions were performed according to standard laboratory methods [84]. In brief, heat-induced antigen retrieval was performed after dewaxing and rehydration, followed by blocking of endogenous peroxidase with 3% H_2O_2 in methanol. Incubation with the primary antibodies BCRP (Abcam; ab24115) and CD31 (Abcam; ab28364) was overnight. Subsequently, CD31 sections were conjugated with Labelled Polymer-HRPAnti-Rabbit Envision (DakoCytomation; K4005) while conjugation of the BCRP sections was performed with Goat-α-Rat-Bio (Santa Cruz; SC-2041) and Streptavidin/HRP (DakoCytomation; P0397) respectively. Visualization was carried out with a diaminobenzidine solution. All washing procedures were conducted in phosphate-buffered saline. Slides were counterstained with haematoxylin.

Table 1. patient characteristics

Specimen	Tumor Type	Sex	Age (yrs.)	Tumor Localisation	Brcp
L1399	NF1 MPNST	F	16	upper leg, right	positive
L4304	NF1 MPNST	F	29	second thoracic vertebra, left	negative
L4309	NF1 MPNST	F	15	mandible angle, right	positive
L4326	NF1 MPNST	F	27	flank region, left	negative
L1537	sporadic MPNST*	M	22	back, middle	negative
L1448	sporadic MPNST	M	51	inguinal region, left	negative
L1219	sporadic MPNST	F	35	gluteus region, right	negative
L1503	sporadic MPNST	F	58	upper leg, right	negative
L1509	sporadic MPNST	M	17	upper arm, left	negative
L1867	sporadic MPNST	M	57	upper leg, left	negative
L2056	sporadic MPNST	M	24	inguinal region, left	positive
L2170	sporadic MPNST	M	22	brachial plexus, left	negative
L4303	sporadic MPNST	F	41	brachial plexus, left	positive
L4320	sporadic MPNST	M	22	back, middle	negative
L4322	sporadic MPNST	F	48	brachial plexus, left	negative
L4325	sporadic MPNST	F	35	fifth cervical vertebra, left	negative
L4327	sporadic MPNST	M	68	retroauricular region, right	positive
L4328	sporadic MPNST	V	26	fifth cervical vertebra, left	positive
L4305	NF 1plexiform neurofibroma	F	33	foot, right	negative
L4321	NF1 plexiform neurofibroma	M	23	skin of neck, left	positive
L4330	NF1 plexiform neurofibroma	V	42	supraclavicular region, right	positive
L4331	NF1 plexiform neurofibroma	M	30	upper leg, right	positive
L4332	NF1 plexiform neurofibroma**	M	30	occipital region, middle	positive
L4333	NF1 plexiform neurofibroma	M	24	cheek, left	positive
L4335	NF1 plexiform neurofibroma	F	31	neck region, left	positive
L4302	sporadic plexiform neurofibroma	F	27	median nerve left	positive
L4329	sporadic plexiform neurofibroma	V	26	axilla, right	positive
L4334	sporadic plexiform neurofibroma	F	51	femoral nerve, right	positive
L1493	sporadic schwannoma	F	39	cerebellopontine angle, right	positive
L3580	sporadic schwannoma	M	58	cerebellopontine angle, right	negative
L3583	sporadic schwannoma	M	47	cerebellopontine angle, left	positive
L3586	sporadic schwannoma	F	48	cerebellopontine angle, right	negative
L3590	sporadic schwannoma	M	43	cerebellopontine angle, right	positive
L3593	sporadic schwannoma	F	53	cerebellopontine angle, left	positive
L3604	sporadic schwannoma	F	43	cerebellopontine angle, right	positive
L4306	sporadic schwannoma	M	69	cerebellopontine angle, left	positive
L4307	sporadic schwannoma	F	57	cerebellopontine angle, right	positive
L4308	sporadic schwannoma	M	73	cerebellopontine angle, right	positive

see next page >>

Table 1. Continued

Specimen	Tumor Type	Sex	Age (yrs.)	Tumor Localisation	Brcp
L4310	sporadic schwannoma	F	59	cerebellopontine angle, right	positive
L4311	sporadic schwannoma	F	56	cerebellopontine angle, left	positive
L4312	sporadic schwannoma	F	56	cerebellopontine angle, left	positive
L4313	sporadic schwannoma	M	43	cerebellopontine angle, right	negative
L4314	sporadic schwannoma	F	59	cerebellopontine angle, left	positive
L4315	sporadic schwannoma	F	61	cerebellopontine angle, right	positive
L4316	sporadic schwannoma	F	67	cerebellopontine angle, left	positive
L4317	sporadic schwannoma	F	50	cerebellopontine angle, left	positive
L4318	sporadic schwannoma	M	55	cerebellopontine angle, left	negative
L4319	sporadic schwannoma	F	72	cerebellopontine angle, right	positive
L4323	sporadic schwannoma	M	62	cerebellopontine angle, right	negative
L4324	sporadic schwannoma	F	54	cerebellopontine angle left	negative

* recurrent tumor from specimen L4325; ** separate tumor from the same patient as L4331

Microscopic analysis

After staining the TMA was scanned using a Pannoramic MIDI Digital Slide Scanner (3DHISTECH Ltd, Budapest, Hungary). Analysis of the digital slides took place with Pannoramic Viewer software version 1.15.3. Scoring was performed by two observers who were unaware of the clinico-pathological data. Staining of tumor specimens was classified as either positive or negative. Differently assessed cases were discussed to reach consistent scoring results.

Abbrevations

ABC	ATP binding cassette
ABCB1	ATP-binding cassette sub-family B member 1
ABCG2	ATP-binding cassette sub-family G member 2
BBB	Blood-brain-barrier
BCRP	Breast Cancer Resistance Protein
BNB	Blood-nerve-barrier
MPNST	Malignant peripheral nerve sheath tumors
NF 1	Neurofibromatosis type I
NF 2	Neurofibromatosis type II
P-gb	permeability glycoprotein
PNST	Peripheral nerve sheath tumors
TMA	Tissue micro array
VS	Vestibular schwannomas

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CHAPTER 7

M-CSF and IL-34 expression as indicators for growth in Sporadic Vestibular Schwannoma

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Abstract

Objective: assess whether macrophage colony stimulating factor and IL-34 are associated with clinical vestibular schwannoma progression.

