SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the genes that code the tumour suppressor, protooncogene, cytokeratin and protease proteins involved in the progression of the cholesteatoma

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 4 September, 2020 1.00 p.m.

Introduction

Cholesteatoma is a relatively frequent, disease of the middle ear that often develops as a result of chronic inflammatory process, it might have serious, rarely even fatal consequences. The prevalence of chronic cholesteatoma otitis media varies worldwide; it is lower in more developed countries and higher in the socially, economically deprived regions. The incidence of the disease in Europe is 3/10000 in children and 9/10000 in adults.

Many keratinous cysts are known in medicine, including the epidermal cyst (atheroma) and cholesteatoma. Cholesteatoma is an ectopic keratinous cyst in the middle ear, which is characterized by local invasiveness. Histologically, the wall of the cyst is a multilayered keratinized epithelium, which is called matrix. The keratin produced by the cells of the matrix, which constantly reproduces itself and peels off, forms detritus inside the cyst, thereby the size of the cholesteatoma increases and becomes more extensive. This growth is the cause of its invasiveness that can lead to many complications of varying severity in the middle ear. The most common consequences are the destruction of the auditory ossicles, otorrhea, dizziness, and intracranial complications. The therapy of the cholesteatoma is surgical removal. However, even after careful surgical removal, the disease is prone to relapse or recidivism, especially in pediatric cases. Therefore, its removal and complete treatment remains a major microsurgical challenge for the otorhinolaryngologists up to now.

Atheroma - or epidermal cyst - is a benign tumor, which is histologically very similar to cholesteatoma, but its clinical behavior is different. After its surgical excision, the epidermal cyst heals almost without complications.

The pathomechanism of cholesteatoma development is not fully understood, even today. Many theories can be found in the literature, about the origin and development of cholesteatoma. According to most of them changes in the physiological and anatomical conditions of the structures in the middle ear are in the background of the development of the cholesteatoma. Two etiological groups of the cholesteatoma are distinguished: the more common acquired and the rare congenital forms which develops behind an intact ear drum. Recent studies have investigated the molecular and cytogenetic changes underlying the development, the irregular spread and proliferation typical of cholesteatoma, hoping that their results will provide a more accurate understanding of the development and behavior of cholesteatoma. The studies published so far however, still do not explain the complete pathomechanism of the disease.

An association can be seen between the development and growth of cholesteatoma and the inflammatory process present in cholesteatoma, as the result of immune response. Inflammatory cells (e.g., monocytes, macrophages, and infiltrating leukocytes) in the matrix and perimatrix release a variety of angiogenic growth factors (e.g., vascular endothelial growth factor, epidermal growth factor, platelet-derived growth factor, interleukin-8, and cyclooxygenase 2). These angiogenic factors subsequently promote angiogenesis, which paves the way for sustained migration of keratinocytes into the middle ear cavity through the provision of a new vascular network. Collectively, the recruitment of inflammatory cell populations in the matrix and perimatrix and their associated angiogenic growth factors are an important force driving the proliferation and aggressiveness of cholesteatoma. In addition, bone resorption is a mechanism that may explain the increase in osteolysis associated with acquired cholesteatoma. Several upregulated cytokines in cholesteatoma have

been shown to promote inflammatory bone resorption, including interleukin-1, interleukin-6, interleukin-17, interferon-beta, and parathyroid-hormone-related proteins. Recent studies have revealed that the matrix-metalloproteinases (MMPs) play a pivotal role in the destruction of bony tissue by cholesteatomas. Furthermore, degradation of the extracellular matrix has been shown to be associated with the upregulation of MMPs (e.g., MMP1, MMP9, MMP10, and MMP12) as well as downregulation of the tissue inhibitor metalloproteinases (TIMPs).

Many researchers have sought for genetic differences behind the invasiveness and aggressive behavior of the cholesteatoma. An interesting but still controversial idea is that cholesteatoma can be considered a low-grade neoplastic change.

Genetic abnormalities, characteristic of tumors, were detected by several molecular biological studies. These include conventional cytogenetic, flow cytometric, molecular cytogenetic and molecular genetic test procedures. There is no clear genomic instability in the background of cholesteatoma, however, using fluorescence in situ hybridization (FISH) tests, many researchers have found chromosomal aberrations in cholesteatoma.

