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Correlation of Unilateral Sporadic Vestibular Schwannoma Growth Rates with Genetic and Immunohistochemical Abnormalities

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Abstract

Background: Unilateral sporadic vestibular schwannomas (USVS) are caused by inactivating somatic mutations of both alleles of the neurofibromatosis 2 (*NF*2) tumor suppressor gene. Unilateral sporadic vestibular schwannomas have a widely-varying growth patterns whose causes are poorly understood. Objective: We examined the relationships between an index of USVS growth, and genetic abnormalities and pathological growth indices. Subjects and Methods: Single-strand conformational polymorphism analysis and heteroduplex methods were used to screen for mutations in all 17 exons of the *NF*2 gene in USVS from 63 patients. Loss of heterozygosity (LOH) analyses were also carried out. An index of USVS growth (clinical growth index, CGI) was calculated as maximum tumor diameter divided by duration of symptoms. The immunohistochemical growth indices were based on monoclonal antibodies to Ki-67 and another tumor cell proliferation marker (platelet-derived growth factor (PDGF)). Results: CGI was highly variable and did not significantly decrease with increasing age at diagnosis. Either somatic *NF*2 mutations or LOH was found in 88% of tumors. PDGF and Ki-67 increased significantly with increasing age at diagnosis, and PDGF was lower in tumors with LOH than in those without LOH. In multiple linear regression analysis, CGI was significantly higher in people with higher PDGF, after accounting for age at diagnosis and LOH. Conclusion: An index of USVS growth increases with increasing PDGF, after accounting for age and LOH.

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Keywords

Vestibular Schwannoma, *NF*2 Gene, Immunohistochemistry

1. Introduction

Acoustic neuromas or vestibular schwannomas are intracranial, extra-axial tumors that arise from the Schwann cell sheath investing either the vestibular or cochlear nerve. As acoustic neuromas increase in size, they eventually occupy a large portion of the cerebellopontine angle. Acoustic neuromas account for approximately 80% of tumors found within the cerebellopontine angle [1]. Clinically diagnosed vestibular schwannomas occur in 0.7 - 1.0 people per 100,000 population. The incidence may be rising, a reflection of the increasing frequency with which small tumors are being diagnosed with the more widespread use of MRI and almost certainly due to better diagnostic imaging [2]. The great majority of VS are unilateral sporadic vestibular schwannomas (USVS), which usually develop between ages 40 and 60. About 5% of VS cases are bilateral VS that occurs in neurofibromatosis 2 (*NF*2), an autosomal dominant genetic disease. The *NF*2 tumor suppressor gene, which was isolated in 1993 [3] [4], was inactivated in both *NF*2-associated VS and USVS [5].

The growth rates of USVS are highly variable, ranging from slow (0.2 mm diameter/year), to medium (2 mm/year), and to fast (10 mm/year) [6]. About 13% of tumors are fast-growing [7]-[9]. Some studies have reported that fast-growing tumors tend to occur in younger individuals [10]-[12], while other studies have reported that USVS growth rates do not change with age [7] [9].

Genotype-phenotype correlations have not been found in *NF*2-associated VS growth rates, although there are genotype-phenotype correlations for other aspects of *NF*2 disease severity [13] [14]. There are differences in the pathology of *NF*2-associated VS and USVS. Treatment options includes wait and rescan, to Radiotherapy to Surgery in larger tumors [15] [16]. The purpose of the present study was to examine the relationship between USVS growth, molecular abnormalities in the NF2 gene, and immunohistochemical markers of cell proliferation in a large series of USVS patients.

2. Methods and Materials

The 63 patients in the study were seen at the neuro-otology clinic of the Manchester Royal Infirmary. These 63 patients were specifically selected because of their clinical characteristics. This was experimental study, and no control group was selected. Since USVS growth may be higher in younger patients, we included 27 patients in the study who were aged $\leq$ 30 years at onset of symptoms. At the time of surgery, none of the patients had a family history of *NF*2 or other neurogenic tumors. The young patients were examined for cutaneous tumors and for pre-senile cataracts using slit lamp biomicroscopy to exclude clinical features of *NF*2.

Magnetic resonance imaging (MRI) with T1-weighted, gadolinium-DTPA enhancement was done using a 1.5 Tesla MR scanner (ACS-NT PT6000 Philips Medical Systems, Best, NL). All scans were acquired using a multi-slice spin echo sequence (3 mm slice thickness) and a 3D gradient echo sequence. Tumor measurements were taken in anteroposterior and mediolateral dimensions including both intra-canalicular and extra-canalicular portions of the tumor.