Background: investigating the biology behind vestibular schwannoma progression helps understanding tumor growth. Inflammation is important in the microenvironment of neoplasms. Macrophages are major players in the intratumoral infiltrate. These tumor associated macrophages are known to stimulate angiogenesis and cell growth. M-CSF and IL-34 are cytokines that can regulate tumor infiltrating macrophages. They are expressed by tumors and form potential targets for therapy. The goal of this study was to investigate these cytokines in vestibular schwannomas and to see if their expression is related to angiogenesis, macrophage numbers, cystic degeneration and volumetric tumor progression.

Methods: immunohistochemical expression of M-CSF and IL-34 was analysed in ten fast-growing vestibular schwannomas and in ten slow-growing vestibular schwannomas. Expression M-CSF and IL-34 were compared between fast versus slow growing, and cystic versus non-cystic tumors. Data on macrophage numbers and microvessel density, known from earlier research, was also included.

Results: all tumors expressed M-CSF and its expression was higher in fast growing tumors ($p=0.003$) and in cystic tumors ($p=0.035$). CD163 expression was higher in tumors with strong M-CSF expression ($p=0.003$). All tumors expressed IL-34 as well, but no significant differences were found in relation to clinicopathological characteristics.

Conclusion: this study demonstrated the expression of M-CSF and IL-34 in vestibular schwannomas. The results suggest that M-CSF is related to macrophage activity and tumor progression, making it a potential target for therapy. If a similar assumption can be made for IL-34 remains unclear.

Introduction

Vestibular schwannomas (VS) are benign neoplastic proliferations recapitulating the differentiation repertoire of the myelin-forming Schwann cells of the vestibular branch of the vestibulocochlear nerve in the internal auditory canal or the cerebellopontine angle. These tumors often display a slow and self-limiting growth pattern but there are also variants that progress more rapidly and persistently. In these patients ongoing tumor progression can eventually cause brainstem compression or paralysis of adjacent cranial nerves. In most cases (>90%) VS occur as unilateral sporadic tumors[1], whereas bilateral tumors are pathognomonic for the hereditary disorder neurofibromatosis type 2 (NF2)[2]. Loss of function of the tumor suppressor protein merlin, encoded by the *NF2* gene, is an essential step in schwannoma pathogenesis[3,4]. Heterozygous germline inactivating mutations affecting the *NF2* gene cause neurofibromatosis type 2 while biallelic somatic mutations of *NF2* are found in sporadic VS[4]. Recent years showed an increase in the number of newly diagnosed VS to approximately 20 per million people per year [5-7]. This phenomenon most probably is the result of more frequent use of magnetic resonance imaging scanning (MRI), which in turn leads to the identification of more subclinical cases of VS.

Management of these VS comprises several options. The initial policy for smaller tumors is to wait and see by performing sequential MRI scans. In case of large tumors or when tumors rapidly progress active treatment is needed. Current therapeutic management of VS consists of microsurgery or radiotherapy. In selected cases of NF2 related tumors pharmacotherapeutic options are also applied [8-10]. This kind of therapy is not used for sporadic VS. One of the clinical dilemmas in selecting the most suitable treatment policy for VS is the unpredictable behavior these tumors can display. Some tumors remain stable for decades while others double in size within less than a year. So far, cystic degeneration is the only known prognostic marker for progressive tumor volume growth[11]. Better prediction of tumor volume progression will improve the accuracy of determining the correct moment and modality of therapeutic intervention. More understanding of tumor behavior requires more insight into tumor biological factors influencing tumor development. Investigating VS biology not only benefits the understand ding of its growth pattern, it will also contribute to the identification of potential therapeutic targets.

In two earlier papers on the inflammatory microenvironment in VS we demonstrated a relationship between tumor associated macrophages (TAM) , angiogenesis and tumor growth[12,13]. These results were in line with the emerging notion that intratumoral inflammation is a major driving force behind the volumetric progression of tumors [14-16]. The fact that TAM may form a target for therapy emphasizes their potential clinical importance [17]. TAM consist of a heterogeneous population of, mainly alternatively activated, M2 type macrophages that seem to have tumor promoting characteristics [16,18]. Inhibiting the formation of M2 macrophages may therefore have a negative effect on tumor progression. An important

regulator within the inflammatory microenvironment capable of polarizing macrophages towards an M2 like phenotype is a cytokine known as the macrophage colony stimulating factor, or M-CSF[19]. The exact role of M-CSF in macrophage associated tumor development remains to be elucidated but its function as a promoter of tumor progression has been indicated in several tumor models [20-22]. Another regulating protein that seems to be capable of skewing the microenvironment into a tumor promoting direction is interleukin-34 (IL-34). This cytokine was first described in 2008 by Lin et. al. [23] and displays common features with M-CSF in such a way that they appear to have synergistic functions[24,25]. Consistent with these findings relatively recent studies have indicated that IL-34 seems to be associated with tumor progression in osteosarcoma and lung cancer[26,27].
The aim of this study was to analyze the expression of M-CSF and IL-34 in our earlier described cohort of twenty sporadic vestibular schwannoma patients and to determine whether their expression can be related to clinicopathologic characteristics and tumor growth.

Materials and methods

We performed immunohistochemical stains against M-CSF and IL-34 on formalin-fixed paraffin-embedded vestibular schwannoma tissue. To investigate whether there is a relationship between the presence of these proteins and VS progression we analyzed their expression pattern in the same selection of tumors that we previously analyzed and described for the expression of tumor associated macrophages[12]. This selection of tumor samples consisted of ten radiologically observed fast growing tumors and ten radiologically observed slower growing tumors. The expression patterns of M-CSF and IL-34 within these groups were compared with each other. The already published data on angiogenesis and macrophage expression was also included into this analysis, as well as radiological data regarding cystic degeneration.

Patient selection

A retrospective selection was made from vestibular schwannoma database of the Leiden University Medical Center. This group of patients had been consecutively treated for a proven sporadic vestibular schwannoma from January 2006 to December 2011. Two different cohorts were compiled out of a total of forty-six consecutively treated patients. All patients had at least two preoperative MRI scans on the basis of which volumetric tumor growth was measured. The first cohort included the ten patients with the slowest growing tumors, while the second cohort included the ten fastest growing tumors. The decision for surgical treatment had been based upon symptoms (e.g. vertigo, hearing loss and tinnitus), tumor size, tumor growth and patients' personal preference. No NF2 related tumors were included in the analysis.

