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MAIN ARTICLE

Role of cyclins D1 and D3 in vestibular schwannoma

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Abstract

Background: Vestibular schwannomas in younger patients have been observed to be larger in size and grow more quickly.

Objective: This study aimed to evaluate the expression of three important cell cycle proteins, cyclin D_1, cyclin D_3 and Ki-67, in vestibular schwannoma patients separated into two age groups: ≤40 years or >40 years.

Method: Immunohistochemical detection of cyclin D_1, cyclin D_3 and Ki-67 was undertaken in 180 surgically resected vestibular schwannomas.

Results: The proliferation index of vestibular schwannomas was statistically higher in the ≤40 years age group compared to that in the >40 years age group (mean of 4.52 *vs* 3.27, respectively; $p = 0.01$). Overexpression of cyclin D_1 and cyclin D_3 was found in 68 per cent and 44 per cent of tumours, respectively.

Conclusion: There was an increased Ki-67 proliferation index in the younger age group that appears to correlate with clinical behaviour. Vestibular schwannomas in both age groups show increased expression of cyclin D_1 and cyclin D_3.

Key words: Acoustic Neuroma; Neurofibromatosis 2; Cyclin D1; Cyclin D3; Ki-67 Antigen; Age Of Onset

Introduction

Vestibular schwannomas are benign tumours that arise from Schwann cells of the vestibular portion of the VIIIth cranial nerve. They account for approximately 6 per cent of intracranial tumours, and occur in two distinct populations: unilateral vestibular schwannomas, which are predominately sporadic,[1,2] and bilateral vestibular schwannomas that occur almost exclusively in patients with germline neurofibromatosis type 2 (NF2).[3,4]

A frequent mutation in vestibular schwannoma is a mutation in the NF2 coding region located on chromosome 22 band q11-13.1. It encodes a 595-amino-acid protein that has been named 'merlin' (moesin-ezrin-radixin-like protein).[5] Sporadic and NF2 vestibular schwannomas are associated with loss of functional merlin in both alleles, in 66 per cent and 33 per cent respectively.[5] Merlin is a known regulator of cell signalling pathways, encompassing cell–matrix adhesion, proliferation and survival. Merlin's tumour suppressor function is mainly anti-proliferative, and is mediated through the inhibition of cell cycle regulators such as downstream target cyclin D_1.[6] Mutations in merlin have been discovered in a spectrum of central nervous system tumours including schwannomas, meningiomas and ependymomas.

Cell cycle deregulation through the activation of oncogenes and inactivation of tumour suppressor genes is well described in both benign and malignant tumours.[7–14] The D-type cyclins (D_1, D_2 and D_3) are induced by mitogens during the G_1 cell cycle phase, and continued synthesis throughout the cell cycle depends on continuous growth factor stimulation. Cyclin D_1 is a protein derived from the CCND1 gene on chromosome 11q13, and cyclin D_3 is a protein closely related to cyclin D_1, derived from the CCND3 gene on chromosome 6p21. The role of cyclin D_1 in a number of human neoplasias such as mantle cell lymphoma is well established.[15] Cyclin D_3 is also speculated to have a role in tumourigenesis.[16]

Ki-67 is a nuclear protein expressed by an individual cell when it is in the replication cycle.[17] The percentage of tumour cells that express Ki-67 immunohistochemistry is termed the Ki-67 proliferation index. This is considered to be a surrogate marker for tumour proliferation, and in current medical practice it is used as an independent prognostic determinate in a variety of tumours.[18–20]

Previous studies of cell cycle regulatory proteins in patients with vestibular schwannoma have revealed inconsistent results.[21–23] This study aimed to further

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evaluate the role of cyclin D_1 and cyclin D_3 proteins in vestibular schwannoma. A secondary aim was to determine whether proliferation index as measured by Ki-67 was associated with patient age at diagnosis.

Materials and methods

Patients

Following ethics committee approval, 180 consecutive patients with previously untreated vestibular schwannoma, who underwent surgical excision over a 15-year period (1998 to 2013), were identified for subsequent tissue sample retrieval from the case records of the Department of Head and Neck Surgery at St Vincent's Hospital, Sydney, Australia. There were 84 females and 96 males; patients were aged 14–81 years at the time of surgery (mean age of 54 years).

Tissue processing and microarray

The archived formalin-fixed paraffin-embedded tumour material was retrieved. The tissue was cut into 4-µm-thick sections for haematoxylin and eosin staining and Ki-67 immunohistochemistry. Representative areas were chosen, and biopsies of 2 mm diameter were taken from these donor blocks and included in recipient tissue microarray blocks using a precision tissue array instrument (Tissue-Tek Quick-Ray System, Sakura Finetek USA, Torrance, California, USA). From this tissue array block, 4-µm-thick sections were prepared for further immunohistochemistry analysis.

Immunohistochemistry

Tissue microarray sections were used for cyclin D_1 and cyclin D_3 immunohistochemistry, whereas sections of the original formalin-fixed paraffin-embedded tumour material were used for Ki-67 immunohistochemistry. Tissue sections of 4 µm thickness were cut from formalin-fixed, paraffin-embedded tissue. Immunohistochemistry staining was performed using the Ventana Benchmark Ultra automated slide stainer (Ventana Medical Systems, Oro Valley, Arizona, USA).