The development of the tumor necessarily accompanied with changes in the expression of proto-oncogenes and tumor suppressors. The two reported “high-throughput” microarray and proteomic experiments, identified hundreds of differently expressed genes in cholesteatoma samples (LCN2, MMP1, MMP9, MMP10, MMP12, BCL2L1, CEACAM6, S100A7, S100A9, PAX, SERPINB3, SERPINB4, KRT6 A/B, KRT18, KRT19, KRT8STS, PRTN3, ELANE, MPO, HTRA1, S100A7, S100A16/18, S100A12, S100A7, S100A8, COL18A1, NID2, KRT4, KRT 7/8/19, PFN2). According to the “field cancerization” theory tumorigenesis is a multistep process that requires intrinsic and extrinsic factors to develop. The presence of HPV16 infection might be an extrinsic

factor in cholesteatoma, the chronic inflammatory process present in cholesteatoma could serve as an intrinsic factor.

The pediatric and adult cases of cholesteatoma provide a good model system for studying the genetic differences contributing to the development of cholesteatoma, because tumors with very similar histological structures exhibit partially different biological behavior in the two age groups. Unlike cholesteatomas in adults, the cholesteatomas in pediatric are more aggressive in behavior and more prone to the development of recurring tumor, therefore, the pediatric cholesteatoma samples may be especially suitable for studying the molecular changes that contribute to the invasiveness.

By studying the expression of six genes, we have aimed for a more complex and more accurate identification of the process involved in the progression of the cholesteatoma.

Aims

The objective of my work was to examine the genetic factors behind the development of the cholesteatoma.

1. Our goal was to measure and compare the expression levels of the c-MYC proto-oncogene in surgical samples of cholesteatoma, atheroma and of healthy skin removed from the retroauricular region, in order to uncover any differences in c-MYC expression between the two histologically very similar epidermal cysts and the control sample.
2. Comparison of the c-MYC expression levels measured in surgical samples of cholesteatoma patient groups subdivided according to age and its tendency to recidivism to find relationship between the recidivism and the c-MYC expression level.
3. To determine the expression patterns of three cytokeratin genes in the patient groups subdivided according to age and the tendency to recidivism. Comparative analysis of the cytokeratin gene expression patterns in order to identify cytokeratin(s) that could have some importance in terms of the development of the disease and its prognosis.
4. Comparing the expression level of the MMP9 gene in the patient groups subdivided according to age and the tendency to recidivism, a protease that presumably plays a role in the growth and aggressive and bone destructive behavior of cholesteatoma.
5. Analyzing the expression of the tumor suppressor TP53 gene in cholesteatoma samples, which is known to be important in the regulation of the cell cycle.
6. Comparison of the expression levels of all tested genes in the first and second samples of a patient who recurrently developed cholesteatoma during the study period, in order to identify any gene that might plays a role in the relapse.

Materials and Methods

Patients and Sample Preparation.

All samples were obtained with informed patient consent and with approval from the Research Ethics Committee of University of Debrecen Medical and Health Science Center that approved the clinical protocol and the study (protocol number 3047-2009). Our study populations consisted of 26 patients with acquired cholesteatoma (11 females and 15 males), 15 patients with atheroma (head-neck region), and 5 normal skin samples (retroauricular region). The eardrums were perforated in all cholesteatoma patients, and all patients underwent primary or secondary surgery. The age of the cholesteatoma patients ranged between 4 and 65 years (average: 23.4 years). Patients were divided into a pediatric (15 cases; 0–18 years) and an adult group (11 cases; over 19 years). All samples were surgically removed and the diagnosis of cholesteatoma and atheroma in all specimens was confirmed by histopathologic examination. the surgically removed samples were preserved in RNA*later*® solution and stored at 4 °C until further processing.

RNA Extraction.

The excess RNA*later*® solution was removed from the samples and the matrix of cholesteatoma and the atheroma specimens were manually cleaned from the surrounding tissues. As an average 70 mg tissue sample were cut into thin slices and homogenized manually in TRI Reagent (Molecular Research Center INC, Cincinnati, USA) using a glass-Teflon tissue homogenizer. Total RNA was extracted from the specimens using the RiboPure kit (Ambion (Europe) LTD, Huntingdon, UK). RNA concentration, quality and integrity were evaluated by NanoDropTM 1000A

spectrophotometer (Thermo Fisher Scientific, Wilmington, USA).