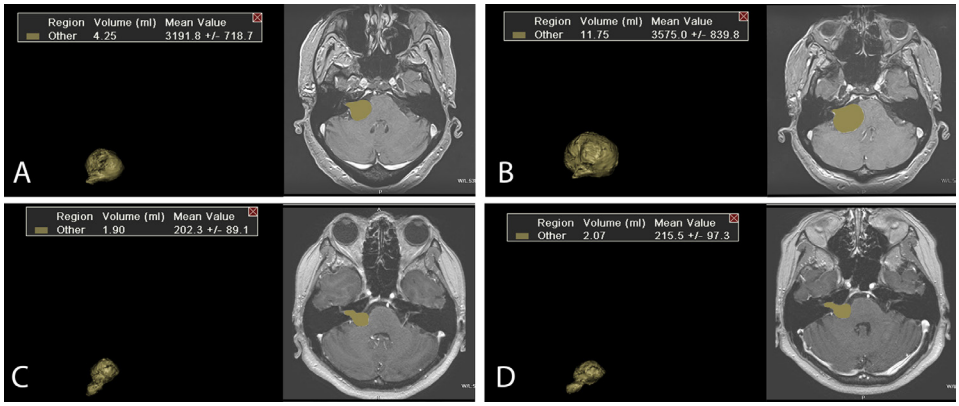

Figure 1. A and **B** show a fast growing tumor with an average growth of 9 ml per year. **C** and **D** show a slower growing tumor with an average growth of 0.2 ml per year.

To ensure patients privacy all samples were managed in a coded fashion and all procedures were conducted according to the Code for Proper Secondary Use of Human Tissue in the Netherlands (Dutch Federation of Medical Scientific Societies).

Tumor measurement

The measurement of all tumors was performed on T1-weithed gadolinium enhanced MRI scans and conducted by one and the same author. Tumor volume was determined with a contour measurement method using Vitrea View software (Vital Imaging, Minnetonka, MN, USA). To increase the accuracy of the measurements, each volume was determined two times per MRI scan. The mean of these two measurements was used for further evaluation of tumor growth. By calculating the difference in tumor volume on sequential MRI investigations, tumor growth rate could be determined. Volumetric growth was expressed as the increase in milliliters per year. Next to measuring tumor growth rate, the presence of cystic degeneration was evaluated. Figure one shows volume measurements of a fast growing and a slow growing tumor.

Immunohistochemistry

Immunohistochemistry was performed on 4 micrometer thick slides acquired form formalin-fixed paraffin-embedded vestibular schwannoma tissue samples. All staining procedures were conducted on one and the same tumor block per tumor sample. The exact materials and methods that were applied for the CD31 and CD163 stains are described in the earlier mentioned paper on tumor associated macrophages [12]. Immunohistochemical stains for M-CSF (pré-incubation and incubation in 5% non-fat milk in PBS/1%BSA , 1:100 diluted, Anti-M-CSF antibody, ab52864, Abcam, Cambridge, MA, USA) and IL-34 (pré-incubation and incubation in 5% non-fat milk in PBS/1%BSA , 1:3000, Anti-Interleukin 34 antibody,

ab224734, Abcam, Cambridge, MA, USA) were performed according to standard laboratory methods[28]. Tonsil served as positive control for M-CSF and hepatocellular carcinoma and prostate carcinoma we used as positive control tissues for IL-34.

Microscopic analysis

Immunostainings were evaluated by two separate observers (M. de Vries and P. Hogendoorn) without knowledge of clinical patient data. M-CSF as well as IL-34 showed a varying staining pattern with areas of strong expression and areas of weak expression within the same tumor. Additionally several tumor specimens showed hemosiderin deposition mimicking positive staining. This made computerized quantification less reliable. For this reason a semi quantitative immunohistochemistry score of the overall staining intensity was made for each tumor sample. Staining intensity was initially categorized as no staining, weak staining, moderate staining and strong staining. Exact details regarding M2 macrophage (CD163) and angiogenesis (CD31) staining and scoring techniques are described in our earlier raport[12].

Statistical analysis

The difference in M-CSF and IL-34 expression in fast- versus slow growing tumors and cystic versus non-cystic tumors was determined with the Fisher's exact test. The Mann-Whitney U test was used to determine the relation between the expression of M-CSF and IL-34 and the degree of microvessel density and CD163 expression. This test was also used to see if there was a relation between cystic degeneration and CD-163 expression. For all statistical tests a level of significance of $p<0.05$ was taken into account. Calculations were made using SPSS version 16.0, IBM, Inc.

Results

Details regarding patient characteristics are listed in Table 1. There were no significant differences in distribution of age, sex and duration of preoperative follow-up between the two patient groups. As expected, the distribution of tumor growth rate and tumor volume as well as cystic degeneration was significantly different in the two groups. Analysis of the immunohistochemical stainings showed the following results. All samples were M-CSF positive, demonstrating cytoplasmic as well as nuclear expression in an irregular staining pattern (Figure 2.). Nine samples showed weak staining, four samples showed moderate staining and seven samples showed strong staining. Based on these results we simplified the categories of staining to weak and strong staining, the latter being the combination of moderate and strong staining. M-CSF staining was significantly higher in the group of fast growing tumors ($p=0.003$). M-CSF expression is also significantly higher ($p=0.035$) in cystic