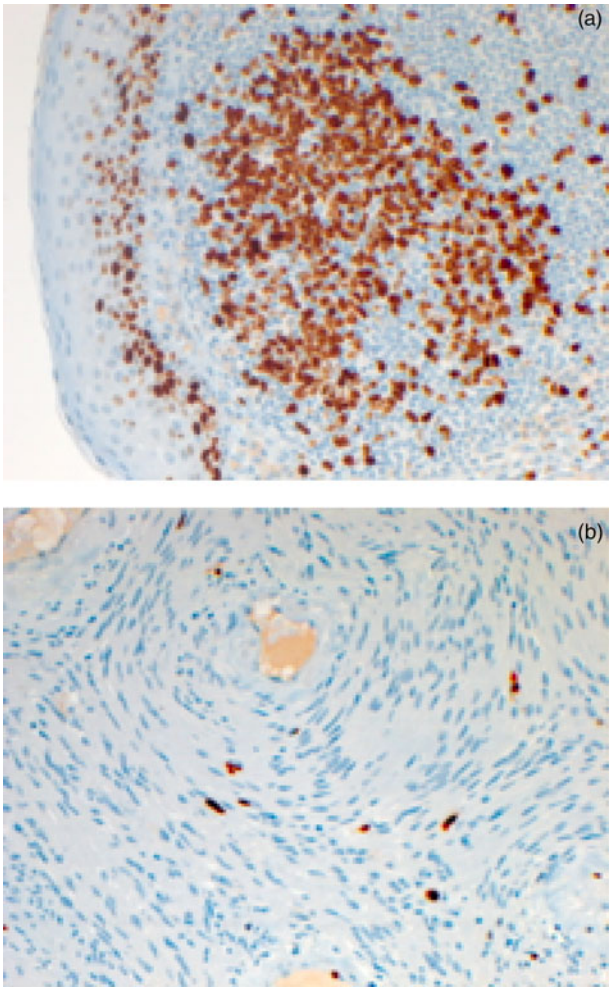

FIG. 1

Immunohistochemical localisation of Ki-67 in a normal tonsil tissue section magnified at ×100 (a) and a vestibular schwannoma tissue section magnified at ×200 (b). Intensity of nuclear labelling is strong in (a) and (b).

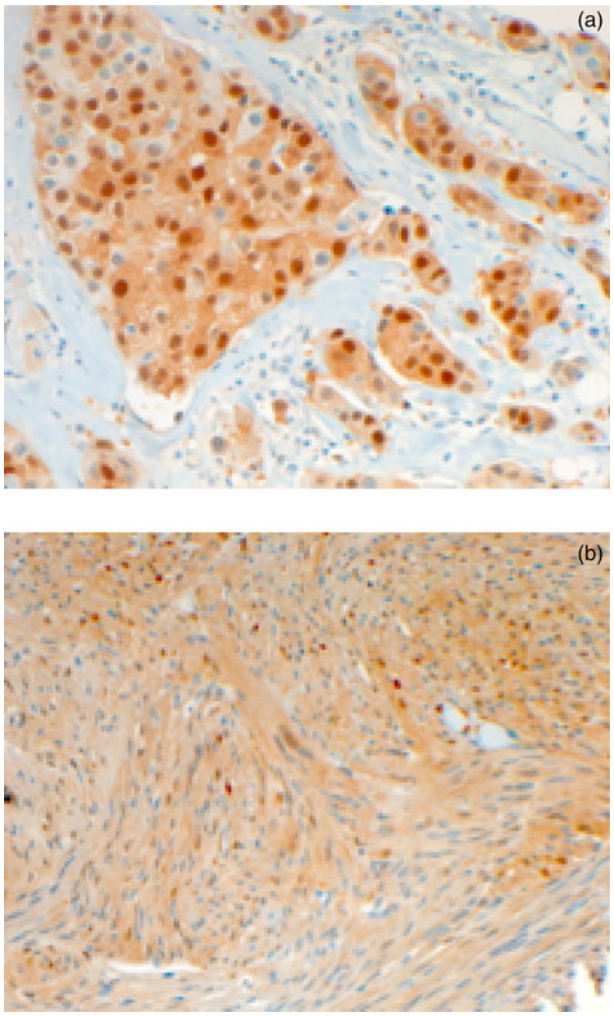

FIG. 2

Immunohistochemical localisation of cyclin D_1 (×200) in a breast carcinoma tissue section (a) and a vestibular schwannoma tissue section (b). Nuclear and cytoplasmic labelling intensity is strong in (a) and mild in (b).