Reverse transcription

A total of 2 µg of RNA was reverse transcribed in a 20 µL reaction volume using the High Capacity cDNA Kit with RNase inhibitor (Thermo Fisher Scientific, Woolston, UK) for cDNA preparation. Briefly, 2 µg total RNA was mixed with 10.0 µL of 2× reverse transcriptase (RT) buffer, 1.0 µL 20 × enzyme mix and nuclease-free water to a total volume of 20 µL. The reaction mix was then incubated first at 25°C for 10 min then at 37°C for 120 min; the reaction was terminated by incubation at 95°C for 5 min and then chilling them immediately on ice for an additional 5 min.

Primer and Probes and real-time PCR Detection

The custom made, gene specific pre-validated TaqMan Gene Expression Assays (Assays-On Demand IDs: MYC: Hs00153408 m1TP53: Hs00153408_m1; KRT1: Hs00196158_m1; KRT10: Hs00166289_m1; KRT19: Hs00761767_m1; MMP9: Hs00957562_m1). Gene expression measurements were carried out with an ABI Prism 7900HT Sequence Detection System (Thermo Fisher Scientific) according to the manufacturer's instructions; briefly 4 ng of cDNA was diluted into a total of 20 µL reaction volume containing 10µl TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific) with AmpliTaq Gold DNA Polymerase and the target gene specific TaqMan Gene Expression Assay mix. Amplification was performed for 40 cycles, including denaturation at 95°C for 15 seconds, annealing at 60°C and extension at 72°C for 60 and 30 seconds, respectively. Relative gene expression levels were calculated by the comparative critical threshold method. The PPIA gene was used as an endogenous reference and results were expressed as relative change compared

to the normal control group.

Statistical Analysis

Results were statistically analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.; San Diego, CA, USA). Descriptive column statistics of each data set were performed and the distribution of data was analyzed by Kolmogorov-Smirnov test. To assess the statistical significance of differences in gene expression between multiple groups the nonparametric one-way ANOVA Kruskal–Wallis test (K-W test) in combination with the *post hoc* Dunn's test to adjust for multiple comparisons was applied. In all tests difference was considered significant as $p<0.05$. Dunn's p values were indicated as: $p < 0.05$(*); $p < 0.01$(**).

Results

Clinical features

In our study, 26 surgically removed acquired cholesteatoma samples were examined using normal skin from 5 retroauricular regions as control sample. We have determined the expression of the c-MYC gene in samples from 15 atheromas, too. Patients were divided into two groups according to their age: children (under 18 years of age, N = 15) and adults (above 18 years of age, N = 11). Based on the clinical data, both age groups were further categorized into acquired (non-relapsing, single appearance) and relapsing groups.

Analyzing the symptoms of cholesteatoma patients based on their clinical data, it can be stated that, the extent of cholesteatoma in the non-relapsing cases was smaller and affected fewer anatomical regions than in the relapsing cases. The spread of the disease affected more than two anatomical regions in the relapsing group. Based on the clinical data, the rate of bone destruction was also higher in the relapsing patients than in the non-relapsing ones. Of the 26 acquired cholesteatomas, 24 patients had auditory ossicle destruction and only 2 patients had intact auditory ossicles. In pediatric cholesteatomas, the recurrence was higher than in adult cases.

Analysis of the expression pattern of the target genes in cholesteatoma samples.

Examination of the expression of-c-MYC gene.

Since the c-MYC gene plays a central role in the regulation of the cell cycle and proliferation, the active growth of cholesteatoma and the immunohistochemical results reported in the literature suggest the possible involvement of c-MYC in the

proliferative process in cholesteatoma samples. We have stratified the cholesteatoma patient group into a pediatric (younger than 18 years old) and an adult (above 18 years] group then both groups were further classified based on the clinical data into primary acquired (non-recurrent) and recurrent groups. In addition to cholesteatoma, the c-MYC expression was also measured in atheroma, too. The c-MYC expression was low in all samples and there was no significant difference between the expression values of the control and the benign atheroma samples. However, the mRNA expression values measured in the cholesteatoma were significantly higher than both the control and the atheroma expression values. The normalized expression values measured in pediatric samples were higher than the mRNA levels measured in adult samples. For multiple sampling correction, there was a significant difference only between the control and the pediatric cholesteatoma values. Comparing the two cholesteatoma (pediatric, adult) groups with the control group individually, the expression values of both cholesteatoma groups were significantly higher than that of the control group. There was no significant difference between the two cholesteatoma groups. In the final comparison, the expression values of the four cholesteatoma groups were analyzed. The highest value was detected in the pediatric recurrent group and, interestingly, the lowest normalized mRNA expression levels were measured in the adult recurrent samples. When multiple testing correction was applied none of the patient groups showed significant differences compared to the control values, however, when the expression values of the four patient groups were individually compared to the control group, both pediatric patient groups had significantly higher expression levels than the control group.

