ORIGINAL ARTICLE

Expression of p63 and p73 in Acoustic Neuroma and Its Possible Clinical Relevance

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KEYWORDS

p63; p73; Immunohistochemistry; Acoustic neuroma; Schwannoma

Abstract

Objectives: Assess p63 and p73 expression in acoustic neuroma and its correlation with clinical and radiological findings.

Materials and methods: medical records of 34 patients who were operated on for acoustic neuroma during a 3-year period (2001–2003) were evaluated retrospectively. Immunohistochemical analysis of the schwannoma was performed with p63 and p73 antibodies and clinical patient characteristics were correlated with the immunoreactivity results.

Results: 41% of the acoustic neuroma specimens showed p63 and p73 staining. Correlation between both proteins was 100%. Age of the patients tended to be older when staining was positive, but no statistical significance was achieved. Likewise, tumour size was bigger for positive tumours but, again, this difference was not statistically significant. There was no correlation between gender and immunostaining.

Discussion and conclusions: Expression of p63 and p73 was demonstrated in almost half of the patients studied. Although both proteins were more prevalent in older patients and bigger tumours, this difference was not statistically significant, probably due to the reduced sample size. No differences were found in laterality, gender or audiogram. However, the expression of these two proteins in almost half of the tumours shows that they can play a role in the development and progression of acoustic neuromas, although further studies are needed.

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**PALABRAS CLAVE**
p63; p73; Inmunohistoquímica; Neurinoma acústico; Schwannoma

**Expresión de p63 y p73 en neurinomas del acústico y estudio de su posible relevancia clínica**

**Resumen**

**Objetivos:** Determinar la expresión de p73 y p63 en muestras de neurinomas del acústico y estudiar su posible relevancia clínica.

**Material y método:** Se realiza un estudio retrospectivo de 34 neurinomas del acústico intervenidos durante un periodo de tres años (2001-2003). Se revisan sus historias clínicas y se realiza un estudio inmunohistoquímico para valorar la expresión de p63 y p73 en las muestras histológicas y la correlación con los hallazgos clínicos más relevantes.

**Resultados:** En el 41% de las muestras se observó una sobre-expresión de p63 y p73 siendo la correlación entre ambas proteínas del 100%. La edad de los pacientes con tinción positiva era mayor que la de los pacientes con tinción negativa con una diferencia a ser estadísticamente significativa. El tamaño tumoral era mayor en los casos positivos, pero la diferencia no ha llegado a niveles significativos. No existe correlación entre la expresión de los marcadores y el sexo, lado anatómico ni audiométrica.

**Discusión y conclusión:** Se observa inmunotinción positiva para p63 y p73 en casi la mitad de los neurinomas estudiados. A pesar de encontrar que estas proteínas están más expresadas en casos de mayor edad y mayor tamaño, la diferencia no ha llegado a valores significativos, posiblemente por el número limitado de muestras en el análisis. Sin embargo, la expresión de estas proteínas en casi la mitad de los pacientes tratados, puede significar que juegan un papel en el desarrollo y progresión de estos tumores, aunque sería necesario realizar estudios futuros para determinar este papel.

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**Introduction**

Acoustic neuromas (AN) are rare, slow-growing tumours that originate from Schwann cells of the 8th cranial nerve, generally from the vestibular nerve. Their molecular biology is still poorly understood, that hinders both the understanding of their origin and evolution and the development of a possible medical treatment.

A group of patients who develop AN also suffer an inherited, autosomal dominant disease known as type II neurofibromatosis (NF 2), which is clinically characterised by bilateral AN and other intracranial and spinal tumours. The NF2 gene, located on chromosome 22q, is a tumour suppressor gene that functions as a critical regulator of the growth of Schwann cells. Thus, NF2 gene inactivation is an essential step in the tumourigenesis that occurs in these patients. The loss of chromosome 22q has been demonstrated in 45% of sporadic cases and biallelic inactivation in almost 100% of AN in NF2 patients. Sporadic forms account for 95% of all diagnosed AN.

In addition to the NF2 gene, there are other genes involved in AN genesis. The genes of the neuregulin family appear to act as Schwann cell mitogens. These proteins act via the c-erb family of receptors, with c-erb 2 and 3 being the main receptors in Schwann cells. Fibroblast growth factors and their receptors (FGFR) also have a mitogenic effect on Schwann cells. A relationship has been described between the expression of FGFR and tumoral growth. The expression of various cytokines and other factors that may regulate the proliferation of Schwann cells has been described in several studies as listed below: studies on Ki-67, proliferating cell nuclear antigen, nerve growth factor receptor, transforming growth factor receptor, fibroblast growth factor, endothelial growth factor, epidermal growth factor and cyclin D1 and D3, among others.