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In brief, dewaxing and rehydration, followed by the blocking of endogenous peroxidase with a 3 per cent hydrogen peroxide and methanol mixture, was carried out. After undergoing heat-induced epitope retrieval using Ventana buffer (type CC1; Ventana Medical Systems), 36 minutes for cyclin D_1, 92 minutes for cyclin D_3 and 36 minutes for Ki-67, primary monoclonal antibodies with either cyclin D_1 (1:100) (code M3642; Dako, Golstrup, Denmark), cyclin D_3 (1:50) (CyclinD3; Leica Biosystems, Buffalo Grove, Illinois, USA) or Ki-67 (a ready-to-use antibody with no specified dilution) (Confirm Ki-67 (30-9); Ventana Medical Systems), were incubated, for 48 minutes for cyclin D_1, 60 minutes for cyclin D_3 and 28 minutes for Ki-67, at 36°C. Slides were then stained with a Ventana Ultraview Detection Kit. An additional amplification step was performed for cyclin D_3 using a Ventana Amplification Kit. All washing procedures were performed in phosphate buffered saline. Slides were then counterstained with Ventana Hematoxylin I, followed by Ventana Bluing Solution (Ventana Medical Systems). Tissues expressing cyclin D_1, cyclin D_3 or Ki-67 protein were identified by deposition of brown chromogen, with the haematoxylin counterstain staining light blue.

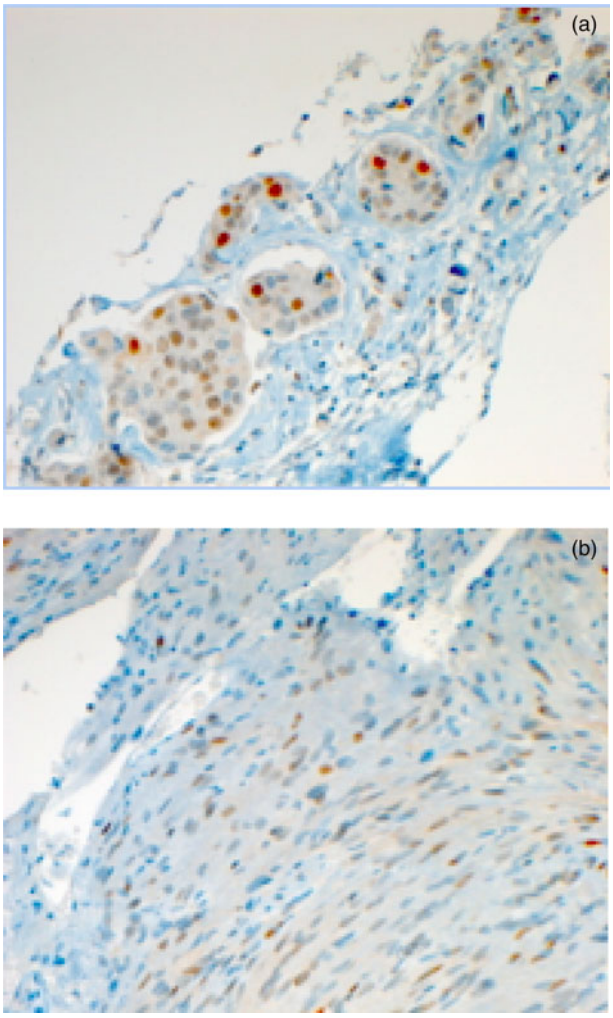

FIG. 3

Immunohistochemical localisation of cyclin D_3 (×200) in a breast carcinoma tissue section (a), and a vestibular schwannoma tissue section (b). Intensity of nuclear labelling is strong in (a) and mild in (b)

Expression quantification

Normal tonsil tissue was used as a positive control for Ki-67, and breast carcinoma was used as a positive control for cyclin D_1 and cyclin D_3 (Figures 1a, 2a and 3a, respectively).[24–26]

Distribution of cyclin D_1 or cyclin D_3 nuclear expression was graded as the following: <10 per cent (absent), 11–25 per cent, 26–50 per cent or 51–100 per cent.[27,28] For intensity of nuclear labelling of the cyclin D expression, a four-tier grading scale consisting of the following was used at high power magnification (×200) (field of view area: 0.15 mm^2): 0 (absent), + (mild), ++ (moderate) or +++ (strong).[28] For cyclins D_1 and D_3, the immunoreactivity was predominately nuclear; however, mild cytoplasmic labelling was also observed in cyclin D_1. Within each patient tumour sample, the percentage of cell labelling with maximum nuclear intensity ('Pos-Max') was scored as follows: 0 (absent), <10 per cent, 10–50 per cent or >50 per cent.

Proliferative activity

The Ki-67 proliferation index was determined using an Olympus X51 microscope at an original magnification of ×200. A cell was classified as positive when any degree of specific chromogen deposition was localised to the nucleus. The mean Ki-67 proliferation index was determined by counting the number of positive tumour cell nuclei in three chosen fields divided by the total number of cells in the same three fields (approximately 1000 cells in total).[29] The three chosen fields of preference were the three highest areas of proliferation or Ki-67 staining.

Statistical analysis

The relationships between Ki-67 proliferation index, and cyclin D_1 and cyclin D_3 protein immunohistochemistry distribution and intensity were compared for the two age groups, using the independent samples *t*-test for continuous variables and the chi-square test for categorical variables. Differences were considered significant at a level of $p < 0.05$.

