

Immunohistochemical profile of various neurotransmitters, neurotrophins and MIB-1 in cholesteatomas of the petrous bone

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Abstract. Compared to the normal epidermal epithelium, cholesteatomas have altered growth properties characterized by the excessive growth of keratinocytes leading to mucosal destruction. Either congenital or acquired, these lesions, which grow in the middle ear space, the petrous apex or the mastoid of temporal bones, are mostly considered benign skin tumoral lesions. However, many questions remain concerning their pathophysiology. Numerous studies have been proposed to identify those cholesteatoma lesions at risk of recurrence, a possible event that may cause hearing loss. We examined patients with petrous apex or mastoid cholesteatoma in order to analyze the expression of various neurotransmitters, neurotrophins and their receptors and the Ki-67 antigen for identification of a possible relationship between clinical outcome and histopathological behaviour in terms of the proliferative activity of cholesteatomas. Expression of the analyzed molecules was studied using immunohistochemical methods in seven adult patients with petrous apex cholesteatoma who underwent surgical removal of the lesion. Our results, in accordance with published data, confirm that Molecular Immunology Borstel-1 (MIB-1) and certain neurotransmitters could be useful in the prognostic evaluation of the risk of recurrence of aggressive forms of cholesteatoma.

Introduction

When Johannes Muller coined the misnomer cholesteatoma in 1838, he described 'a layered pearly tumor of fat which was distinguished from other fat tumors by the presence of

biliary fat or cholesterol that is interspersed among sheets of polyhedral cells'. Cholesteatomas are considered to be benign tumoral skin lesions growing in the middle ear space (1). It is now well known that cholesteatomas do not contain fat and are composed of an outer matrix comprised of fully-differentiated keratinizing squamous epithelium resting on a collagenous perimatrix, which surrounds layers of desquamated epithelium. Various theories have been advanced to account for the destructive bone resorption commonly seen as a consequence of these lesions, including activation of osteoclastic bone destruction, mechanical pressure necrosis and bone degradation by enzymes such as collagenases and lysozymes (2).

Cholesteatomas may be classified as either congenital or acquired, the congenital forms developing behind an intact tympanic membrane without previous history of infection or breach of the tympanic membrane. This definition has recently been challenged, as approximately 70% of pediatric patients have had at least one episode of otitis media. The acquired form of cholesteatoma is much more common. Primary acquired disease arises from a skin-lined retraction pocket within which retained keratin debris accumulates. It occurs most commonly in the posterior-superior quadrant of the *pars tensa* and in the *pars flaccida*. Secondary acquired cholesteatoma develops from an ingrowth of skin through a tympanic membrane perforation retained within the middle ear, mastoid or both. The lesions can be complicated by facial nerve palsy, fistulas, labyrinthitis, lateral sinus thrombosis and infections such as meningitis and cerebellar or cerebral abscesses (3).

The pathogenesis of cholesteatoma growth is still poorly understood, and multiple theories are currently found in the literature. A common denominator does appear to be Eustachian tube dysfunction, such as functional obstruction, which represents a predisposing factor to high negative middle ear pressures. The most susceptible tympanic membrane areas are the *pars flaccida* and the posterior-superior regions. The expansion or growth of cholesteatoma follows well-defined pathways determined by ligaments and folds, recognizable with reference to embryologic development. Between the third and seventh month of development, the gelatinous tissue of the middle ear space is absorbed, and a primitive tympanic cavity develops by growth of an endothelium-lined pouch extending from the Eustachian tube to the middle ear cleft, with four

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primary sacs budding into the cleft (4). The remnants of these sacs direct the growth direction of the cholesteatoma along predictable pathways in the middle ear, expanding in one of three directions: the posterior (most commonly seen), inferior or anterior route. The presence of cholesteatoma usually requires surgical intervention. Most cholesteatomas are asymptomatic in their early development; children may come for medical attention due to otorrhea, but will rarely complain of decreased hearing. Microscopic examination of the ear is imperative if cholesteatoma is to be identified and delineated. Audiometric evaluation is part of the standard preoperative work-up, and computerized tomography may be used to delineate the extent of the disease, to check the aeration of the mastoid and to help rule out intratemporal and/or intracranial complications (5).