At present, the role played by p53, a tumour suppressor gene found mutated in over 50% of all cancers, in the genesis of AN is unknown. While several studies have shown that the contribution of p53 in AN genesis is unlikely, another recent study indicates that loss of heterozygosity of the p53 gene occurs in a significant number of cases studied.

The p63 and p73 genes belong to the p53 family and have numerous structural similarities. Their presence has been demonstrated in different types of human tumours, with a negative prognostic value in some of them, such as breast, bladder and hepatocellular carcinomas or B-chronic lymphocytic leukaemia.

In this study we attempted to observe the expression of p63 and p73 by immunohistochemistry (IHC) in samples of AN intervened at our centre. We also attempted to assess the possible relationship of the expression of this protein with certain data obtained from the medical records of patients.

**Material and Methods**

**Patients and Review of Medical Records**

We reviewed the medical records of all patients diagnosed and intervened due to AN at the Otolaryngology Service of Donostia Hospital in San Sebastián between January 2000 and December 2002. We collected the demographic data of these patients: age, gender and symptoms, as well as information obtained from physical examinations and surgical
interventions performed: audiogram, tumour size, surgical approach, morbidity and date of last visit.

Immunohistochemistry and Antibodies Used

The IHC studies were performed using mouse monoclonal antibodies developed at the laboratory of the Head and Neck Surgery Department of the University of California at San Diego in collaboration with Imgenex, San Diego, California. Both antibodies were used at a 1:100 dilution in PBS. We used samples of head and neck squamous cell carcinoma as positive controls, we used the same AN tissue as negative controls, but without adding the antibody, and we used nerve tissue from cadavers (8th cranial nerve) as normal controls.

Tissue samples were fixed in formaldehyde solution buffered at 4% and embedded in paraffin. Sections of 4 μm were made from the blocks and mounted on 3-aminopropyltriethoxysilane slides, specially prepared for IHC staining. They were incubated for 1 h at 60 °C, deparaffinized in xylene and successively rehydrated with different grades of ethanol. Antigenic amplification was performed by incubating the samples at maximum pressure with DAKO Target Retrieval solution. Endogenous peroxidase was removed with 3% H2O2. In order to avoid non-specific binding, samples were incubated for 30 min in a solution designed to block biotin–avidin non-specific binding (Protein Block Serum-Free, DAKO, Carpinteria, CA, USA). Background staining was reduced by incubation in goat serum (1:20) for 60 min. We added the primary antibody and incubated for 1 h at room temperature. Subsequently, we used secondary antibodies conjugated with streptadivine/HRP (LSAB2, DAKO, Carpinteria, CA, USA). Counterstaining was performed with Gill II haematoxylin.

Staining Assessment

Cases were considered positive if the staining was at least double (2+) on a scale of 1 to 4+ and if 20% or more of the tumour cells were stained over the healthy background or the negative control. We assessed only the areas away from the edges that were not cauterised or overly wrinkled, or did not contain artefacts. Nuclear immunostaining was evaluated as positive.

Statistical Study

We used the Student t test to associate age and tumour size with gene expression and the Chi-square test to assess the association between the gender of patients, audiometry or anatomical side and the expression of both genes.

Results

Data Obtained From Medical Records of Patients

We studied a total of 34 patients (Table 1).

The mean age of the sample was 49.5 years (range between 25 and 72 years), with a male/female ratio of 19:15. In total, 20 tumours were on the left side and 14 on the right side. There were no cases of bilateral tumours in this cohort of patients.

The most common symptom was hearing loss, followed by tinnitus and imbalance. Four patients suffered hypoaesthesia in the trigeminal area at the time of diagnosis and there was facial nerve palsy in 2 cases (both grade III in the House-Brackmann scale). The audiometric data were identified in 31 of the 34 cases studied. All of them presented asymmetric sensorineural hearing loss, which was profound in 18 cases. The remaining 13 patients had audiometric curves between 35 and 65 dB. We performed speech audiometry and calculated the verbal reception threshold (VRT) in 20 of the 34 cases intervened and observed VRT curves below 50 dB in 15 of these 20 cases.

All patients underwent magnetic resonance imaging (MRI) and we also performed computed tomography (CT) in addition to MRI in 12 cases. Tumour size is shown in Table 1. The mean was 17.1 mm (range between 3 and 40 mm), taking the maximum tumour diameter as measurement.

In 4 patients, the approach was by the middle fossa, the approach was retrosigmoid in 10 cases and the approach was translabyrinthine in the remaining 20 cases. The choice of approach depended on tumour size (when this was larger than 1 cm or if there was extension beyond the internal auditory canal [IAC], the middle fossa approach was not used), hearing or possibility of preserving hearing (if hearing was good or useful and it was possible to conserve it, the middle fossa or retrosigmoid approaches were used, depending on the size) and, lastly, the lateral extension of the tumour into the IAC was taken into account (if it extended laterally beyond the medial portion of the IAC, we did not opt for a retrosigmoid approach).