Results

In total, 180 patients (aged 54 years ± standard deviation (SD) 13.9; range, 14–81 years) were studied. Patients were divided into 2 age groups: 35 patients (18 males and 17 females) were ≤40 years of age and 145 patients (78 males and 67 females) were >40 years of age.

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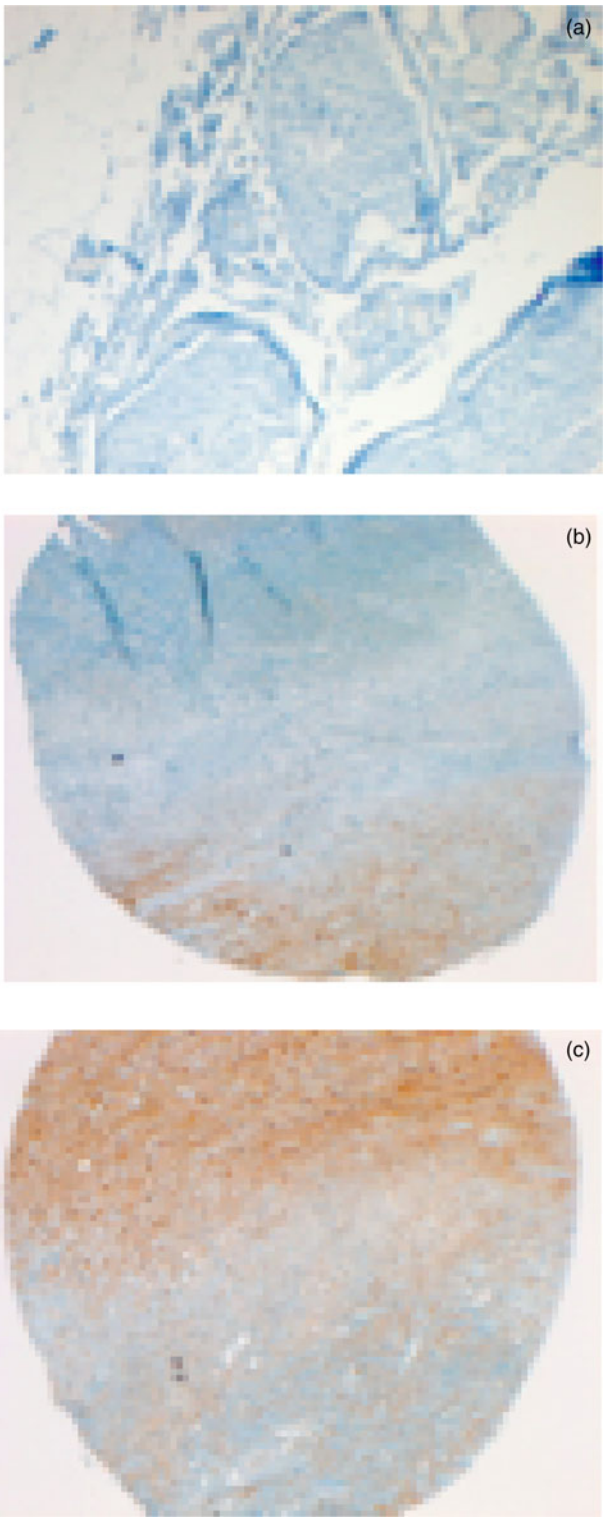

FIG. 4
Grading scale of cyclin D_1 distribution of labelling in a normal saphenous nerve tissue section magnified at ×100 (a), and vestibular schwannoma tissue sections magnified at ×40 showing: 11–25 per cent (b), 26–50 per cent (c) and 51–100 per cent grading (d).

Proliferation index

The proliferation was statistically significantly higher index ($p = 0.01$) in the ≤40 years age group (mean 4.52 ± SD 2.56) compared to the >40 years age group (mean 3.27 ± SD 1.79). There was no statistically

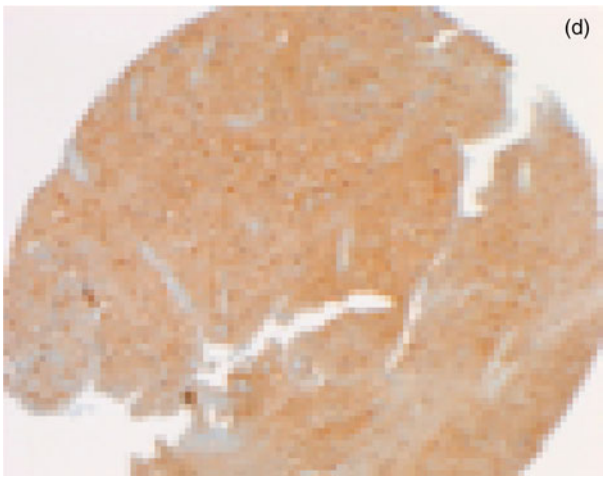

FIG. 4
(continued)

significant difference ($p = 0.62$) between male-to-female ratios in relation to proliferation index. Respective immunohistochemistry immunoreactivity data for Ki-67 are shown in Figure 1.

