Tumor biological characteristics of Vestibular Schwannoma

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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

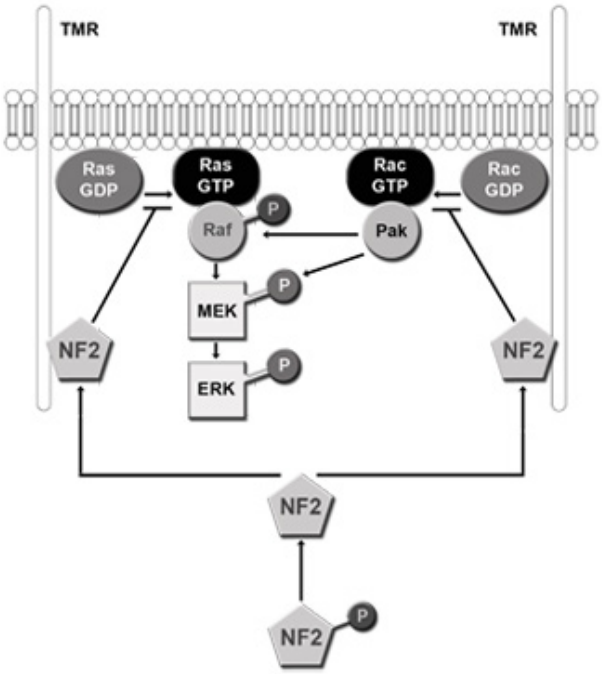

Figure 1. NF2 involvement in the MAPK/ERK pathway. NF2 becomes active through dephosphorylation. Dephosphorylated NF2 binds to a transmembrane receptor (TMR). NF2 then blocks the activation of Ras and Rac thereby inhibiting phosphorylation of Raf and MEK by PAK. NF2 also inhibits signaling from constitutively 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.

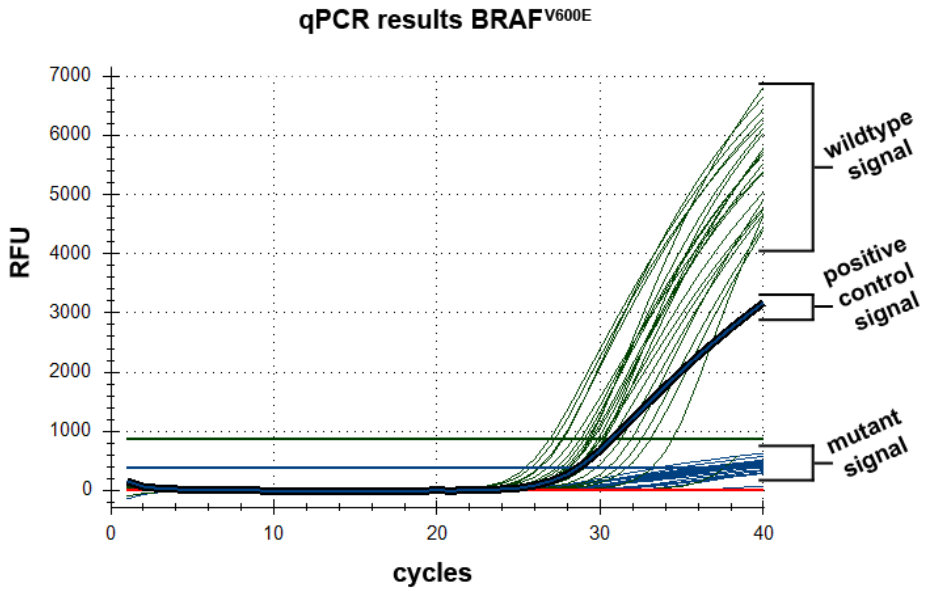

Figure 2. Example of qPCR results for the BRAF assay.

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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