Expression of Tumour Suppressors p63 and p73 in Acoustic Neuromas

The expression of p63 in AN samples was observed in 14 of the 34 preparations (41.2%). The expression of p73 was exactly the same, with the concordance between both proteins being 100%. That is, all p63 positive cases were p73 positive and they were also nearly identical in intensity (Fig. 1A and B).

The mean age for cases with positive immunostaining (both markers, p63 and p73) was 53.1 years, whereas that of cases with negative immunostaining was lower, 46.9 years. However, this difference in expression related to age did not reach statistical significance (P=.22).

When comparing tumour size in cases of positive immunostaining (19.8 mm) with respect to negative immunostaining (15.2 mm), we found larger tumours among the positively stained samples. However, this difference did not reach significant values (P=.19).

As for the laterality of the tumours, there were more cases on the left side than on the right, 20 versus 14. Among the left tumours, 55% were p63/73 positive, whereas among the right, only 21% were positive. Nevertheless, this difference was not statistically significant.

Patients were divided into 2 groups depending on the audiometry. Those in the first group presented a mean value for the 3 frequencies (500–1000–2000 Hz) higher or better at 60 dB. For those in the second group, this result was worse
Table 1  Demographic Data of Patients.

<table>
<thead>
<tr>
<th>No.</th>
<th>Gender</th>
<th>Age, years</th>
<th>Side</th>
<th>Maximum Size Ø, mm</th>
<th>Tonal Audiometry (Mean Frequencies 0.5–1–2 kHz)</th>
<th>Audiometry (VRT)</th>
<th>Approach</th>
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<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>25</td>
<td>L</td>
<td>15</td>
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<td>&lt;90 dB</td>
<td>Translabyrinthine</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>68</td>
<td>L</td>
<td>10</td>
<td>35 dB</td>
<td>40 dB</td>
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</tr>
<tr>
<td>3</td>
<td>F</td>
<td>45</td>
<td>L</td>
<td>25</td>
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<td>35 dB</td>
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</tr>
<tr>
<td>4</td>
<td>F</td>
<td>63</td>
<td>L</td>
<td>12</td>
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<td>&lt;90 dB</td>
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</tr>
<tr>
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</tr>
<tr>
<td>6</td>
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<td>L</td>
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<td>75 dB</td>
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</tr>
<tr>
<td>7</td>
<td>M</td>
<td>72</td>
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<td>65 dB</td>
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</tr>
<tr>
<td>8</td>
<td>M</td>
<td>60</td>
<td>R</td>
<td>20</td>
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<td>&lt;80 dB</td>
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<tr>
<td>9</td>
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<td>56</td>
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<td>&lt;90 dB</td>
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<tr>
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<td>F</td>
<td>60</td>
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<td>12</td>
<td>&lt;90 dB</td>
<td>&lt;90 dB</td>
<td>Translabyrinthine</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>57</td>
<td>L</td>
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</tr>
<tr>
<td>12</td>
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<td>45</td>
<td>R</td>
<td>3</td>
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<td>&lt;90 dB</td>
<td>Middle fossa</td>
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<td>L</td>
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<td>14</td>
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<td>32</td>
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</tr>
<tr>
<td>15</td>
<td>M</td>
<td>59</td>
<td>L</td>
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<td>&lt;90 dB</td>
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<td>16</td>
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<tr>
<td>17</td>
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<td>20 dB</td>
<td>20 dB</td>
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</tr>
<tr>
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<td>M</td>
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<td>L</td>
<td>10</td>
<td>&lt;90 dB</td>
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<tr>
<td>20</td>
<td>M</td>
<td>53</td>
<td>R</td>
<td>7</td>
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<td>&lt;90 dB</td>
<td>Middle fossa</td>
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<tr>
<td>21</td>
<td>M</td>
<td>57</td>
<td>R</td>
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<td>&lt;90 dB</td>
<td>Middle fossa</td>
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<tr>
<td>22</td>
<td>F</td>
<td>58</td>
<td>L</td>
<td>15</td>
<td>50 dB</td>
<td>50 dB</td>
<td>Translabyrinthine</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>28</td>
<td>R</td>
<td>30</td>
<td>&lt;90 dB</td>
<td>&lt;90 dB</td>
<td>Retrosigmoid</td>
</tr>
<tr>
<td>24</td>
<td>M</td>
<td>61</td>
<td>R</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td>Retrosigmoid</td>
</tr>
<tr>
<td>26</td>
<td>F</td>
<td>26</td>
<td>R</td>
<td>40</td>
<td>&lt;90 dB</td>
<td>&lt;90 dB</td>
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</tr>
<tr>
<td>27</td>
<td>F</td>
<td>25</td>
<td>L</td>
<td>32</td>
<td>&lt;90 dB</td>
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<td>Retrosigmoid</td>
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<td>28</td>
<td>M</td>
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<td>L</td>
<td>25</td>
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<td>M</td>
<td>48</td>
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<td>Translabyrinthine</td>
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<td>30</td>
<td>M</td>
<td>59</td>
<td>R</td>
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<td>Retrosigmoid</td>
</tr>
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<td>31</td>
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<td>?</td>
<td>?</td>
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<td>33</td>
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</tr>
<tr>
<td>34</td>
<td>F</td>
<td>34</td>
<td>R</td>
<td>10</td>
<td>50 dB</td>
<td>50 dB</td>
<td>Translabyrinthine</td>
</tr>
</tbody>
</table>