Cyclin D_1 expression

Cyclin D_1 was overexpressed in 68 per cent of the patients. There was overexpression of cyclin D_1 immunoreactivity in 63 per cent of patients (22 out of 35) in the ≤40 years age group and in 69 per cent (100 out of 145) in the >40 years age group. The cyclin D_1 immunoreactivity distribution scoring system is demonstrated in Figure 4a. There was no statistically significant difference ($p = 0.49$) in the distribution of labelling between the two age groups when negative labelling (≤10 per cent) was compared with positive labelling (>10 per cent).

The cyclin D_1 nuclear labelling was dichotomised into low intensity (0, +) or high intensity (++, +++). Figure 5 summarises the grading scale used for intensity of nuclear labelling. There was no statistically significant difference in intensity of cyclin D_1 nuclear labelling between the two age groups ($p = 0.49$).

Similarly, the cyclin D_1 protein Pos-Max was dichotomised into low per cent distribution (0, <10) and high per cent distribution (10–50, >50), with no statistically significant difference between the two groups ($p = 0.54$).

Tables I–III summarise the cyclin D_1 protein immunoreactivity between the two groups.

Cyclin D_3 expression

Cyclin D_3 was overexpressed in 44 per cent of the patients. Cyclin D_3 protein expression exhibited strong nuclear immunoreactivity (Figure 3b). There was overexpression of cyclin D_3 immunoreactivity in 57 per cent of patients (20 out of 35) in the ≤40 years age group and 41 per cent (60 out of 145) in the >40 years age group ($p = 0.09$). The distribution

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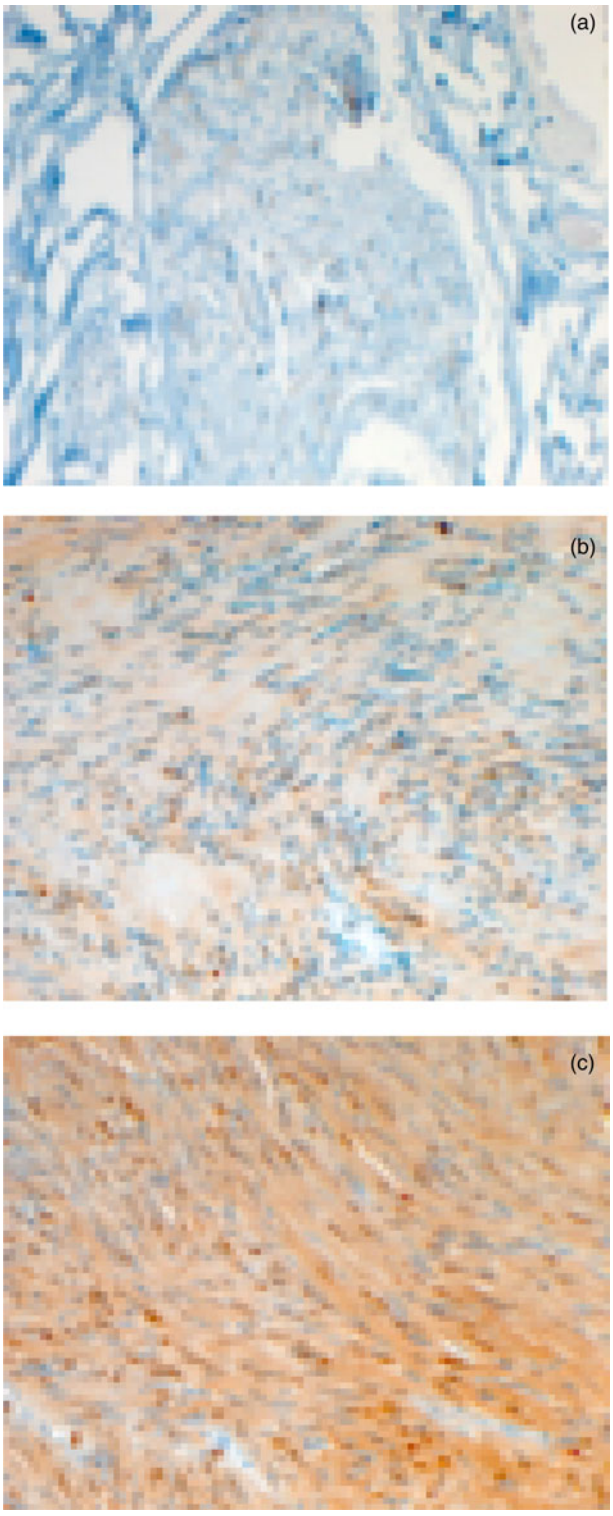

FIG. 5
Grading scale of cyclin D_1 (×200) intensity of nuclear labelling in a normal saphenous nerve (score 0) tissue section (a), and vestibular schwannoma tissue sections showing mild (+) (b), moderate (++) (c) and strong (+++) grading (d).

of cyclin D_3 labelling of tumours in the ≤40 years age group ranged from 11 to 100 per cent, compared to <26 per cent in all tumours in the >40 years age group.

Cyclin D_3 was dichotomised into low intensity (0, +) and high intensity (++, +++) immunoreactivity.