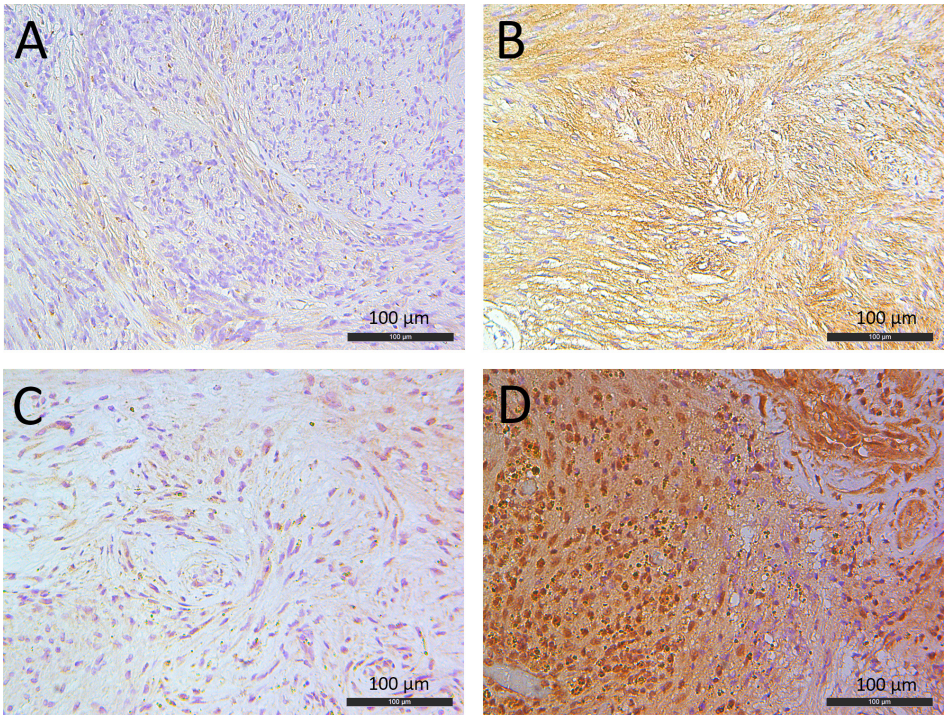

Figure 2. A. weak expression of M-CSF, **B.** strong expression of M-CSF, **C.** a tissue area with weak IL-34 expression, **D.** a tissue area with strong IL-34 expression

tumors (table 2). CD163 expression was significantly higher in tumors with strong M-CSF expression (p=0.003). There was no significant relation between micovessel density and M-CSF expression.

Analysis of IL-34 showed that all tumors displayed nuclear as well as cytoplasmic immunopositivity for this protein (Figure 1). Similar to M-CSF the intratumoral staining pattern of IL-34 was irregular. Eight tumors showed moderate staining and the remaining twelve tumors showed strong staining. Statistical analysis of our scoring results showed no significant differences in IL-34 expression when it comes to tumor growth, cystic degeneration, CD-163 expression or microvessel density.

Finally, cystic tumors showed a significantly higher degree of CD-163 expression compared to non-cystic tumors (p =0.005).

Table 1. patient characteristics

Case	Sex	Age (yrs)	Tumor volume (ml)	Growth Rate (ml/yr)	Preoperative mri follow up (months)	Cystic
Slow growing group						
L3721	M	60	0.35	0.06	13	no
L3742	F	54	0.27	0.21	7	yes
L3773	F	52	0.22	0.07	6	no
L3774	F	50	0.88	0.19	27	no
L3775	M	53	0.69	0.17	10	no
L3779	F	51	1.46	0.18	32	yes
L3780	F	56	0.74	0.25	11	no
L3781	F	44	0.53	0.19	12	no
L3787	M	58	0.52	0.18	12	no
L3797	F	60	2.13	0.23	12	no
mean (±SD)		53.8 (±4.96)	0.77 (±0.59)	0.17(±0.06)	14.2 (±8.46)	
Fast growing group						
L3725	F	62	3.26	1.33	11	no
L3731	F	81	14.41	4.41	19	yes
L3733	F	76	11.66	8.84	10	no
L3740	F	52	2.27	2.50	7	no
L3741	F	71	7.25	1.83	21	yes
L3745	F	56	5.62	2.18	6	yes
L3746	M	46	30.73	43.98	4	yes
L3792	F	75	6.92	3.43	21	yes
L3793	F	39	7.32	5.52	10	yes
L3805	F	39	23.05	2.90	9	yes
mean (±SD)		59.7 (±15.65)	11.24 (±9.16)	7.69 (±12.94)	11.8(±6.26)	
difference fast vs. slow (p)	0.26a	*0.41b*	*<0.0001b*	*<0.0001b*	0.31b	*0,007a*

a Chi-square test, b Mann-Whitney U test, results at $p \leq 0.05$ are shown in italics

Discussion

Inflammation is an important feature of almost every type of neoplasm. The inflammatory process that takes places within tumors is often characterized by the abundance of tumor associated macrophages[16]. The influx of these cells and their immunomodulating capacities allow the progression of tumor cells by stimulating processes such as of angiogenesis and cell survival[15]. M-CSF and IL-34 are cytokines that regulate macrophage recruitment, proliferation and differentiation [24]. More specifically, M-CSF and recently also IL-34 are identified as important factors that polarize macrophages towards a protumoral M2 phenotype[22,27]. High M-CSF expression and subsequent increased macrophage levels have

Table 2. Fisher's exact test for M-CSF and IL-34 expression

	M-CSF staining		**IL-34 staining**	
	Weak	Strong	Moderate	Strong
Growth rate				
Slow	8	2	4	6
Fast	1	9	4	6
Total	9	11	8	12
P-value		*0.003*		*0.675*
Cystic degenration				
No	7	3	5	5
Yes	2	8	3	7
Total	9	11	8	12
P-value		*0.035*		*0.325*

results at $p \leq 0.05$ are shown in italics

been associated with disease progression and unfavorable outcome in several types of tumors[20,21,29] and similar findings are reported for IL-34[26,27].

Our earlier studies showed that VS can contain large quantities of TAM, and their presence seems to be related to tumor expansion. In this study we attempted to find out if M-CSF and IL-34 are part of this process as well by comparing their expression pattern in progressive versus more indolent VS. A necessary condition to examine tumor tissue is that patients are surgically treated. One of the main indications for surgery is tumor growth. Even the group of slow growing tumors showed a certain form of volumetric growth and therefore did not fully represent the truly indolent tumors. This unavoidable selection bias makes the identification of biological differences between indolent versus progressive variants of VS more challenging.

In this report we demonstrate the presence of M-CSF and IL-34 in VS. The expression of M-CSF is higher in fast growing VS compared to slower growing VS and, in accordance with its supposed function, the presence of M-CSF seems to be related to the expression of TAM within VS. Furthermore, the expression of M-CSF as well as the number of CD-163 positive macrophages appears to be higher in cystic tumors. We therefore postulate that intratumoral inflammation might also contribute to the pathogenesis of cystic degeneration. We could not demonstrate a relation between IL-34 expression and clinicopathologic characteristics of VS in our study group. Nevertheless it should be noted that this protein was at least moderately expressed by all tumors, more than half of which even showed high immunopositivity. These relatively small intertumoral differences in expression of IL-34 together with the above mentioned selection bias may be a reason why we were unable to find a significant difference in the expression pattern of this protein.