For each tumor, a clinical growth index was calculated by dividing the maximum tumor diameter (in cm) by the duration of symptoms (time between onset of symptoms and tumor detection by MRI scan, in months). Fifty-two of the 63 tumors with fresh tissue were stained with H&E and evaluated by a neuropathologist (H.R.) for vessel-related changes such as features of hemorraghic changes and angiogenesis. In these 52 patients, tumor proliferation rate was estimated using monoclonal antibodies to Ki-67 and PDGF. Ki-67 and PDGF immunostaining was done on 4 μm thick sections cut from formalin-fixed or paraffin-embedded tumor samples using the streptavidin-biotin complex peroxidase technique [17]. In the Ki-67 proliferation staining, there were few positive nuclei, indicating that most of the cells had entered the S phase of nuclear division cycle. To calculate the proliferation index, the area with the most staining was selected, at least 200 nuclei were counted, and the index was the percentage of positive nuclei (0 = 0%, 1 = <1%, 2 = 1% - 3%, 3 = 4% - 5%, 4 = 6% - 8%). In the PDGF staining, none of the tumors had diffuse strong positivity. Positivity was graded as 0 (no perivascular staining), 1) (few foci of perivascular staining with few positive cells), 2) (few foci of perivascular staining with moderate

number of positive cells), 3) (moderate number of foci of perivascular staining with few positive cells), or 4) (moderate number of foci of perivascular staining with moderate number of positive cells). These staining methods used were more qualitative and based on the pathologists (H.R) experience. Unfortunately we do not have any material or slides available to show any pictures of the slides and how it was carried out, but the technique used was similar to that of Hsu *et al.* [18]

Genomic DNA was extracted from fresh and paraffin-embedded tumor samples as previously described [17]. Genomic and tumor DNA samples were amplified for the 17 *NF*2 exons by PCR using previously-described primers and conditions [3] [19], except the primer pairs for exons 1, 2, 3 and primers for reverse exon 6 and forward exon 8 were those of Mohyuddin *et al.* [20]. Single-strand conformation polymorphism (SSCP)/heteroduplex analysis (HA) was carried out on 32 cm long 8% (49:1 acrylamide:bis acrylamide ratio) native polyacrylamide gels at 360 V constant voltage for approximately 16 hours at 4˚C and visualized by silver staining [21].

Samples that gave rise to SSCP or heteroduplex shifts that were present at low levels on silver stained SSCP/HA gels (minority alleles) were re-amplified and a larger quantity of the sample was loaded onto a second SSCP/HA gels. The gel was subjected to silver staining as previously described, except that the fourth solution was replaced with 50 mM EDTA (ethylene diamine tetra acetic acid). The gel slices were briefly rinsed in 50 µL sterile water and DNA was eluted by crushing and soaked overnight in 50 µL of sterile water. The minority alleles isolated from the gel were then re-amplified in a PCR reaction. PCR products were purified prior to sequencing using Centricon 100 columns. Forty µL PCR reaction products were mixed with 2 mL of sterile water and added to the columns which were then spun, according to manufacturer's instructions. The purified DNA products were visualized on 2.5% agarose gel. DNA sequencing reactions were carried in both forward and reverse orientations for each sample using Big Dye Terminator version 20 ready reaction kits. Electrophoresis and fluorescent detection of the sequencing reactions was carried out on an Applied Biosystems 377 sequencer with 48 cm well.

LOH was tested using the microsatellite markers D22S275, NF2CA3, D22S268, D22S280 [22]-[25]. NF2CA3 is located within intron 1 of *NF*2, and D22S275, D22S268 and D22S280 are tightly linked to *NF*2 [26]. For the markers D22S275, NF2CA3, and D22S268, LOH was determined on fluorescently-labelled PCR product. The lymphocyte and tumor DNA pairs were amplified at the same time and electrophoresed on the same Genescan gels. Fragment sizing and comparison between lymphocyte and tumor DNA results were carried out using the Genescan version 2.1.1 fragment analysis program. A diminution in relative allele signal strength between lymphocyte and tumor DNA of more than 30% was taken as indicative of LOH. For the marker D22S280, LOH was determined by comparison of lymphocyte and tumor. PCR amplifications were electrophoresed on non-denaturing gels and visualized by silver staining.

Statistical analyses were done using the two-tailed t-test, one-way analysis of variance with test for trend, bivariate correlations (Pearson's for continuous variables and Spearman's for ordinal variables), and multivariate linear regression. Due to the relatively small number of patients, the Ki-67 and PDGF values were collapsed into three categories (0, 1 - 2, and 3 - 4). In regression analyses, the Ki-67 and PDGF indices were categorized as three indicator variables (1 - 2 and 3 - 4, each compared to 0).