The success of any given approach to optimizing cholesteatoma therapy may be measured in terms of the rate of recurrence. Cholesteatoma in children is widely considered to be a more aggressive disease than in the adult population, not only because very extensive forms of disease more frequently affect children than adults, but also because higher rates of residual and recurrent disease have been documented in the pediatric population. Noteworthy, however, is the observation that the incidence of complications arising from cholesteatoma is directly related to the duration of the disease. Consequently, adults show higher complication rates (6).

Many questions remain concerning the pathophysiology of cholesteatoma; whether or not the disease differs in children and adults is still a matter of debate. However, it is clear from the above data that a carefully considered and individualized approach to cholesteatoma is not only the best way to achieve low rates of recurrence, but may also help to identify markers indicative of high proliferative activity and/or recurrence.

A promising marker for monitoring the behaviour of cholesteatoma as proposed by Mallet *et al* (7) is Molecular Immunology Borstel 1 (MIB-1), an antibody developed against the Ki-67 (Kiel clone 67) antigen. Known as a marker of cellular proliferation, it was additionally found overexpressed by Motamed *et al* (8). Other molecules have been tested, such as p53. However, as Motamed *et al* previously demonstrated, p53 was not significantly expressed in cholesteatoma lesions, although this protein could prove to be a useful marker. Previous studies have also identified the possible involvement of metalloproteinase 1, found overexpressed in cholesteatoma lesions (9). We also analyzed neurotrophins (NTs), neurotrophic signalling polypeptides including nerve growth factor (NGF), brain-derived growth factor (BDNF) and neurotrophin-3 (NT-3). The biological actions of NTs are mediated by their binding with two families of membrane receptors, the high affinity tyrosine kinase (Trk) and the low affinity protein 75 (p75^{NTR}) receptor (10). The Trk family includes the TrKA, TrKB and TrKC receptors, whereas the p75^{NTR} receptor belongs to the transmembrane molecules, serving as a receptor for tumor necrosis factor and for cytokines (11). TrKA is specifically activated by NGF, whereas TrKB and TrKC are primarily receptors for BDNF and NT-3, respectively. The physiological role of NTs in the development, maintenance and regeneration of the sympathetic and sensory nervous system has been well established (12). Furthermore, NTs control the differentiation and proliferation of certain cell

types derived from all three germ layers (13), and NGF induces differentiation and decreases growth rate in a variety of neoplastic cells. Although a rare observation, there is increasing evidence that signalling between neurotransmitters and various tissues can also be mediated by stromal cells and their secreted cytokines and surface molecules, resulting in an even wider complexity of interactions. There is also mounting evidence that NTs may act with a paracrine mechanism in the regulation of the functional activity of neuronal and non-neuronal structures. Many findings support the theory that NTs are also produced by target organs of the nervous system, in which they modulate several biological functions through their receptor activity (14).

Materials and methods

Immunohistochemical analysis. We analyzed the surgical biopsies of seven patients. Four patients had petrous apex cholesteatoma, three of which were of aggressive form, and three patients had mastoid cholesteatoma, two of which were of aggressive form. Only cholesteatomas collected during surgical ear procedures that contained all the epithelial layers with stroma were included in the study.

The following molecules were investigated: NGF, BDNF, calcitonin gene-related peptide (CGRP), NT-3, substance P (SP), vasointestinal peptide (VIP), neuronal nitric oxide synthase (nNOS) and various NT receptors, such as tyrosine kinases A (TrKA), B (TrKB), C (TrKC), p75 and MIB-1.