It is important to note that the results of our comparisons are observations of association.

There is always the possibility that these findings are epiphenomena of a larger biological growth process and therefore not directly related to each other. The fact remains that the results of this study seem to be in line with our earlier presented data on macrophage expression in VS. These current observations provide additional support for our hypothesis that the inflammatory microenvironment plays an essential role in the progression of these tumors. By modulating the characteristics of this microenvironment through the inhibition of M-CSF, and maybe IL-34, the progression of VS may in turn be decreased. For M-CSF several clinical trials with inhibitors such as PLX3397 (Pexidartinib) and RG7155 (Emactuzumab) showed promising results in a variety of neoplasms [30,31]. IL-34 might be a potential target as well but clinical evidence remains to be provided[32]. Before applying anti-*M-CSF* or anti-*IL-34* therapy in VS patients in vivo schwannoma models would be very helpful to further elucidate the biological mechanisms that are involved in the associations observed in this study. Unfortunately the current lack of sporadic vestibular schwannoma cell lines hampers such functional studies.

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CHAPTER 8

Summary and concluding remarks

Vestibular schwannomas are benign tumors recapitulating the differentiation repertoire of the Schwann cells that are part of the nerve sheath that insulates the vestibular portion of the eighth cranial nerve. These tumors classically arise within the internal auditory canal and usually progress into the cerebellopontine angle. One of the clinical hallmarks of these tumors is their capricious growth pattern. A significant portion of tumors are limited in size and remain indolent after they have been diagnosed. In contrast to this relatively harmless phenotype there are also tumors with a faster and more extensive growth pattern that are capable of causing brainstem compression or paralysis of adjacent cranial nerves[1]. This unpredictable behavior is one of the main clinical problems in the optimal management of vestibular schwannomas. Current treatment comprises observation by sequential MRI scanning for smaller and indolent tumors. For larger tumors, tumors that display rapid growth or in case of major symptoms, such as invalidating vertigo, active treatment in the form of radiotherapy or microsurgery is indicated. Pharmacotherapeutic alternatives are emerging but they are not part of standard treatment yet.
The different studies on which this thesis is based have the general aim to increase current insights into the intratumoral biological dynamics associated with vestibular schwannoma progression. Enlarging our tumor biological knowledge helps to interpret tumor growth patterns which in turn is paramount to the search for prognostic markers and targets for therapy.

Chapter one serves as a general introduction to vestibular schwannomas with regard to their aetiology, symptoms and the diagnostic tools that are used for their detection. This chapter also provides an overview of current therapeutic management and it discusses the clinical dilemmas involved in the treatment of these tumors that have resulted in the research questions which form the basis of this thesis.

Chapter two is a review of the literature on the tumor biology of vestibular schwannomas. It describes current knowledge on the genetic profile of these tumors and provides an overview of the cell signalling pathways associated with vestibular schwannoma progression. The role of angiogenesis as an important factor in tumor growth is discussed, as well as its potential function as a target for therapy. Other targets for therapy are also summarized and potential subjects of future research are briefly addressed.

Chapter three investigates a selection of intratumoral processes that take place within vestibular schwannomas in order to identify biological factors that contribute to tumor progression. Tumor specimens of sixty-seven vestibular schwannoma patients were immunohistochemically analysed for cell proliferation (Ki-67, Histone-H3), neovascularisation (CD31) , macrophage expression (CD68) and the presence of intratumoral bleeding (hemosiderin). In this study we find no association between cell proliferation and clinical

characteristics of tumor growth. On the other hand, factors like neovascularisation and macrophage expression do seem to be related to tumor size and the tumor growth index. Additionally we find that intratumoral bleeding occurs to a higher degree in tumors that show cystic degeneration, which in turn is a known process contributing to tumor expansion[1]. We therefore hypothesize that growth of vestibular schwannomas is not based on cell proliferation alone, factors like degenerative changes, angiogenesis and inflammation influence progressive tumor growth as well.

Chapter four describes a gene expression assay for the thirteen most frequent mutations affecting *BRAF, EGFR, PIK3CA* and *KRAS.* The products of these genes are members of the MAPK/ERK cell signalling pathway. Increased activity of this pathway is associated with progression of different types of tumors including vestibular schwannomas[2,3]. This knowledge combined with the finding of *BRAF* mutations in a number of sporadic non head and neck schwannomas [4] lead us to investigate the hypothesis that vestibular schwannoma progression is influenced by the occurrence of accessory oncogenic mutations related to the MAPK/ERK pathway. We performed a gene expression assay in a selection of forty-eight vestibular schwannomas, all of which turned out to be negative for any of the mutations that were tested. This finding therefore doesn't support the hypothesis that vestibular schwannoma progression is accelerated by additional oncogenic mutations that arise during the development of these tumors.

Chapter five is a study that further investigates the role of inflammation within vestibular schwannomas. As described in chapter three, some vestibular schwannomas contain large amounts of macrophages. During the past decades it has become clear that in various types of neoplasms the inflammatory microenvironment has many tumor promoting effects[5]. Macrophages are one of the major determinants of this microenvironment[6]. These so called tumor associated macrophages can roughly be divided into two groups consisting of the classically activated M1 type macrophages versus the alternatively activated M2 type macrophages. Of these two categories the M2 type macrophages are the ones with the tumor promoting characteristics such as stimulating angiogenesis and dampening of the antitumor immune response[7]. Using immunofluorescent stains against CD163, a specific marker for M2 type macrophages, we determined the expression of M2 macrophages in tumor samples of twenty retrospectively analysed vestibular schwannoma patients. We also determined the degree of angiogenesis within these tumors. The results of this study indicate that some vestibular schwannomas contain large amounts of M2 type macrophages and additionally there seems to be a positive relation between the expression of these macrophages and angiogenesis as well as tumor growth rate. These results imply that tumor infiltrating M2 type macrophages may stimulate the progression of vestibular schwannomas, which in turn makes them a potential target for therapy.

