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LOSS OF HETEROZYGOSITY OF THE APC GENE IN A CASE OF VESTIBULAR SCHWANNOMA ASSESSED BY TWO INTRAGENETIC MARKERS

Nives Pećina-Šlaus[1,2]

[1]Laboratory of Neurooncology, Croatian Institute for Brain Research, [2]Department of Biology, School of Medicine, University of Zagreb, Zagreb, Croatia

SUMMARY – Schwannomas are benign encapsulated tumors of Schwann cells, the main peripheral glia cells. The majority of schwannomas arise spontaneously and account for 8% of intracranial tumors. Those involving the cerebellopontine angle are schwannomas in 90% of cases. A case is presented of the loss of heterozygosity of the adenomatous polyposis coli (APC) gene in a female patient with cranial schwannoma from Croatia. The observed change of the APC gene was investigated by use of two intragenetic markers. In the light of novel findings on merlin connection to the wnt signaling reported in the literature, the finding of gross deletion in a patient with cranial vestibular schwannoma is a relevant genetic event.

Key words: *Schwannoma; APC gene; Loss of heterozygosity; Neurinoma*

Introduction

Although it is well known that the main cause of transformation of Schwann cells into schwannomas is ascribed to the inactivation of the neurofibromin 2 (NF2) gene and the consecutive loss of its protein merlin, the intracellular mechanism of this transformation still needs to be elucidated. Recent research on NF2 gene demonstrates that merlin is a tumor suppressor that is capable of modulating a wide range of signaling pathways that influence cell growth, motility and apoptosis[1,2]. Novel reports indicate merlin connection to the wnt signaling pathway[3-5]. A study by Lau *et al*.[4] demonstrated the relationship between merlin and wnt signaling in human glioma cells. They showed that merlin re-expression in human glioma cells decreased the quantity of frizzled-1 (FZD1) receptors (FZD1 binds wnt ligands and activates wnt pathway), and increased the expression of molecules that inhibit wnt signaling, dickkopf-1 (DKK1) and dickkopf-2 (DKK2). So merlin re-expression reduced wnt signaling. Another paper by Bosco *et al*.[3] report on a significant increase in transcriptionally active nuclear beta-catenin upon merlin deletion. Beta-catenin is the main signaling effector molecule of this pathway and its activation and nuclear transfer starts up the wnt signaling. In this contribution, the authors show that when merlin is lost, the TCF/LEF/beta-catenin transcription activity is increased. Also, NF2-/- cells contained an increased active beta-catenin level, and finally elevations in transcriptional activity of downstream targets of activated beta-catenin were evident in the absence of merlin. A paper by Zhou *et al*.[5] demonstrates that canonical wnt signaling is activated in primary human schwannoma cells and that activated beta-catenin localizes in the nucleus.

In wnt signaling, APC has the function of beta-catenin destruction[6]. Namely, this tumor suppressor has many cellular functions: as a component of the wnt signal transduction pathway, as a component of

Corresponding author: *Nives Pećina-Šlaus*, Laboratory of Neurooncology, Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Šalata 12, HR-10000 Zagreb, Croatia
E-mail: nina@mef.hr

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adherens junctions, and as a component of the cytoskeleton stabilization. Involvement of the adenomatous polyposis coli (APC) gene as a general tumor suppressor gene in a great variety of human tumors has been known for a long time. The gene (chromosome 5q21) is organized in 15 translated exons that encode a large multidomain protein. We tested gross deletions of the APC gene by use of Msp I and Rsa I obtained genetic markers inside exon 15 and exon 11. Since the relationship between merlin and wnt signaling has been demonstrated, our finding contributes to the overall genetic profile of schwannoma.

Methods

DNA extraction

Autologous blood sample was collected from the patient. The schwannoma tissue was frozen in liquid nitrogen and transported to the laboratory, where it was immediately transferred at -75 °C. The peripheral blood sample was collected in EDTA and processed immediately. The sample was diagnosed at the Department of Pathology, Sestre milosrdnice University Hospital Center, Zagreb, Croatia. The local Ethics Committee approved our study and the patient gave her informed consent.

Approximately 0.5 g of tissue was homogenized with 1 mL extraction buffer (10 mM Tris HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and incubated with proteinase K (100 μg/mL; Sigma, USA; overnight at 37 °C). Phenol chloroform extraction and ethanol precipitation followed. Blood was used to extract leukocyte DNA. Five mL of blood was lysed with 7 mL distilled water and centrifuged (15 min/5000 g). The pellet was then processed as for DNA extraction from tissue samples.