Experiments were conducted in compliance with Italian laws and guidelines concerning the informed consent of patients. Surgical samples of the cholesteatomas were removed, washed in PBS, fixed in formalin and embedded in paraffin. Serial 10- μ m sections were obtained using a rotatory microtome, mounted on gelatin-coated slides and processed for immunohistochemistry as described elsewhere (15). For the immunohistochemical detection of neurotransmitters, NTs and NT receptors, the following antibodies were used: i) rabbit polyclonal TrkA immunoglobulin (Santa Cruz, CA, USA), which recognized an epitope corresponding to amino acids 763-777, mapping adjacent to the carboxy terminus of human trkA p140, non-cross-reactive with TrkB or TrkC; ii) rabbit polyclonal TrkB immunoglobulin (Santa Cruz), which recognized an epitope corresponding to amino acids 794-808 of mouse TrkB p145, non-cross-reactive with TrKA and TrKC; iii) rabbit polyclonal TrkC immunoglobulin (Santa Cruz), which recognized an epitope corresponding to amino acids 798-812 of porcine TrkC p140, non-cross-reactive with TrKA and TrKB; iv) goat polyclonal antibody to human p75^{NTR} receptor (Santa Cruz), which recognized the amino acid sequence mapping the carboxy terminus of the p75^{NTR} receptor precursor of human origin, non-cross-reactive with other growth factor receptors; v) rabbit anti-NGF polyclonal antibody (Santa Cruz), which displayed <1% cross-reactivity against recombinant human NT-3, NT-4 and BDNF; vi) rabbit polyclonal antibody anti-BDNF (Santa Cruz), which recognized the amino-terminal of mouse BDNF coupled to ovalbumin and did not cross-react with NT-3 or NGF; vii) rabbit polyclonal antibody anti-NT-3 (Santa Cruz), which was raised against the amino-terminal of mouse NT-3 coupled to BSA and did not cross-react with BDNF or NGF;