3. Results

There were 33 females and 32 males in the study. The mean ± SE age of onset of symptoms was 40 ± 2 years, and at diagnosis, 42 ± 2 years. Since age at onset of symptoms and age at diagnosis were highly correlated (r^2 = 0.97), only the results for age at diagnosis are reported for subsequent analyses.

At least one specific somatic *NF*2 mutation was identified in 48 (74%) of the 65 tumors. Of the 53 somatic mutations (both first and second hits), 30 were frame-shift mutations, 12 were non-sense mutations, eight were splice-site mutations and three were in-frame deletions. LOH was found in 39 (60%) of tumors. Either a somatic *NF*2 mutation or LOH were found in 88% of the tumors; both mutational hits were identified in 34 (52%) of tumors and one mutational hit in 23 (35%) of tumors.

CGI was highly variable and did not significantly decrease with increasing age at diagnosis (**Figure 1**; r^2 = 0.00). Ki-67 and PDGF were highly correlated (Spearman's rho = 0.60, $P < 0.001$), and each index was significantly correlated with age at diagnosis. The mean age at diagnosis in people with tumors having Ki-67 = 0 was 27 ± 1 years; Ki-67 = 1 - 2, 43 ± 2 years; and Ki-67 = 3 - 4, 51 ± 4 years (one-way analysis of variance, test for trend, $P < 0.001$). The mean age at diagnosis in people with tumors having PDGF = 0 was 25 ± 1 years; PDGF = 1 - 2, 40 ± 2 years; and PDGF = 3 - 4, 48 ± 4 years (test for trend, $P < 0.001$).

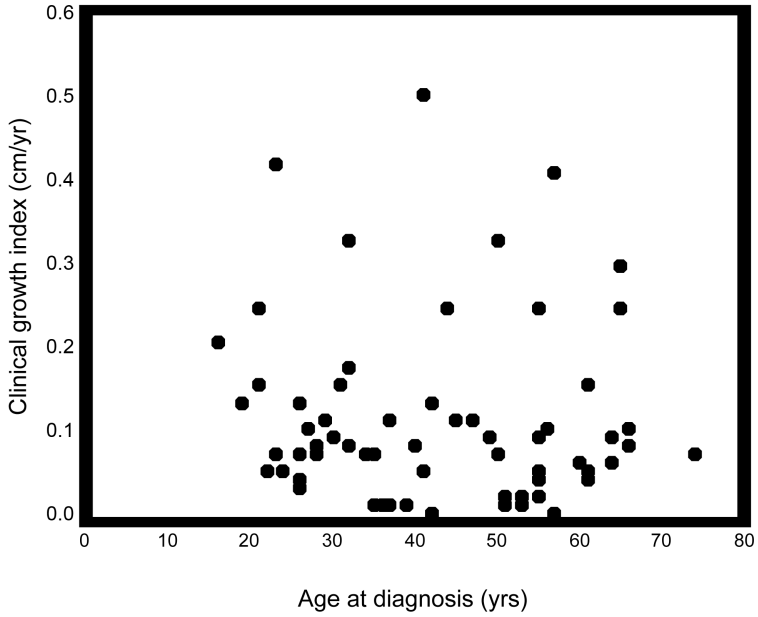

Figure 1. Clinical growth index and age at diagnosis of USVS.

CGI, PDGF, and Ki-67 were not significantly different between tumors with at least one non-truncating mutation (splice-site mutation or in-frame deletion), and tumors with either two truncating mutations (nonsense or frame-shift mutations) or one truncating mutation and LOH. However, irrespective of mutation type, people with tumor LOH were significantly younger at diagnosis than those without LOH (with LOH, 38 ± 2 years; without LOH, 48 ± 3 years; two-tailed t-test, P = 0.010). PDGF indices were lower in tumors with LOH than in those without LOH (Spearman's rho = −0.25, P = 0.054). CGI was significantly higher in tumors with higher PDGF indices, but not in those with higher Ki-67 indices. The mean ± SE CGI in tumors with PDGF = 0 was 0.10 ± 0.01; in tumors with PDGF = 1 - 2, 0.10 ± 0.01; and in tumors with PDGF = 3 - 4, 0.20 ± 0.04 (test for trend, P = 0.030). We used multiple linear regression to examine the associations of covariates with CGI (**Table 1**). People with PDGF indices of 3 - 4 had significantly higher CGI than those with a PDGF index of 0, after accounting for age at diagnosis and LOH.