Polymerase chain reaction (PCR)

For the amplification of APC exon 11 (length of the obtained fragment 133 bp) the optimal reaction mixture (25 μL) was: 10 pmol of each primer (5'-GGACTACAGGCCATTGCAGAA-3' and 5'-GGCTACATCTCCAAAAGTCAA-3'), 200-400 ng template DNA, 2.5 μL 10X buffer II, 1 mM $MgCl_2$, 2.5 mM of each dNTP, 0.2 μL (1U) of Taq polymerase (AmpliTaq Gold, Applied Biosystems, USA). PCR conditions were: initial denaturation, 4 min/95 °C; denaturation, 1 min/94 °C; annealing, 2 min/58 °C; extension, 1.5 min/72 °C; 35 cycles. For the amplification of fragment of exon 15 of the APC gene (length of the obtained fragment 550 bp) we used in a volume of 25 μL: 5 pmol of each primer (5'-ATGATGTTGACCTTTCCAGGG-3' and 5'-CTTTTTTGGCATTGCGGAGCT-3'), 150 ng template DNA, 2.5 μL 10X buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 2.5 mM of each dNTP, 0.2 μL (1 U) of Taq polymerase. PCR conditions were: initial denaturation, 5 min/95 °C; denaturation, 30 s/95 °C; annealing, 30 s/57 °C; extension 45+1s/72 °C; final extension 7 min/72 °C, 35 cycles. The PCR products were analyzed on 2% agarose gels.

Restriction fragment length polymorphism (RFLP) and loss of heterozygosity

Loss of heterozygosity (LOH) of the APC gene was detected on the basis of RFLP of the PCR products using Rsa I and Msp I restriction endonucleases; the method has been previously described in detail[7]. All the PCR experiments were repeated twice and the LOHs were confirmed.

Case Report

Our patient was a 62-year-old female admitted to the University Department of Neurosurgery, Sestre milosrdnice University Hospital Center, manifesting symptoms of raised intracranial pressure including severe headache and nausea, dizziness and imbalance caused by a brain tumor. The symptoms lasted for 79 months. The patient was without clinical NF1, NF2 or schwannomatosis and had no family history of brain tumors. Using magnetic resonance imaging (MRI), a tumor lesion was found in the left cerebellopontine angle. It was a vestibular cranial schwannoma. During the operative procedure[8], the schwannoma was removed using a microneurosurgical technique. The case was classified as WHO grade I and specified as Antoni B pattern (Fig. 1). The tumor was composed of loosely arranged spindle cells admixed with foamy macrophages. In some tumor cells, nuclear atypia was seen but mitotic activity was not observed.

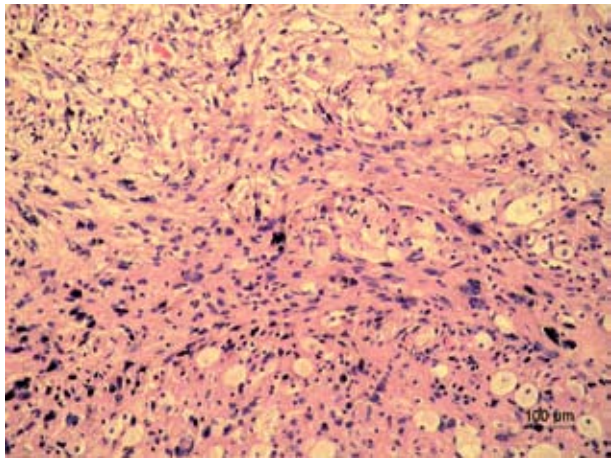

Fig. 1. Vestibular schwannoma, Antoni B. The tumor was composed of loosely arranged Schwann cells admixed with foamy macrophages. In some tumor cells, nuclear hyperchromatism and atypia were seen. Mitoses were not present. (H&E, X200)

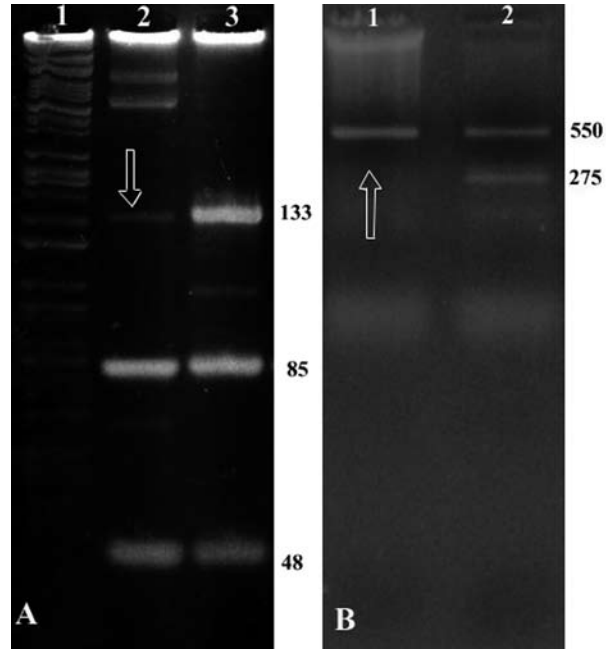

Fig. 2. LOHs of the APC gene in vestibular schwannoma (indicated by arrows): (A) RFLP/Rsa I/exon 11. Lane 1: DNA standard; lane 2: schwannoma sample; lane 3: corresponding blood sample; (B) RFLP/Msp I/exon 15. Lane 1: schwannoma sample; lane 2: corresponding blood sample.