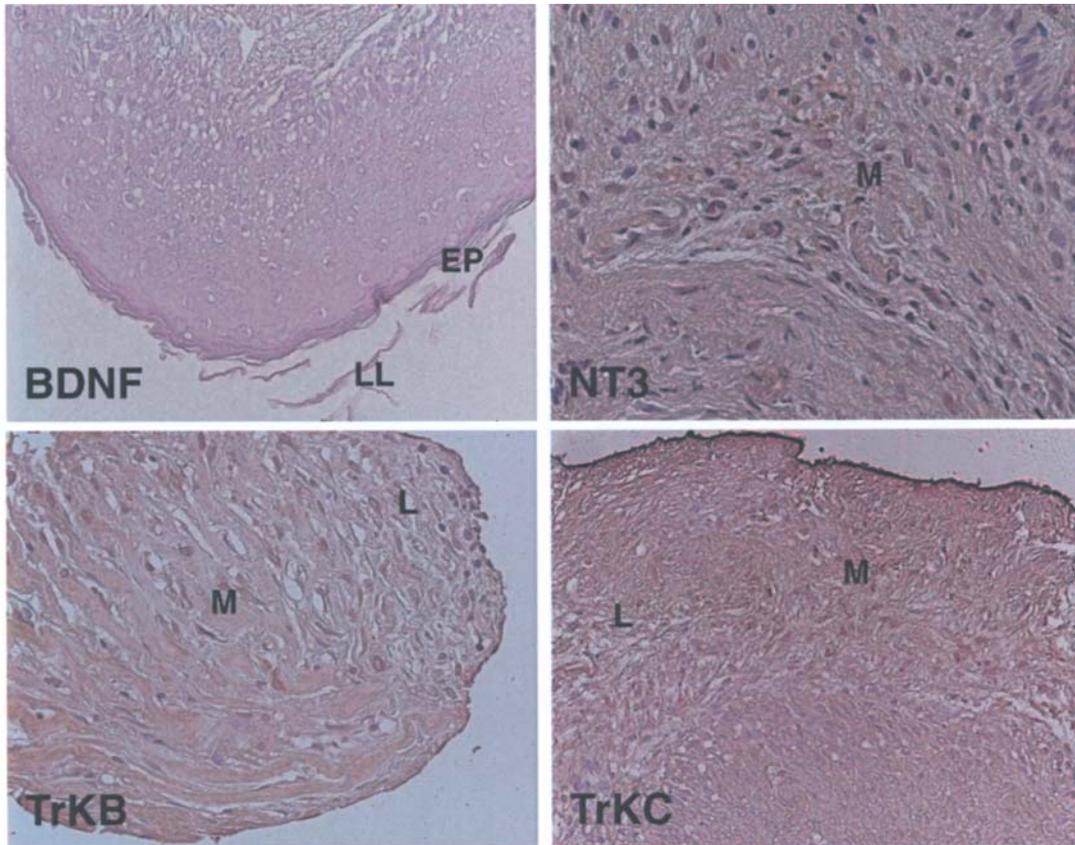


Figure 2. Petrous bone cholesteatoma. Micrographs of BDNF, TrkB, NT-3 and TrkC immunostaining. Moderate immunoreactivity for BDNF is visible. Strong immunoreactivity for TrkB, NT-3 and TrkC is clearly evident. Ep, epithelium; LL, lamellar layer; L, lymphocytes; M, macrophages.

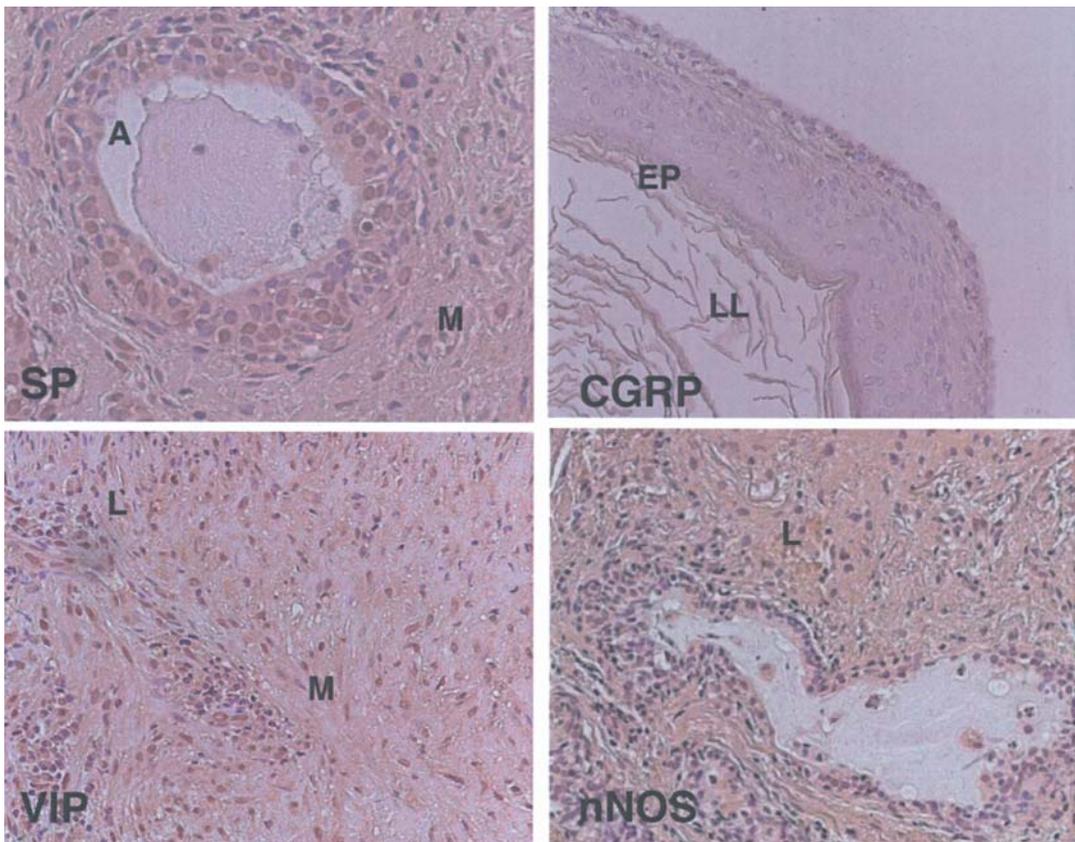


Figure 3. Petrous bone cholesteatoma. Micrographs of SP, VIP, CGRP and nNOS immunostaining. Strong immunoreactivity for SP is visible. Moderate immunoreactivity for VIP, CGRP and nNOS was observed. Ep, epithelium; LL, lamellar layer; L, lymphocytes; M, macrophages; A, artery.