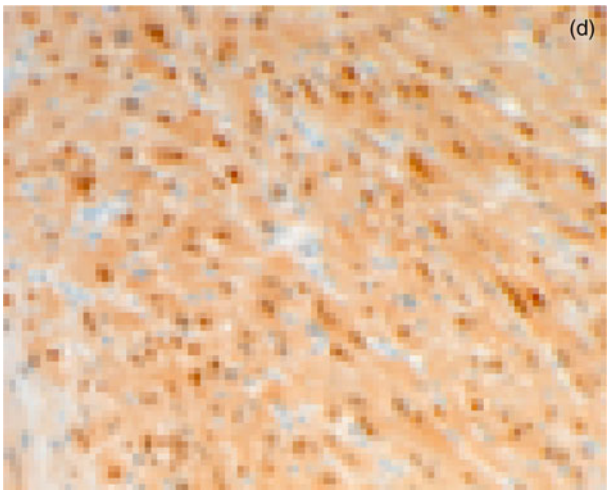

FIG. 5
(continued)

Figure 6 summarises the grading scale used for intensity of nuclear labelling.

The Pos-Max was dichotomised in a similar manner to the cyclin D_1 protein. There was a significant increase in Pos-Max cyclin D_3 nuclear protein staining in the younger age group compared to the older age group ($p = 0.02$).

Tables IV–VI summarise the cyclin D_3 protein immunoreactivity between the two groups.

Discussion

The loss of functional merlin leads to deregulation of a pathway associated with the contact-dependent inhibition of normal Schwann cells, ultimately leading to increased cell growth, tumour formation and tumour cell invasion. Merlin functions as a negative cell cycle regulator of the Rac-dependent signalling pathway.[30,31]

The regulation of merlin is complex and poorly understood, although studies suggest it may be dependent on its molecular conformation.[32,33] Merlin inactivation occurs when it is phosphorylated at S518 by p21-activated kinase (PAK), which is activated by Rac1 (ras-related C3 botulinum toxin substrate 1), interrupting the c-terminal domain and maintaining the folded state.[34,35] The disrupted folded state occurs by a folding of the alpha-helical portion and c-terminal portion of merlin, such that it blocks the four-point-one, ezrin, radixin, moesin ('FERM')-binding domain, which allows it to mediate cell–cell attachment, cell motility, membrane receptor availability and signal transduction.[32,35–39] The Rac pathway has been associated with tumourigenesis, and merlin tumour suppressor function is likely mediated by its dephosphorylated accumulation, inhibiting Rac signalling.[35]

Rac activates a variety of intracellular signalling pathways involved in transcriptional activation, transformation and proliferation. Downstream signalling

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TABLE I
SUMMARY OF CYCLIN D_1 PROTEIN IMMUNOREACTIVITY: DISTRIBUTION DATA

Age group	Nuclear expression % grading scale*				Dichotomised scale†		*p*
	<10	11–25	26–50	51–100	Negative	Positive	
≤40 years	13	10	10	2	13 (37.1)	22 (62.9)	0.49
>40 years	45	70	29	1	45 (31.0)	100 (69)	

*Data represent numbers of patients; †data represent numbers (percentages) of patients

TABLE II
SUMMARY OF CYCLIN D_1 PROTEIN IMMUNOREACTIVITY: INTENSITY OF NUCLEAR LABELLING DATA

Age group	Nuclear expression intensity grading scale*				Dichotomised scale†		*p*
	0	+	++	+++	Low	High	
≤40 years	13	0	2	20	13 (37.1)	22 (62.9)	0.49
>40 years	45	0	20	80	45 (31.0)	100 (69)	

*Data represent numbers of patients; †data represent numbers (percentages) of patients

regulated by Rac includes PAK, mitogen-activated protein kinase (MAPK), MET signalling, Jun-N terminal kinase (JNK), p38 and nuclear factor kappa beta (NF-κB).[40,41] Rac activation is important for progression of the G_1/S (synthesis) phase transition of the cell cycle, which occurs through Rac's ability to propagate transcription, and translation of cyclin D_1, driving the retinoblastoma protein (pRb)-cyclin-dependent kinase (CDK) pathway. In contrast, overexpression of merlin induces G_1 cell cycle arrest, highlighting an important association with merlin and cyclin D_1 regulation.[6] In a previous study, the loss of functional merlin in vestibular schwannoma was associated with decreased p21 protein and mRNA levels, when compared to normal myelinated nerve, and subsequently elevated cyclin D_1 protein.[42]

Previous studies have indicated important roles for cyclin D_1 protein in the regulatory control of Schwann cell proliferation.[21–23] The current study is the largest to date investigating the expression of cyclin D_1 and cyclin D_3 in vestibular schwannoma. Cyclin D_1 was overexpressed in 68 per cent of patients. This is consistent with a study of 64 sporadic vestibular schwannomas, in which 67 per cent of the tumours were positive.[21] Cyclin D_3 immunoreactivity in the current study was overexpressed in 44 per cent of patients. Again this is comparable to previous findings; in a study of 15 vestibular schwannomas, cyclin D_3 immunoreactivity was overexpressed in 50 per cent of tumours.[23] Our study provides further evidence to support the potential association between the overexpression of cyclin D_1 protein and merlin deregulation.

