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CORRESPONDENCE

RASSF1A methylation and cyclin D1 expression in vestibular schwannomas

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Inactivation of the neurofibromatosis type 2 tumor suppressor gene is the best known alteration in vestibular schwannomas (VS). In recent years aberrant promoter methylation of tumor-related genes, as well as alterations in growth regulatory genes have been described as possible mechanisms of VS development and progression [2, 5, 8]. *RASSF1A* has been identified as a tumor suppressor gene with a high incidence of epigenetic inactivation in many common human cancers. This gene acts at downstream of the Ras mediated apoptotic pathway [1]. The role of *RASSF1A* in VS has been poorly evaluated. In a recent study hypermethylation of *RASSF1A* was correlated with VS growth [9]. Several studies have established *cyclin D1* as a proto-oncogene, revealing that its amplification and overexpression may contribute to uncontrolled cell growth in many human tumors [4]. *Cyclin D1* elicits its function early in G1 phase, binding to and activating the cyclin dependent kinases CDK4 or CDK6 [3]. In a recent study, Neff et al. [10] found expression of cyclin D3 in seven of 15 VS, whereas no cyclin D1 expression was found in any of these cases. The relationship between *RASSF1A* and *cyclin D1* pathways remains unclear. To our knowledge no studies have previously addressed this issue in VS.

The study group consisted of 21 patients who underwent surgery for removal of unilateral VS between February 2002 and January 2004. To assess the *RASSF1A* methylation profile, genomic DNA was isolated from frozen tissues by standard methods. Bisulfite modification of genomic DNA was performed [6]. Methylation Specific PCR was carried out for the methylated and unmethylated alleles in standard conditions with an annealing temperature of 59°C. We used the primer sets described previously [7]. Immunostaining of VS sections was performed as described [8]. Deparaffined tissue sections were incubated overnight at 4°C with the anticyclin D1 (P2D11F11) monoclonal antibody (Novocastra) at a 1:50 dilution. The tissues were incubated in biotin-labeled goat anti-mouse serum (1:200) for 30 min, rinsed with PBS and incubated with avidin–biotin–peroxidase complex for 1 h. The signal was detected using 3,3-diaminobenzidine as the chromogen. Cyclin D1 immunoreactivity was usually nuclear, with only occasional and faint cytoplasmic immunostaining. Immunoreactivity was semiquantitatively evaluated by scoring both the staining intensity and the percentage of reacting cells. The percentage of reacting cells was evaluated by counting the stained cells in ten high-power fields (HPF×400). Intensity was scored: 0 absent, ± mild (<50 cells/10 HPF), + moderate (50–100 cells/10 HPF), and ++ strong (>100 cells/10 HPF). To analyze the relationship between *RASSF1A* methylation and cyclin D1 expression the Fisher exact test was performed.

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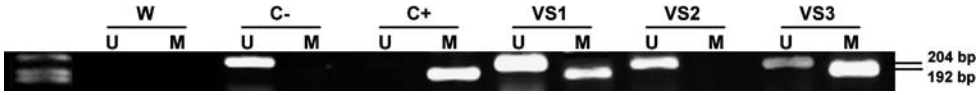

Fig. 1 MSP analysis of *RASSF1A* gene in VS. Agarose *lanes U and M* show amplified product with primers recognizing unmethylated and methylated sequences, respectively. In vitro methylated DNA from control blood lymphocytes was used as a positive control for methylation (C+), and untreated DNA as a negative control (C−). *W lanes* show PCR negative controls. The PCR product sizes of *RASSF1A* gene are shown on the *right*

RASSF1A methylation was found in four cases (23%, Fig. 1). Methylation did not occur in the autopsy samples of nonneoplastic tissue. Eleven cases (52%) showed positive staining for cyclin D1 (Fig. 2). In our study, cyclin D1 expression was inversely associated with *RASSF1A* hypermethylation ($P = 0.03$). None of the tumors with positive cyclin D1 expression showed *RASSF1A* hypermethylation.

It has been suggested that overexpression of *RASSF1A* leads to a blockage of cell progression at the G1 phase by inhibiting the accumulation of cyclin D1. In a study by Shivakumar et al. [12], reintroduction of *RASSF1A* expression in lung and breast tumor-derived epithelial cells resulted in growth arrest by negatively regulating cell cycle progression at the level of G1/S-phase transition. *RASSF1A*-induced cell cycle arrest was accompanied by loss of cyclin D1 accumulation and could be relieved by ectopic expression of *cyclin D1* cDNA. In a similar study, Whang et al. [13] showed that exogenous expression of *RASSF1A* inhibited the phosphorylation of c-Jun and reduced the expression of cyclin D1, thus inducing cell cycle arrest at the G1 phase. Pizzi et al. [11] investigated the prevalence of *RASSF1A* promoter methylation, and expression of cyclin D1 in 62 endocrine tumors from different sites in the gut. The *RASSF1A* promoter was methylated in 32% of foregut tumors, whereas cyclin D1 expression was found in 53% of tumors. Cyclin D1 expression was found in all foregut cases with *RASSF1A* methylation, but was significantly less frequent in tumors without promoter methylation. The reason by which the association between *RASSF1A* promoter methylation and cyclin D1 expression was only found in malignant foregut tumors among endocrine tumors from different sites in the gut was not explained by the authors. Curiously, in the same study a consistent cyclin D1 expression in the absence of *RASSF1A* methylation was found in all rectal carcinoid tumors.

In our study, hypermethylation of *RASSF1A*, which corresponds to loss of *RASSF1A* expression, was associated with negative cyclin D1 expression in a series of 21 VS. This findings are different from those reported in foregut tumors [11] and malignant lung cells [12, 13]. Although limited by the relatively small number of cases investigated, our data do not support the hypothesis by which *RASSF1A* modulates cell cycle progression through pathways regulating accumulation of cyclin D1 protein. In our VS samples an alternative mechanism to inactivation of the *RASSF1A* tumor suppressor gene may be the reason for this finding.

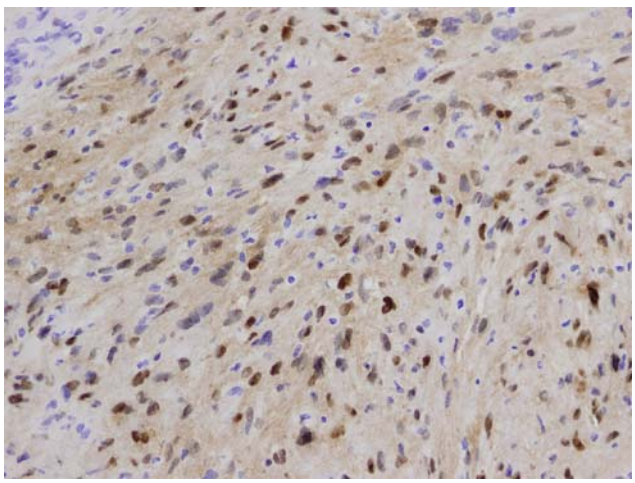

Fig. 2 Cyclin D1 immunostaining. Tissue sections of VS were stained with an anticyclin D1 monoclonal antibody. A strong nuclear staining of Schwann cells is shown

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