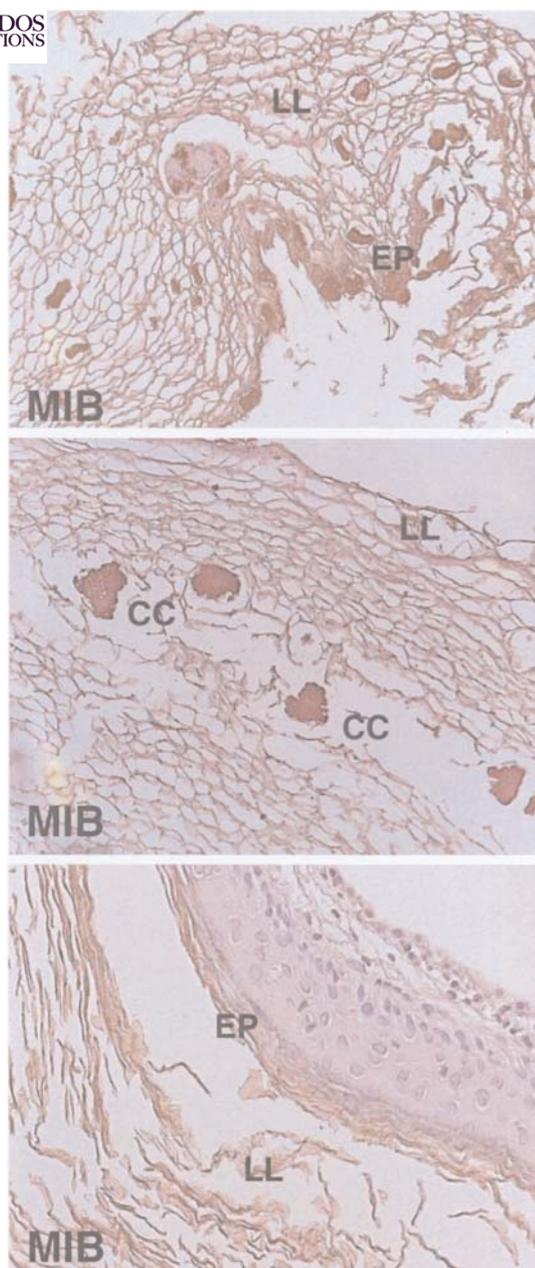


Figure 4. Petrous bone cholesteatoma. Micrographs of MIB immunostaining. Strong immunoreactivity for MIB is clearly visible. Ep, epithelium; LL, lamellar layer; CC, cholesterol crystals.

slightly expressed in the endothelium, but was absent in the epithelium and lamellar layer. TrKC was strongly expressed in the epithelium and widely expressed in the stroma and lamellar layer, but was absent in the endothelium (Fig. 2).

SP and VIP were widely expressed in the stroma and expressed in the endothelium, but were absent in the epithelium and lamellar layer. CGRP was strongly expressed in the stroma, widely expressed in the lamellar layer and expressed in the epithelium, but was absent in the endothelium. nNOS was strongly expressed in the lamellar layer, widely expressed in the stroma and slightly expressed in the endothelium, but was absent in the epithelium (Fig. 3).

MIB-1 was strongly expressed in the epithelium and lamellar layer and widely expressed in the stroma, but was absent in the endothelium (Fig. 4).

To summarize, the epithelium strongly expressed MIB-1, TrKB and TrKC, expressed p75, SP and CGRP and slightly expressed NGF and TrKA, but did not express BDNF, NT-3, VIP or nNOS. The stroma strongly expressed TrKB and CGRP, widely expressed MIB-1, TrKC, SP, VIP and nNOS and expressed NT-3, but did not express NGF, BDNF, TrKA or p75. The lamellar layer strongly expressed MIB-1 and nNOS, widely expressed TrKB, TrKC and CGRP, expressed p75 and slightly expressed NGF, BDNF and SP, but did not express NT-3, TrKC or VIP. The endothelium widely expressed TrKB and p75, expressed VIP and slightly expressed NT-3 and nNOS, but did not express MIB-1, NGF, BDNF, TrKA, TrKC, p75 or CGRP.