While nuclear labelling was required to confirm positive tumour sections for D cyclins, cytoplasmic labelling was present in most tumour sections for cyclin D_1. Cyclin D_1 is regulated by phosphorylation at its threonine 286 site via glycogen synthase kinase 3β (GSK-3β), which allows binding of cyclin D_1 with nuclear exportin CRM1, which places cyclin D_1 in the cytoplasm for proteolysis.[43] As such, we did not consider cytoplasmic positivity as significant.

Cyclin D_3 protein is expressed in variable abundance in proliferating cell populations in several types of human tumours, including breast, colorectal, melanoma, and head and neck cancers.[44] The overexpression in human cancers is unlikely to be as frequent as that of cyclin D_1 immunoreactivity in head and neck cancers, and the reason for this is not well understood. The functional role of cyclin D_3 in vestibular schwannoma pathogenesis remains to be determined. Schwann cell cultures exposed to polypeptide growth factor heregulin and adenylyl cyclase activator forskolin increased steady state levels of CCAAT/enhancer binding protein-b (C/EBPb) in Schwann cells which cyclin D_3 binds to.[45] In cultured melanoma cell lines, cyclin D_3 overexpression is regulated by fibronectin-mediated phosphatidylinositol 3-kinase/AKT signalling, but not

TABLE III
SUMMARY OF CYCLIN D_1 PROTEIN IMMUNOREACTIVITY: POS-MAX DATA

Age group	Pos-max* grading scale†				Dichotomised scale‡		*p*
	0	<10	10–50	>50	Low	High	
≤40 years	13	0	8	14	13 (37.1)	22 (62.9)	0.54
>40 years	45	1	42	57	46 (31.7)	99 (68.3)	

*Refers to percentage of cell labelling with maximum nuclear intensity. †Data represent numbers of patients; ‡data represent numbers (percentages) of patients

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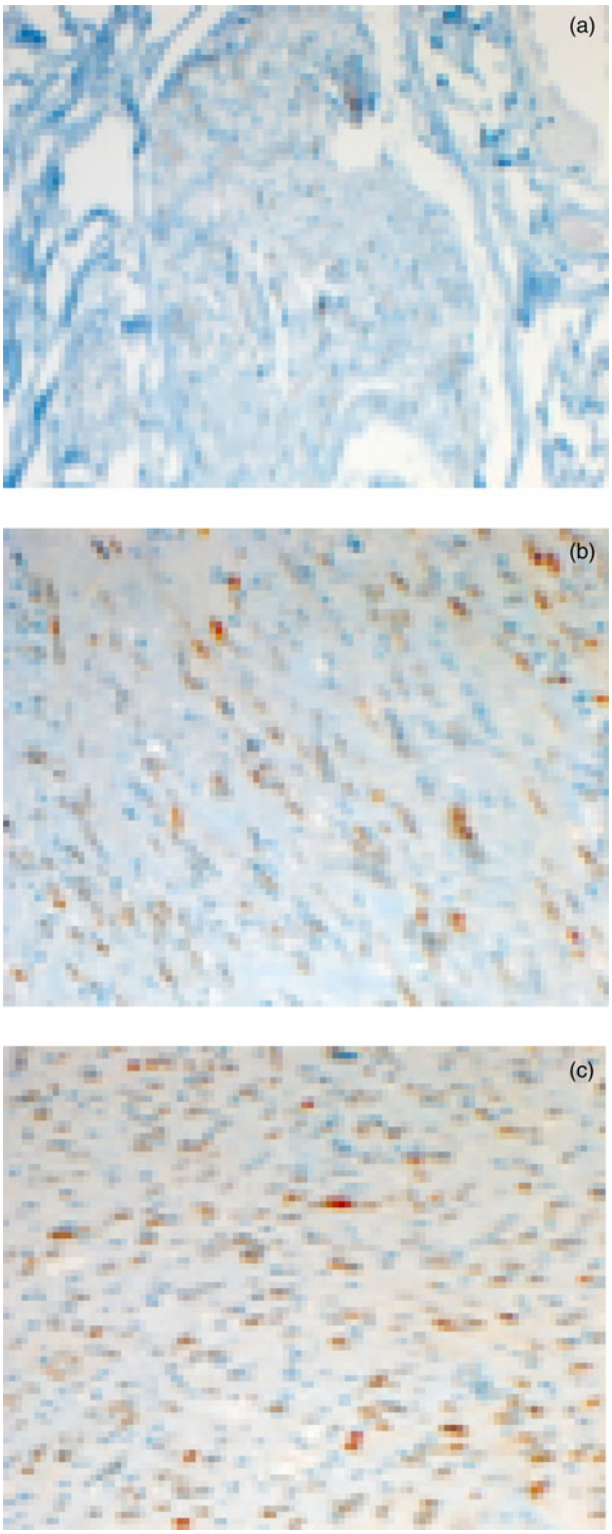