Chapter six describes a study that investigates BCRP expression as a potential cause of drug resistance in a selection of peripheral nerve sheath tumors. BCRP is a transmembrane efflux transporter protein that seems to play an important role in different biological barriers such as the blood-brain-barrier[8,9]. Its presence has also been associated with drug resistance in cancer[10]. An immunohistochemical staining for BCRP was performed on a tissue microarray composed out of twenty-two sporadic vestibular schwannomas, ten plexiform neurofibromas and eighteen malignant peripheral nerve sheath tumors. The findings of this study demonstrate the expression of BCRP in the vasculature of a significant portion of all three tumor types included in the assay. It is possible that this endothelial expression of BCRP is a specific characteristic of these tumors but it may very well also be is a remnant part of the blood-nerve-barrier of the nerves these tumors originate from. Nonetheless these results suggest that BCRP expression may reduce drug exposure to the underlying tissue in these tumors. This may be part of the reason why drug therapy of peripheral nerve sheath tumors, including vestibular schwannomas, often has variable and disappointing effects. If this hypothesis is correct, inhibiting BCRP expression in these tumors could subsequently lead to enhanced susceptibility to drug therapy.

Chapter seven forms the sequel to the analysis of the tumor associated macrophages described in chapter five. This study comprises the same patient cohort and focusses on two cytokines, M-CSF and IL-34. Both these proteins are known to have regulatory functions with regard to macrophage activation. M-CSF stands for macrophage colony stimulating factor, a cytokine that is capable of polarizing macrophages towards a M2 like phenotype. The exact tumor biological characteristics of this protein have not been fully understood yet, but there are several tumor models in which it seems play a role in macrophage associated tumor progression[11,12]. IL-34 is a cytokine that displays common features with M-CSF[13,14]. Relatively recent studies have indicated that IL-34 appears to be related to tumor progression in a manner that resembles M-CSF[15]. Our study demonstrates the expression of these two cytokines in vestibular schwannomas. In accordance with its proposed function we find that M-CSF expression is related to clinical tumor progression and the expression of M2 type macrophages. We were unable to demonstrate similar significant findlings for IL-34, but the fact that this protein has a relatively high expression in all the VS we investigated does suggest it plays a role in VS biology. These observations are in line with the hypothesis that the inflammatory microenvironment is an important factor in the progression of vestibular schwannomas. This makes M-CSF, and maybe IL-34 as well, a potential target for therapy.

Conclusion

The overall aim of this thesis is to shed more light on the biological background of the clinical progression of sporadic vestibular schwannomas. In case of vestibular schwannomas tumor progression is measured by the increase in tumor size. The obvious factors that determine vestibular schwannoma size seem to be cell growth and cystic formation.
The results of our studies indicate that, in different ways, intratumoral inflammation seems to be important in the clinical progression of these tumors. By stimulating angiogenesis and through inhibition of antitumor immune responses tumor associated macrophages may allow some tumors to progress faster and reach a larger volume. Next to permitting ongoing tumor cell proliferation it might be possible that in some tumors the actual bulk of the inflammatory infiltrate also contributes to the expansion of vestibular schwannomas.
M-CSF and IL-34 may play a regulatory role when it comes to macrophage activity within vestibular schwannomas, thereby potentially making them targets for therapy. These outcomes must be interpreted with caution. It is important to note that the results of the comparisons we made are observations of association. There is always the possibility that these findings are epiphenomena of a larger biological growth process and therefore not directly related to one another. Before treating VS patients with drugs capable of modulating the intratumoral microenvironment our findings need to be replicated, and in vitro or animal schwannoma models should be performed. Interesting examples of such drugs are PLX3397 (Pexidartinib) and RG7155 (Emactuzumab)[16,17]. Both are relatively new inhibitors capable of blocking macrophage activity and, considering our hypothesis, potentially form effective agents in vestibular schwannoma therapy.

In the search for new drugs capable of targeting tumor biological factors involved in the progression of vestibular schwannomas it is important to realize that our findings also indicate that these tumors may be protected by barrier proteins such as BCRP. Future research should reckon with this additional obstacle in order to optimize the efficacy of novel treatment strategies.

Next to looking for new pharmacologic ways to treat vestibular schwannomas there are other clinical questions and dilemmas that need to be explored. An important issue that needs further research is the potential superiority of proton radiotherapy over the conventional radiotherapy. Current literature on this topic is not decisive and clinical trials are needed to answer this question. The ongoing development of new therapeutic modalities also has its impact on shared decision making when it comes to choosing the correct form of therapy. For this reason continuous research in terms of quality of live is of great importance in order to tailor future treatment to the individual needs of the patient.

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APPENDICES

Samenvatting en conclusie
Acknowledgements
Curriculum Vitae

Samenvatting en conclusie

Vestibularis schwannomen zijn goedaardige tumoren die ontstaan vanuit een ongeremde deling van Schwanncellen die deel uitmaken van de zenuwschede die de vestibulaire tak van de achtste hersenzenuw omvat. Doorgaans ontstaan deze tumoren in de inwendige gehoorgang waarna ze zich uit kunnen breiden in de brughoek regio. Om deze reden staat dit type tumor ook wel bekend als de brughoek tumor. Eén van de klinische kenmerken van vestibularis schwannomen is het grillige groeipatroon dat ze kunnen vertonen. Een groot deel van deze tumoren blijft nadat ze gediagnostiseerd zijn beperkt in omvang en vertonen weinig tot geen groei. Echter zijn er ook exemplaren die een agressiever gedrag vertonen waarbij aanhoudende groei kan leiden tot hersenstam compressie of uitval van nabijgelegen hersenzenuwen [1] Tot op heden is het grotendeels onduidelijke welke biologische processen aan deze verschillende groeiwijzen ten grondslag liggen. Deze onvoorspelbaarheid in tumor groei is één van de voornaamste klinische problemen in de behandeling van vestibularis schwannomen. Het huidige palet aan behandelopties bestaat uit observatie met behulp van periodieke MRI scans in het geval van kleinere en langzaam groeiende tumoren terwijl grotere of sneller groeiende tumoren worden behandeld met radiotherapie of microchirurgie. Medicamenteuze therapieën zijn in opkomst maar worden nog niet als standaard behandeling voor sporadische vestibularis schwannomen toegepast.
De verschillende onderzoeken die de basis van dit proefschrift vormen hebben als gemeenschappelijke doel om meer inzicht te verkrijgen in de tumorbiologische processen die zich afspelen in vestibulaire schwannomen. Meer kennis over deze processen is nodig voor het interpreteren van de verschillende groeipatronen en een essentiële voorwaarde voor het vinden van prognostische markers en aangrijpingspunten voor therapie.