Discussion

It is well known that cholesteatoma is caused by the abnormal migration of keratinized squamous epithelium progressively extending to the tympanic and mastoid cavities. At times, destruction of the surrounding osseous structures by resorption owing to accelerated abnormal growth of keratinocytes and subsequent mucosal destruction causes local inflammation with granulation tissue or even, ultimately, hyperosteolysis. As some authors have described, this lesion seems to be tumoral and presents the risk not only of complications, but also of recurrence.

Overproliferation of the cholesteatoma matrix has almost always been described in pediatric patients; these forms are known to be more aggressive in terms of outcome - prognosis *quo ad audiendum* and recurrence both - than adult forms. Bujia *et al* (9) demonstrated a relationship between the mitotic index (monitored by analysing MIB-1 expression) and the aggressiveness of the pathology. Molecular prognostic markers are an interesting result of modern genomic analysis and appear in a variety of pathways, including signal transduction, apoptosis, cell cycle regulation, angiogenesis and cell adhesion (16). The emergence of viable prognostic markers indicative of specific tumor types may potentially improve cancer screening methods as well as overall patient survival in terms of recurrence and/or metastasis.

Cholesteatoma has come to be considered a tumoral form only recently, but over the last few years many studies have been carried out to identify one or more prognostic elements that might help clinicians decide which patients have a high risk of recurrence. As hypothesized by Mallet *et al* (7), the rate of cell proliferation in cholesteatoma could be indicative of a future recurrence of the lesion and its osteolytic potential. Consequently, as the expression of MIB-1/Ki-67 appears to be a promising marker of generalized increased proliferation activity, it was analyzed along with various neurotransmitters, NTs and NT receptors.

Ki-67 is a nuclear protein thought to be expressed exclusively in proliferating cells. Closely linked to the cell cycle, it is considered a marker of cell proliferation and is useful for identifying good or poor prognostic categories, even if its correlation with gene expression has not as yet been fully elucidated. Recent studies have affirmed that Ki-67 is physically associated with the chromatin of ribosomal DNA clusters, especially in the promoter and transcribed regions (17). For this reason, it seems possible that this protein plays a role in the early phases of ribosomal RNA synthesis, or that it may be

involved in chromatin remodelling (18). Anti-Ki-67 is an IgG1 class monoclonal antibody discovered by Gerdes *et al* (19) that recognizes a core antigen present in proliferating cells, and is totally absent in quiescent cells. It is expressed in all phases of the cell cycle with the exception of G0 and the early stages of G1. However, the amount of expressed antigen at different phases of the cell cycle may vary, and the precise role of Ki-67 protein is still unclear.

MIB-1 antibody is a monoclonal antibody which identifies Ki-67 antigen in formalin-fixed and paraffin-embedded tissue sections. It also stains the cell membrane of numerous tumor types. The presence of Ki-67 antigen on the cell membrane was initially a diagnostic finding in certain thyroid tumors, and then in other cancer lesions. Using MIB-1 to detect Ki-67 antigen greatly improves the value of Ki-67 antigen detection, and its profile may be considered a powerful tool for the investigation of the clinical predictive role of Ki-67 antigen as a proliferation marker, especially in diagnostic and prognostic procedures. In fact, many studies have shown the Ki-67/MIB-1-labelling index of proliferation activity to be of increasing value as the rate of malignancy increases (20). MIB-1 is considered to be a clinical marker in many tumors, even if its use is always recommended in combination with other factors such as clinical data, imaging and histopathological features. Its predictive value may be enhanced by its use together with other tissue- or tumor-specific markers, depending on the purpose of the clinical study (21). MIB-1 analysis should be considered routine in the case of certain tumors (with notable differences), as it is widely considered to be a marker of cellular proliferation (known as growth fraction). In fact, the MIB-1 proliferative index is considered to be strictly correlated with clinicopathological findings, and is acclaimed as a high index marker in oncological patients with poor outcome, as demonstrated in gastric and gastrointestinal tumors, pheocromocytomas, pituitary tumors, various nervous system tumors, uterine and ocular tumors, laryngeal carcinoma, gingival carcinoma and soft tissue sarcomas (in which the MIB-1-labelling index, known as the MIB-1 system, is the most significant existing immunohistochemical marker of cell proliferation). Occasionally, MIB-1 has also been used as a predictive marker for the possible metastatic evolution of some tumors (as demonstrated in thyroid carcinomas) (22).