The amplified fragment of exon 15 is 550 bp long and in case of heterozygous samples one allele was left uncut and the other with the restriction site was cut to two fragments of approximately 275 bp. Amplification of Rsa I polymorphic site in exon 11 generated a 133- bp fragment that was cut to 85- and 48- bp fragments by Rsa I on the allele with the site present, and remained uncleaved on the allele without the restriction site. Our patient was heterozygous for both intragenetic markers, which means that two bands (550+275 bp) were visible for Msp I RFLP and 3 bands for Rsa I (133-, 85- and 48- bp) in the blood sample of the patient.

The loss of one polymorphic allele in schwannoma compared to the heterozygous autologous blood sample was considered as LOH of the APC gene. Both markers showed LOH of the APC gene in the analyzed case of vestibular schwannoma (Fig. 2).

Discussion

The role of APC gene as a component of the so-called classic wnt signaling is primarily as a negative regulator of beta-catenin, the main signaling molecule of the pathway[6]. When APC is lost, the level of beta-catenin attains oncogenic activity and beta-catenin is transferred to the nucleus[9], where it finds a transcription factor partner TCF/LEF. Bosco *et al.*[3] believe that deregulated canonical wnt signaling is associated with NF2 loss of function. They showed a significant increase in transcriptionally active beta-catenin when merlin protein was deleted. It has been shown previously that cells lacking NF2 loose contact inhibition[10] and are free to proliferate. Associated with the loss of contact inhibition, merlin-lacking cells are also known to contain defective adherens junctions[11]. Bosco *et al.*[3] indicated the relationship of merlin to the wnt as a possible mechanism by which NF2 deficient cells are able to escape contact inhibition. They also showed that elevated nuclear beta-catenin activity in NF2 deficient cells contributed to the growth phenotype of the cells in confluent state. They established the relationship between Rac1 involvement and demonstrated the Rac1 mediated canonical wnt signaling to be essential for the loss of contact inhibition in NF2 deficient cells. Another study on high grade human gliomas by Lau *et al.*[4] discovered an increase in wnt signaling as a result of NF2 loss, specifically the increase of TCF transcription factor activity. To inves-

tigate whether wnt signaling is involved in schwannoma, Zhou *et al*.[5] used normal Schwann cells in comparison to schwannoma cells. They demonstrated that canonical wnt signaling was activated in primary human schwannoma cells because they found activated beta-catenin localized in the nucleus and wnt target genes c-myc and cyclin D1 overexpressed as a consequence of enhanced transcriptional activities. These findings all collectively describe the influence of merlin on wnt signaling.

Since APC is part of the beta-catenin destruction machinery, its deficiency in schwannoma cells could contribute to the excess cytoplasmic beta-catenin levels, which enter the nucleus and activate wnt target genes. Although the exact molecular explanation of APC and merlin functional connection has not yet been established and merlin loss was primarily linked to increased beta-catenin levels, it is possible that parallel events that include APC deficiency also contribute to the accumulation of beta-catenin critical for the increased proliferation of human schwannoma cells.

Our results demonstrate LOH of the APC gene in a case of vestibular schwannoma, supporting reports on the merlin-wnt link. The report of this deletion in schwannoma is a novel finding that contributes to the overall genetic profile of schwannoma in hope for better understanding of its pathology.

Acknowledgments

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Sažetak

GUBITAK HETEROZIGOTNOSTI GENA APC U SLUČAJU VESTIBULARNOG ŠVANOMA PROCIJENJEN DVAMA INTRAGENSKIM BILJEZIMA

N. Pećina-Šlaus

Švanomi (neurinomi, neurilemomi) su dobroćudni tumori podrijetlom od Schwannovih stanica, glavnih perifernih glia stanica. Većina švanoma javlja se spontano, tj. sporadično u populaciji, a pojavnost im iznosi 8% ukupnog broja intrakranijskih tumora. Tumori smješteni u pontocerebelarnom kutu su u 90% slučajeva upravo švanomi. U ovom radu prikazan je nalaz gubitka heterozigotnosti gena APC (*adenomatous polyposis coli*) u bolesnice sa švanomom iz Hrvatske. Velika delecija gena APC istražena je i dokazana uporabom dvaju intragenskih biljega. U svjetlu novih spoznaja o vezi merlina, produkta gena NF2, i signalnog puta wnt čiji je APC sudionik, opisani nalaz o gubitku heterozigotnosti predstavlja vrijedan doprinos genetičkom profilu švanoma.

Ključne riječi: *Švanom; Gen APC; Gubitak heterozigotnosti; Neurinom*