Examination of the expression of KRT1 and KRT10 genes

Although the KRT1 and KRT10 genes are located on different chromosomes, they are co-expressed during the terminal differentiation of the keratinocytes, because their protein products form a functional dimer. As expected, the normalized expression values of the two genes were very similar in all the tested groups, however, the expression level of the KRT10 was higher than that of KRT1. The KRT10 expression was lower in the cholesteatoma group than in the control skin samples, but the difference was not significant. Our RT-PCR data showed that the mRNA expression differed significantly between the control group and the stratified cholesteatoma groups. KRT10 mRNA expression was significantly higher in the control samples compared to the adult recurrent group. The KRT10 expression was also significantly higher in the pediatric recurrent cases than in adult recurrent cases.

Examination of the expression of the KRT19 gene in cholesteatoma samples

The KRT19 was poorly expressed in all samples, and its expression showed no significant difference between the normal skin - and of cholesteatoma samples. The lowest expression level was observed in samples of the two recurrent groups.

Examination of the expression of the MMP9 gene in cholesteatoma samples

In case of the MMP9 gene, the mRNA expression levels were significantly elevated in the cholesteatoma samples compared to the normal skin. In the samples of non-recurrent and recurrent cholesteatoma groups significant difference was found between the control and both recurrent groups. The highest MMP9 expression was measured in the adult and pediatric recurrent patient groups, and the lowest was detected in the adult non-recurrent group. When the mRNA expression values of the four groups were compared individually to the those of the control group, significant difference was detected between the expression values of the control group and

those of the pediatric - and adult recurrent patients. However, taking multiple testing into account, the differences were no longer significant.

Examination of the expression of the TP53 gene in cholesteatoma samples

The TP53 gene expression was higher in recurrent cases than in the non-recurrent patients. From the four cholesteatoma groups, the highest TP53 expression was detected in the recurrent pediatric cholesteatoma samples, while slightly lower values were measured in the adult non-recurrent group. However, none of our comparisons showed significant differences between the mRNA expression values of the groups.

Case study

A recurrence has occurred in a pediatric patient during the sample collection period of the study. This allowed us to compare the expression levels of all tested genes in the primary and recurrent cholesteatoma samples of the same person. All the genes had increased expression levels in the recurrent sample, except for the KRT19, which showed much lower expression level in the recurrent cholesteatoma sample.

Discussion

Our results confirm the difference seen in characteristics of the pediatric and adult cholesteatoma patients on molecular level and at the same time, provide new data about the differences in the expression of the six genes, which might play a role in the development and progression of cholesteatoma.

The quantitative measurement of the expression of c-MYC, KRT1, KRT10, KRT19, MMP9 and TP53 genes was carried out in surgical samples of cholesteatoma patients using RT-QPCR methodology. Normal skin samples derived from the retroauricular region of healthy individuals were used as control for all measurements- The c-MYC expression was also measured in atheroma samples.

Our results show that the c-MYC proto-oncogene in cholesteatoma samples shows significantly different expression compared to benign atheroma and the control samples. Based on this observation, we presume that the increased expression of the c-MYC in the cholesteatoma causes altered cell cycle regulation. The highest levels of c-MYC detected in the pediatric cholesteatoma cases can be related to the tendency of frequent recurrence observed in this age group.

In case of the cytokeratin genes KRT1 and KRT10 showed identical expression patterns in cholesteatoma samples. The expression level of KRT1 and KRT10 genes in the pediatric recurrent group was higher than that of the control and non-recurrent pediatric groups but the difference was not significant. The expression of KRT1 and KRT10 suggests an ongoing keratinocyte differentiation and keratinization process in pediatric recurrent cases. The significantly reduced expression of KRT1 and KRT10 in the adult group might shift the proliferation-differentiation balance of cells toward

dedifferentiation and proliferation.

The differential expression of the KRT19 gene seems to be especially interesting. Its expression level was lower in the more invasive recurrent cases in our pediatric and adult groups, too. Since its reduced level might be associated with elevated cell proliferation, its lower expression level might be used as a prognostic factor to predict the clinical behavior of the cholesteatoma and its tendency for recurrence.