NTs, also known as neurotrophic factors, constitute a family of dimeric proteins that work as polypeptidic growth factors, acting as extracellular ligands. NTs, including NGF, BDNF and NT-3, are involved in vertebrate neuronal cell development, differentiation, survival and functional activities. They are also conserved as distinct genes over large evolutionary distances (23). The biological role of NTs is widely expressed throughout the lifetime of neuronal cells, both during development and in the function and organization of the adult central nervous system, as well as in the neural control of different activities related to the vegetative innervation of several organs (10,24-28). This study aimed to investigate the functional relationships between different cytotypes (epithelial and endothelial cells, stroma and lamellar layers) and the neural compartment. It did so by analysing the expression of various neurotransmitters and NTs with their own receptors in human cholesteatomas, so as to identify possible relationships between innervation and tumoral signalling.

Significant expression was observed for TrKB, MIB-1 TrKC, CGRP (absent only in the endothelium) and nNOS (absent in the epithelium and endothelium). Expression of these could be integrated as a multiple marker prognostic index, useful in monitoring the clinical outcome of aggressive cholesteatomas. As we have seen, the epithelial layer and the corneous lamellar region show a similar expression pattern of NGF, NT-3, TrKB, TrKC, p75 and CGRP. Moreover, a ubiquitous localization of MIB-1 was observed in the epithelium, connective stroma and lamellar region, producing strong immunohistochemical reactivity. The above findings suggest that the outer regions of the epithelium and the corneous layer maintain a continuous and dynamic molecular signalling system for cell communication, possibly due to the influence of NTs and their receptors. This also involves the connective tissue, with particular regard to TrKB, TrKC and p75 expression.

Our data corroborate that of previous studies, suggesting that MIB-1 also represents a useful prognostic marker for cholesteatoma, similar to that observed in other tumoral lesions, as its increase represents a generic index of cell proliferation. Our observations also stress the importance of more recent reports regarding neurotransmitters and NTs, which have been identified as mediators of functional signals in various tissues, such as lymphoid and respiratory epithelium. These observations confirm the prominent role of growth factors in the complex regulation of not only immune functions but also, more extensively, of the cell signalling pathways, particularly in the light of the complex events that occur when there is malignant cell survival and progression.

Our data also confirm the presence, described in previous studies, of MIB-1 in cholesteatomas, interpreting it as the expression of Ki-67 antigen on the membrane of abnormal cells. Since this antigen seems to be absent in the normal cell membrane, its presence may be significant for the prediction of an abnormal evolution of the cell cycle towards possible tumoral progression. This supports the notion that MIB-1 may be a powerful indirect tool, capable of being used as a marker for predicting tumoral progression. In fact, in our study, it was strongly expressed in each layer of the analyzed cholesteatomas.

In light of the above-mentioned data, we hypothesize that a relationship exists between the expression of some of the analyzed molecules and the degree of aggressiveness and proliferation ratio of cholesteatoma. This hypothesis particularly concerns MIB-1 expression, which also seems to be related to the probability of recurrence in cholesteatomas. In conclusion, we can confirm the general data reported in the literature, which considers MIB-1 to be a potentially good marker of cell proliferation in the follow-up of surgically-treated cholesteatoma patients. Its laboratory and immunohistochemical profile may be useful for the prompt detection of a likely recurrence of the lesion. Moreover, the integration of the combined data regarding other overexpressed molecules in these patients may enhance the prognostic significance of MIB-1 alone.

Considering the very small number of patients analyzed in this study, further studies with a statistically significant number of patients, including those with both petrous apex and mastoid cholesteatomas, may allow for a better prognostic evaluation of these lesions.

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