F: female; L: left; M: male; R: right; VRT: verbal reception threshold.

Figure 1  Expression of p63 (A) and p73 (B) by immunohistochemistry in acoustic neuromas.
Expression of p63 and p73 in Acoustic Neuroma and Its Possible Clinical Relevance

than 60 dB. In the first group, 46% were positive for the proteins studied; in the second group, this figure was 39%. There were no significant differences in either case.

Neither was there any correlation between the expression of the markers and patient gender.

Discussion

The molecular biology of AN is still scarcely known and it is clear that such knowledge would make us better understand the genesis, development and progression of these tumours. Furthermore, the more we know about the molecular biology of these tumours, the more likely we shall be to develop new therapies to treat them.3,7,23

At present, patients with AN are offered one of the following options: (1) surgical removal of the tumour, (2) stereotactic radiation or (3) observation and monitoring by MRI. In recent decades, there has been a great development of surgical techniques. However, there is a group of patients with a base condition in whom the surgery, despite such progress, may be contraindicated. Moreover, the number of patients who are diagnosed in a fortuitous manner has increased. In addition, patients without symptoms and even those with AN in a single ear or bilateral AN would benefit enormously from the possibility of pharmacological treatment. Even if only a partial response was obtained, the avoidance of surgery in this specific group of patients would represent a success and a significant breakthrough.

In this study we used IHC to demonstrate the presence in about half of the patients studied (41%) of p63 and p73, tumour suppressor genes of the p53 family, with which they share many structural similarities. This structural similarity is even higher in the former pair (that is, between p63 and p73). In this study, we observed that their expression in the 34 cases showed a 100% concordance. Understanding the function of each of these genes in AN better would be necessary to explain the reason of this concordance, as well as to know which isoform acts in these tumours.

The role that p63 and p73 play in the development and regulation of neural cells and in the neurogenesis of the central nervous system (CNS) has recently been demonstrated.24 There are several isoforms of each of these genes that may act in a pro-apoptotic (Tap63 and Tap73) or anti-apoptotic manner (ΔNp63 and ΔNp73). The TA (transcriptional activation) isoform mimics the functions of p53 and induces apoptosis, while the ΔN isoform suppresses the TA as well as the p53, thus fulfilling an oncogenic function. Various studies have shown that the TA isoform of p73 (Tap73) determines a sensitivity of tumour cells to various chemotherapeutic agents in cases of head and neck squamous cell carcinomas, as well as thyroid anaplastic carcinoma.25,26 Unfortunately, there is no possibility of distinguishing between these 2 isoforms of the protein by IHC, so we do not know which one is expressed in the 41% of positive cases in this study.27

We wanted to observe if the expression of these proteins could play a clinical or developmental role in the disease by analysing data from medical records, such as tumour size and age, fundamentally, as well as from the anatomical side, audiometry data and gender of patients. Although we observed that p63/p73 positive cases were correlated with older patients with larger tumours, none of these differences reached statistically significant levels, possibly due to the limited number of samples in the analysis. We also observed a higher percentage of positive cases among tumours on the left side, but this fact did not reach significant values; we believe that it holds no particular interest in our sample. Nevertheless, some publications have tried to relate mobile phone use with the development of AN,28 and have thus examined the anatomical side more closely. Finally, we can observe that the data studied do not make it possible to correlate the expression of proteins and audiometry, at least in a significant manner. However, tumour size was correlated with poorer audiometric results in this sample.

Nevertheless, the fact that nearly half of all patients studied showed expression of these proteins may mean that they play a role in the development and progression of these tumours, although further studies would be needed to determine this role.

Conflict of Interests

The authors have no conflicts of interest to declare.

References