Hoofdstuk één is een algemene introductie over de etiologie, symptomen en diagnostiek van het vestibularis schwannoom. Daarnaast bevat dit hoofdstuk een overzicht van de huidige behandelopties en het beschrijft een aantal klinische dilemma's die in de praktijk een rol spelen en de basis vormen voor de onderzoeksvragen van onze studies.

Hoofdstuk twee is een review van de literatuur over de tumor biologie van vestibularis schwannomen. Het geeft een beschrijving van de huidige kennis van het genetische profiel van deze tumoren en het bevat een overzicht van de verschillende cell signalling pathways die een rol lijken te spelen in tumor groei van vestibularis schwannomen. De functie van angiogenese als groeifactor en daarmee aangrijpingspunt voor therapie wordt besproken evenals een aantal andere potentiele doelwitten voor medicamenteuze behandeling.

In **hoofdstuk drie** wordt een aantal intratumorale processen onderzocht die plaatsvinden in vestibularis schwannomen en die een mogelijke bijdrage leveren aan tumor progressie.

Tumor weefsel van in totaal zevenenzestig patiënten is met behulp van immunohistochemie onderzocht op cel proliferatie (Ki-67, Histone H3), neovascularisatie (CD31), macrofagen expressie (CD68) en intratumorale hematoomvorming (hemosiderine). Deze studie toont geen associatie tussen proliferatie en klinische kenmerken van tumor groei. Echter valt neovascularisatie en expressie van macrofagen wel te relateren aan tumor omvang en de tumor growth index. Tevens lijkt intratumorale hematoomvorming in hogere mate voor te komen in tumoren met cysteuze degeneratie, een proces waarvan bekend is dat het bijdraagt aan tumor expansie[1]. Op basis van deze resultaten baseren we de hypothese dat de groei van vestibularis schwannomen niet alleen het gevolg is van toegenomen cel proliferatie. Factoren zoals cysteuze degeneratie, angiogenese en ontsteking lijkten ook van invloed op tumor groei.

Hoofdstuk vier beschrijft een gen expressie analyse naar de dertien meest voorkomende mutaties in de genen *BRAF, EGFR, PIK3CA* en *KRAS.* De eiwitten waar deze genen voor coderen maken deel uit van MAPK/ERK cel signaleringscascade. Toegenomen activiteit van deze cascade wordt gezien in verschillende tumoren waaronder ook vestibularis schwannomen [2,3]. Deze kennis en de vondst van *BRAF* mutaties in sporadische, niet hoofd-hals, schwannomen [4] gaf aanleiding de hypothese dat groei van vestibularis schwannomen beïnvloed kan worden door secundaire oncogenetische mutaties met betrekking op de MAPK/ERK cascade te onderzoeken. Een selectie van achtenveertig sporadische vestibularis schwannomen is gescreend op de aanwezigheid van deze mutaties waarbij geen enkele mutatie is aangetroffen. Deze uitkomst geeft daarmee geen ondersteuning aan de hypothese dat de groei van vestibularis schwannomen versneld kan worden door het optreden van secundaire oncogenetische mutaties.

Hoofdstuk vijf is een studie die verder ingaat op de rol van ontsteking in vestibularis schwannomen. Zoals ook beschreven in hoofdstuk drie lijkt het zo te zijn dat sommige tumoren grote hoeveelheden macrofagen bevatten. Gedurende de afgelopen jaren is in toenemende mate duidelijk geworden dat in verschillende typen tumoren het inflammatoire micromilieu een bevorderend effect kan hebben op tumor progressie[5]. Macrofagen hebben een groot aandeel in de samenstelling van dit micromilieu[6]. Deze zogenaamde tumor associated macrophages kunnen grofweg in twee categorieën verdeeld worden, namelijk de klassiek geactiveerde M1 macrofagen en de alternatief geactiveerde M2 macrofagen. Van deze twee categorieën zijn het de M2 macrofagen die tumorgroei bevorderende eigenschappen bezitten zoals stimulatie van angiogenese en het onderdrukkend van de immuunreactie die zich juist tegen de tumor richt[7]. Met behulp van immunofluorescente kleuringen tegen CD163, een specifieke marker voor M2 macrofagen, is de expressie van M2 macrofagen in twintig retrospectief geanalyseerde vestibularis schwannomen bepaald. Daarnaast is de mate van angiogenese gescoord. De resultaten van deze kleuringen laten

zien dat sommige vestibularis schwannomen grote hoeveelheden M2 macrofagen bevatten. Tevens lijkt er een positieve relatie te bestaan tussen de mate van macrofagen expressie en angiogenese evenals tumorgroei snelheid. Deze resultaten zijn in lijn met de gedachte dat tumorinfiltrerende M2 macrofagen de progressie van vestibularis schwannomen bevorderen en daarmee vormen deze ontstekingscellen een potentieel aangrijpingspunt voor therapie.

Hoofdstuk zes omvat een studie naar de rol van BCRP expressie als een mogelijke oorzaak van medicatie resistentie in een selectie van verschillende perifere zenuwschede tumoren. BCRP is een transmembran efflux transporter protein dat een belangrijke functie lijkt te bekleden in verschillende biologische barrières zoals de bloed-hersen-barrière [8,9]. De expressie van dit eiwit is ook geassocieerd met medicatie resistentie in kanker[10]. Een immunohistochemische kleuring van BCRP is verricht op een tissue microarray die is samengesteld uit tweeëntwintig sporadische vestibularis schwannomen, tien plexiforme neurofibromen en achttien maligne perifere zenuwschede tumoren. De uitkomsten van deze studie demonstreren de expressie van BCRP in het vaatendotheel van een groot aantal van alle drie de soorten tumoren. Mogelijk is deze endotheliale expressie van BCRP een specifiek tumorkenmerk, maar het zou ook een overblijfsel van de bloed-zenuw-barrière kunnen betreffen. Hoe dan ook suggereren deze resultaten dat BCRP mogelijk de blootstelling van het onderliggende weefsel aan medicatie kan verlagen. Dit kan een onderdeel van de verklaring vormen waarom medicamenteuze therapie voor perifere zenuwschede tumoren, waaronder vestibularis schwannomen, wisselende effecten laat zien. Als deze hypothese klopt dan kan de inhibitie van BCRP eventueel de gevoeligheid voor medicatie van deze tumoren vergroten.