Based on the data of our gene expression measurements, it can be concluded that the expression level of the MMP9 gene is proportional to the bone destruction and the invasiveness of cholesteatoma since the expression of MMP9 gene was significantly elevated in the recurrent cholesteatoma group compared to the control sample. The highest MMP9 mRNA level was found in adult recurrent cases. We hypothesize that the elevated level of the c-MYC proto-oncogene might be the cause of the increased MMP9 expression since c-MYC binding site is present in the promoter region of the MMP9 gene.

For the TP53 gene, there was no significant difference between the cholesteatoma and the control samples, however, slightly increased expression could be observed in the relapsing samples.

During the course of our experiments, we had the opportunity to perform the genetic analysis of primary and relapsing samples collected from the same patient; the changes in the gene expression level in the two samples properly represent our above-described findings.

In summary, it can be stated that pediatric and adult cholesteatomas have different characteristics and recurrence tendencies which behavior might be supported by the different expression patterns of c-MYC, KRT1 and 10, KRT19, MMP9 and TP53 genes

also shown in our samples. Our experimental results partially support the probability of the neoplastic malformation of the cholesteatoma.

We have detected several similarities in the expression patterns of the examined genes - c-MYC, KRT1, KRT10, KRT19, MMP9, TP53 – subdivided into groups according to clinical data, which are, however, histologically identical cholesteatomas, although also striking differences were identified. This knowledge allows us to understand the molecular differences involved in the development and growth of the cholesteatoma more precisely.

New results of the dissertation

Our study is the first to describe the genetic abnormalities that affect the cell cycle's operation and it helped to clarify the pathomechanism and the clinical behaviour of the cholesteatoma:

1. As the first step, we have studied the expression patterns of the c-MYC, KRT1/10, KRT19, MMP9 and TP53 genes in the surgical cholesteatoma samples of patients grouped by age and tendency to recidivism using quantitative real-time PCR. This study allowed the quantitative analysis of the expression of the genes, which allowed us to determine the role of these six genes we have studied, in the pathomechanism of the disease more precisely.

2. Our research group was the first to compare the expression values of the c-MYC gene measured in the samples of cholesteatoma and another epidermal cyst (atheroma), using normal skin samples as control samples. The significant increase in the expression of the c-MYC protooncogene in the cholesteatoma suggests that the c-MYC gene plays a cell proliferation stimulating role in the pathomechanism of the cholesteatoma, which is required for growth of the tumour.

As there is c-MYC binding place in the promoter of the TP53 and MMP9 genes, we have investigated, the overexpression of the c-MYC may also have a positive effect on the TP53 and the MMP9 genes' expression levels. This explains the increased expression of these genes in our experiments. Thus, the overexpression of the c-MYC may also affect the recurrence indirectly.

3. The role of the cytokeratin 19 as tumour suppressor has been proven in the

recent years. In the relapsing cases of cholesteatoma, the level of the KRT19 mRNA we have determined was lower than in the non-relapsing cases, thus, we were the first to prove the role of the cytokeratin 19 as tumour suppressor in the cholesteatoma, which influences the progression of the cholesteatoma.

4. In our study, we were the first to detect the differences in the pattern of the c-MYC, KRT1/10, KRT19, TP53 and MMP9 genes in patients categorized according to clinical data and tendency to recidivism, in the different groups. In part, the differences in such patterns may explain the differences of the invasiveness and the aggressiveness seen in the two age groups.

5. The described case study clarifies the genetic abnormalities typical of the relapsing cases, which influence the increased cellular activity, the potential for invasiveness, which suggest worse prognosis.

6. With our study, we have proved the altered expression of several regulatory genes involved in the tumorigenesis of the cholesteatoma, suggesting the neoplastic malformation of the cholesteatoma.

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List of publications related to the dissertation

1. **Palkó, E.**, Póliska, S., Sziklai, I., Penyige, A.: Analysis of KRT1, KRT10, KRT19, TP53 and MMP9 expression in pediatric and adult cholesteatoma.
   Plos One. 13 (7), 1-12, 2018.
   DOI: http://dx.doi.org/10.1371/journal.pone.0200840
   IF: 2.776
2. **Palkó, E.**, Póliska, S., Csákányi, Z., Katona, G., Karosi, T., Helfferich, F., Penyige, A., Sziklai, I.: The c-MYC Protooncogene Expression in Cholesteatoma.
   BioMed Research International. 2014, 2014.
   DOI: http://dx.doi.org/10.1155/2014/639896
   IF: 1.579

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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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