4. Discussion

The primary result of this study was that CGI increased with increasing PDGF, after accounting for age at diagnosis and LOH. PDGF is found in α-granules of human platelets and is associated with an increased rate of protein synthesis in fibroblasts, smooth muscle cells, and glial cells [27]. The exact function of these growth factors is not known, but they may be associated with events leading to DNA replication and cell cycle control, especially in tumors showing features of angiogenesis [28]. In the present study, all USVS were immunohistochemically positive for PDGF in a focal perivascular pattern (either in endothelial cells or in nuclei and cytoplasm of surrounding schwannoma cells), indicating that growth was perivascularly oriented.

The association between USVS growth rates (as inferred from CGI) and PDGF in particularly is interesting with respect to the findings of a recent longitudinal study of VS growth rates in *NF*2 [14]. In multiply affected members of similar ages in the same *NF*2 family, VS growth rates varied by as much as 58-fold. This means that factors other than the type of first mutational hit (such as growth factors [29]) may be important determinants of post-second hit VS growth rates. The present study provides evidence that growth factors are associated with USVS growth rates *in vivo*.

The association between CGI and PDGF requires further research, but it should be noted that immunohistochemical indices of tumor proliferation may overestimate growth because they do not reflect cell death (*i.e.*, in all growing tissues, there is an imbalance between cell production by mitosis, and cell loss by apoptosis or necrosis). Also, CGI is only a surrogate for volumetric USVS growth rates determined from serial MRI. Patient recall may be inexact regarding onset of symptoms, which may not necessarily relate to onset of tumor development in

Table 1. Results of linear regression analyses with CGI as the outcome.

Model and covariates	$b \pm SE_b$	P
PDGF model		
LOH	−0.012 ± 0.029	0.69
Age at diagnosis (per year)	−0.001 ± 0.001	0.26
PDGF (compared to PDGF = 0)		
PDGF = 1 - 2	0.015 ± 0.041	0.71
PDGF = 3 - 4	0.117 ± 0.051	0.03
Ki-67 model		
LOH	−0.030 ± 0.030	0.31
Age at diagnosis (per year)	−0.001 ± 0.001	0.26
Ki-67 (compared to Ki-67 = 0)		
Ki-67 = 1 - 2	0.024 ± 0.043	0.58
Ki-67 = 3 - 4	0.071 ± 0.053	0.18

every individual. Most medially-arising tumors can attain a large size before becoming symptomatic, while laterally-arising tumors can become symptomatic earlier in their development. This may produce a bias towards apparent faster growth in laterally-arising tumors. Consideration should also be given to non-mitotic factors in tumor growth, such as cystic degeneration and intraluminal hemorrhages.

Ki-67 nuclear antigen is expressed in all human proliferating cells throughout the cell cycle. Expression of this antigen occurs preferentially during the late G1, S1, G2, and M phases of the cell cycle, while the G0 (non-cycling) phase consistently lacks the Ki-67 antigen [30]. Charabi *et al*. noted a strong correlation between symptom duration and Ki-67 expression in 21 USVS [31] [32], but we did not find such a correlation in this study (test for trend, P = 0.58). Transforming growth factor-beta (TGF-β)/TGF-β R1 and R2 system is present in human schwannoma, but its biologic role for tumor development and growth remains unclear [33].

In general, tumors in older people are slower-growing than those in younger people due to a reduced rate of somatic cell growth or smaller proportion of growing cells. In the present study, CGI did not significantly decrease with increasing age, possibly because CGI was extremely variable. This characteristic variability may be due to growth factors, other genes, or stochastic processes that remain to be identified.

As in many previous studies of USVS and *NF*2, the most common *NF2* mutations in this study were frameshift, nonsense, and splice-site mutations. Two thirds of the mutations were on or flanking the first eight *NF*2 exons. This preponderance has been noted previously [34] [35], and it is possible that some mutations within the terminal *NF*2 exons preserve part of the C-terminus and result in a more stable protein with less likelihood of promoting tumorigenesis [36].

We found either LOH or a *NF*2 mutation in 88% of tumors. SSCP/HA does not detect mutations within the intronic or promoter regions of the *NF*2 gene, or deletions that remove an entire exon. *NF*2 expression may be suppressed by methylation or mutation of the promoter elements [37], or by mutations at another regulatory locus. Point mutations and methylation at CpG sites significantly decrease promoter activity and expression of *NF*2 mRNA. Some *NF*2 gene mutations can act in a dominant negative manner, in which abnormal gene products interfere with the wild-type allele product. However, mutational inactivation and allelic loss in the *NF*2 gene appear to be the causal event in the majority of vestibular schwannomas [34].

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