Hoofdstuk zeven vormt het vervolg op de macrofagen analyse die in hoofdstuk vijf is beschreven. Deze studie omvat dezelfde patiënten groep en focust op twee cytokinen, M-CSF en IL34. Van deze twee eiwitten is bekend dat ze een regulerende functie hebben met betrekking tot het activeren van macrofagen. M-CSF staat voor macrophage colony stimulating factor en is een cytokine dat macrofagen richting een M2 fenotype kan polariseren. De exacte tumor biologische karakteristieken van dit eiwit zijn nog niet geheel duidelijk maar er zijn verscheidenen tumor modellen waarbinnen het een rol lijkt te spelen bij macrofaag geassocieerde tumor progressie [11,12]. IL-34, interleukine-34, is een eiwit dat veel overeenkomstige kenmerken met M-CSF vertoond [13,14]. Relatief recente studies laten zien dat IL-34 op een vergelijkbare wijze als M-CSF tumor progressie kan stimuleren[15]. Onze studie demonstreert de expressie van deze twee cytokinen in vestibularis schwannomen. In overeenstemming met de verwachte functie van M-CSF wordt een relatie gezien tussen de mate van M-CSF expressie, de aanwezigheid van M2 macrofagen en tumor groei. Voor IL-34 konden geen significatie verbanden worden aangetoond, echter is

de observatie dat IL-34 in alle tumoren in aanzienlijke mate aanwezig is toch suggestief voor het feit dat ook dit eiwit een rol speelt in de tumor biologie van het vestibularis schwannoom. De resultaten van deze studie lijken in lijn met de hypothese dat het inflammatoire micromilieu binnen het vestibularis schwannoom een belangrijke rol speelt in de progressie van deze tumoren. Daarmee vormen M-CSF, en wellicht ook IL-34, potentiële aangrijpingspunten voor toekomstige therapie.

Conclusie

Het algemene doel van dit proefschrift is het ontrafelen van de biologische achtergrond van de klinische progressie van sporadische vestibularis schwannomen. In het geval van vestibularis schwannomen wordt progressie gemeten op basis van toename in tumor omvang. De meest voor de hand liggende factoren die het volume van vestibularis schwannomen bepalen zijn cel groei en cyste vorming. De resultaten van onze studies duiden er op dat intratumorale ontsteking op verschillende vlakken de progressie van deze tumoren beïnvloed. Door het stimuleren van angiogenese en de inhibitie van de immuunreactie gericht tegen tumorcellen stellen tumor associated macrophages sommige tumoren mogelijk in staat snellen en tot een groter volume te groeien. Daarnaast zou het goed mogelijk kunnen zijn dat de bulk van het ontstekingsinfiltraat op zichzelf al bijdraagt aan het volume van de tumoren. Het lijkt er op dat M-CSF en IL-34 een rol spelen in het reguleren van deze ontstekingsreactie waardoor ze potentieel een aangrijpingspunt kunnen vormen voor medicamenteuze therapie. Deze uitkomsten dienen uiteraard omzichtig geïnterpreteerd te worden. Het is belangrijk om te benoemen dat de resultaten van de vergelijkingen die we gemaakt hebben bestaan uit observaties van verschillende associaties. Het is daarbij altijd mogelijk dat deze resultaten nevenverschijnselen van een overkoepelend proces betreffen en in dat geval niet direct aan elkaar gerelateerd zijn. Voordat experimentele behandeling van patiënten met medicijnen die het intratumorale ontstekingsproces moduleren gestart kan worden is het dan ook nodig deze bevindingen in in vitro- en diermodellen te bevestigen. Mogelijke voorbeelden van dit type medicijnen zijn PLX3397 (Pexidartinib) en RG7155 (Emactuzumab) [16,17]. Beide zijn in staat macrofagen activiteit in tumoren te blokkeren en vormen om die reden ook potentiele behandelopties voor vestibularis schwannomen.

In de zoektocht naar nieuwe medicatie die tumorgroei van vestibularis schwannomen kan beïnvloeden is het belangrijk om mee te nemen dat de bevindingen van deze studie laten zien dat deze tumoren mogelijk “beschermd” worden door barrière eiwitten zoals BCRP. Toekomstig onderzoek moet rekening houden met dit potentiele extra obstakel om de effectiviteit van nieuwe behandelingen te kunnen optimaliseren.

Naast het vinden van nieuwe farmacologische manieren om vestibularis schwannomen te behandelen zijn er nog een aantal andere klinische dilemma's die meer onderzoek behoeven. Een belangrijk onderwerp dat verder onderzocht moet worden is de potententiele superioriteit van protonen bestraling boven de reguliere fotonen bestraling. De huidige literatuur over dit onderwerp is tot op heden niet doorslaggevend en meer klinische trials zijn nodig om deze vraag te beantwoorden. De verdere ontwikkeling van nieuwe therapeutische behandel opties heeft ook effect op de gezamenlijke besluitvorming tussen arts en patiënt. Om deze besluitvorming blijvend te optimaliseren is onderzoek naar kwaliteit van leven van groot belang zodat de uiteindelijke behandeling het beste op de individuele behoeften van de patiënt aan zal sluiten.

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Curriculum Vitae

Maurits de Vries (08-08-1985) was born in Utrecht, The Netherlands. In 2003 he graduated from Het Nieuwe Lyceum in Bilthoven. The same year he was admitted to Leiden University's medical school and obtained his medical qualification in 2011. During his studies he worked for Stichting BIS, a national organisation responsible for the explantation and allocation of human tissues in the Netherlands. After completing medical school het worked as a full-time PhD student at the department of Pathology of the Leiden University Medical Center. This is where he started his research on vestibular schwannoma biology under supervision of Prof. P.C.W. Hogendoorn and Dr. A.G.L van der Mey. During this period he successfully accomplished several research related courses such as the BROK course, the Basic Methods in Reasoning in Biostatistics course and the PhD introductory Meeting. In 2012 he commenced his clinical traineeship as a resident at the department of Otorhinolaryngology of the Leiden University Medical Center under supervision of Prof. J.H.M. Frijns and dr. A.G.L. van der Mey. In 2018 he obtained his registration as an otolaryngologist and continued his work at the Leiden University Medical Center under prof. P.P.G van Benthem.