FIG. 6
Grading scale of cyclin D_3 (×200) intensity of nuclear labelling in a normal saphenous nerve (score 0) tissue section (a), and vestibular schwannoma tissue sections showing mild (+) (b), moderate (++) (c) and strong (+++) grading (d).

by the mitogen-activated kinase kinase (MEK) pathway.[46] In the same study, RNA interference experiments demonstrated that cyclin D_3 contributed to G_1/S cell cycle progression and proliferation. In our study,

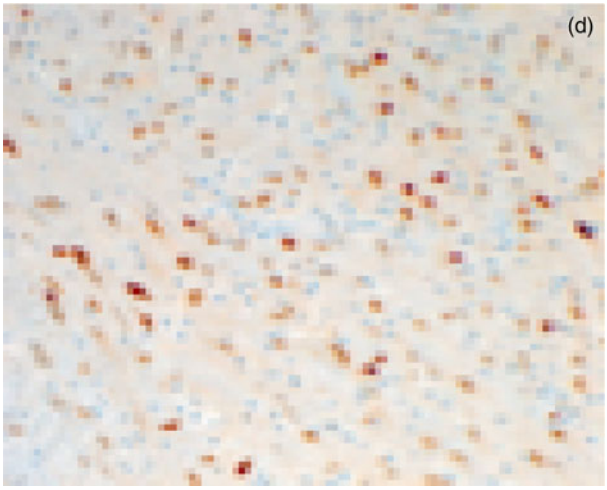

FIG. 6
(continued)

the younger age group had a higher expression of cyclin D_3 compared to the older age group, but only when assessed by the Pos-Max method.

Previous imaging and clinical studies have shown a statistically significant higher tumour growth rate in younger individuals (≤40 years) compared to those who are older (>40 years).[47,48] This is the first study to demonstrate an association between patient age and tumour proliferation index as measured by Ki-67. Cyclin D_1 and cyclin D_3 expression in this study did not correlate with the Ki-67 proliferation index, which is consistent with previous studies.[12,49]

- **Loss of functional merlin is common in both sporadic and neurofibromatosis type 2 related vestibular schwannomas**
- **This loss contributes to cell cycle pathway deregulation, with loss of Rac signalling inhibition**
- **This may lead to cyclin D protein overexpression, promoting vestibular schwannoma growth**
- **Younger patients have increased Ki-67 proliferation index compared to older patients, corresponding to increased tumour diameter and growth**
- **Overexpression of cyclin D_1 and cyclin D_3 was found in 68 and 44 per cent of tumours, respectively**
- **Elucidating molecular targets in vestibular schwannoma will aid treatment, enabling residual disease and radioresistant tumour morbidity to be addressed**

A better understanding of the tumour biology of vestibular schwannoma will enhance the management paradigm of this condition. Identifying predictors of growth may in future enable more selective

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TABLE IV
SUMMARY OF CYCLIN D_3 PROTEIN IMMUNOREACTIVITY: DISTRIBUTION DATA

Age group	Nuclear expression % grading scale*				Dichotomised scale†		p
	<10	11–25	26–50	51–100	Negative	Positive	
≤40 years	15	17	2	1	15 (43)	20 (57)	0.09
>40 years	85	60	0	0	85 (59)	60 (41)	

*Data represent numbers of patients; †data represent numbers (percentages) of patients

TABLE V
SUMMARY OF CYCLIN D_3 PROTEIN IMMUNOREACTIVITY: INTENSITY OF NUCLEAR LABELLING DATA

Age group	Nuclear expression intensity grading scale*				Dichotomised scale†		p
	0	+	++	+++	Low	High	
≤40 years	15	2	9	9	17 (49)	29 (51)	0.06
>40 years	85	0	32	28	85 (59)	60 (41)	

*Data represent numbers of patients; †data represent numbers (percentages) of patients

TABLE VI
SUMMARY OF CYCLIN D_3 PROTEIN IMMUNOREACTIVITY: POS-MAX DATA

Age group	Pos-max* grading scale†				Dichotomised scale‡		p
	0	<10	10–50	>50	Low	High	
≤40 years	15	1	17	2	16 (46)	19 (54)	0.02
>40 years	85	2	58	0	85 (59)	60 (41)	

*Refers to percentage of cell labelling with maximum nuclear intensity. †Data represent numbers of patients; ‡data represent numbers (percentages) of patients

management strategies for patients with this condition. Furthermore, this understanding is critical if potential new targeted molecular therapies are to be considered as treatment modalities in the future. Studies are currently underway to further evaluate the role of multiple molecular proteins and determine their importance in vestibular schwannoma pathogenesis.

Conclusion

Vestibular schwannomas show a small but statistically significant increase in Ki-67 proliferation index in patients ≤40 years old compared to those aged >40 years. Overexpression of cyclin D_1 and D_3 proteins is a common feature in vestibular schwannomas, and may have a role in tumour biology. Further investigations of the Rac signalling pathway in vestibular schwannoma are indicated, and may lead to the identification of molecular targets to augment current treatment modalities.

Acknowledgements

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