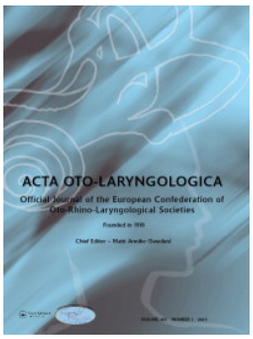

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RESEARCH ARTICLE

Somatic genetic alterations of oncogenes and tumor suppressor genes in cholesteatoma

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ABSTRACT

Background: Cholesteatoma is a proliferative disease that affects the tympanic cavity and temporal bone. Despite many studies and various theories, the etiopathogenesis of cholesteatoma has not been fully elucidated. Features such as invasion, migration, uncontrolled proliferation, and lack of differentiation are observed in both cholesteatoma and neoplasia.

Aims/Objectives: The aim of this study is to investigate somatic genetic alterations in known proto-oncogenes and tumor suppressor genes in cholesteatoma.

Material and Methods: 60 different known proto-oncogenes and tumor suppressor genes were comparatively analyzed in cholesteatoma and peripheric blood samples from 15 middle ear cholesteatoma patients using next-generation sequencing.

Results: JAK3 c.2164G>A, TP53 c.284delC, and KRAS c.377A>T alterations were observed in cholesteatoma tissue but not in normal tissue. In addition, 12 different germline variants were also identified in 8 patients.

Conclusions and Significance: In this study, the presence of changes in cancer-related genes in cholesteatoma was determined and these changes were discussed in terms of possible clinical applications. We hope that the genetic alterations that emerged in this study, will be beneficial in guiding future research in this field.

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Cholesteatoma; neoplasia; JAK3; TP53; KRAS

Introduction

Cholesteatoma is a progressive disease characterized by squamous epithelial proliferation in the middle ear and temporal bone, with an annual incidence of 4–12.6 per 100,000 [1,2]. Hearing loss, vestibular dysfunction, facial paralysis, sigmoid sinus thrombosis, epidural abscess, and meningitis are among the most common complications of cholesteatoma leading to morbidity and mortality [3].

Cholesteatoma is clinically classified as congenital, acquired, and unclassifiable [4]. Theories for etiopathogenesis differ between congenital and acquired cholesteatomas. For congenital cholesteatoma, the most accepted theory is the retention of embryologic epidermoid formation in the middle ear [5]. Whereas there is no single standout theory for acquired cholesteatoma.

There are four prominent theories regarding the etiopathogenesis of acquired cholesteatomas: invagination, migration, squamous metaplasia, and basal cell hyperplasia [6]. Each of these theories explains several aspects of the pathogenesis to some degree, but none of them led to a comprehensive formulation or a consensus to this date.

Histopathologically, cholesteatoma is considered benign in the literature; but, there are significant similarities with cancer such as invasion, migration, uncontrolled proliferation, lack of differentiation, aggressive course, osteolysis, and recurrence [6,7]. In addition, there are several publications reporting the cooccurrence of cholesteatoma and middle ear carcinoma [8–12]. Despite dozens of studies and different theories, a clear picture of the etiopathogenesis of cholesteatoma has not been revealed.

Our study aims to reveal the role of genetic alterations in genes, that are pivotal for carcinogenesis, in etiopathogenesis of acquired cholesteatoma. For this purpose, genetic variations in 60 different known proto-oncogenes and tumor suppressor genes were comparatively analyzed in cholesteatoma and peripheric blood samples from acquired middle ear cholesteatoma patients.

Materials and methods

The study was conducted in the Otorhinolaryngology and Medical Genetics departments of our hospital and was approved by the Clinical Trials Ethics Committee (protocol no: 2020/2581).

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/00016489.2024.2433138.

Patients

15 acquired cholesteatoma patients with an indication for surgical resection were included in the study. All the participants had given written informed consent. The patients were between the ages of 18-65 and the mean age was 38.46 years. 40% ($n=6$) of the patients were female and 60% ($n=9$) were male. 27% ($n=4$) of the patients underwent recurrent surgery whereas 73% ($n=11$) of them underwent primary surgery (see Supplementary Table-1).

Sample collection

Cholesteatoma tissues, routinely removed during cholesteatoma resection operation from the volunteers who decided to participate in the study, were placed in tubes containing RPMI 1640 (Lonza, Verviers, Belgium) medium immediately after the resection. Concurrently, peripheral blood samples were taken to tubes with Ethylene Diamine Tetra Acetic Acid (EDTA). All samples were stored at −80 °C.

DNA isolation

Frozen tissue and blood samples were thawed at room temperature. Then, the tissues were divided into small pieces manually in a sterile environment with the help of a scalpel. Afterwards, DNA isolation from both tissue and blood samples was performed using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany). For cholesteatoma tissue samples, proteinase K treatment was adjusted to 16 h at +55 °C to optimize tissue digestion.

Next-generation sequencing

For next-generation sequencing, Celemics Solid Tumor Kit (Celemics, Seul, South Korea) and its recommended protocol were used. The kit contains primers for ABL1, AKT1, ALK, APC, ATM, BRAF, BRCA1, BRCA2, CDH1, CDKN2A, CSF1R, CTNNB1, DLC1, EGFR, ERBB2, ERBB4, ESR1, FBXW7, FGFR1, FGFR2, FGFR3, FTSJ3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KCNB2, KDR, KIT, KRAS, MET, MLH1, MYC, MYCN, NOTCH1, NRAS, NRXN1, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RBAK, RET, ROS1, SMAD4, SMARCB1, SMO, SMURF1, SRC, SSFA2, STK11, TP53, VHL, and ZNF594 genes (see Supplementary Table-2). Afterwards, Illumina Miniseq (Illumina, San Diego, California, USA) platform was used for sequencing and the sequencing datasets were obtained as FASTQ format.

Data analysis

Datasets in FASTQ format were aligned using the SEQ (Genomize, Istanbul, Turkey) platform over the GRCh37(h19) reference genome. Sequence data in Binary Alignmment Map (BAM) format obtained after this process were evaluated using the Integrative Genomics Viewer (IGV) 2.9.4 software [13]. Variants different from the reference genome were confirmed manually *via* IGV 2.9.4. For germline variants, variants with an allelic fraction between 35-65% were considered heterozygous, and variants >65% were considered homozygous. For somatic alterations, variants with an allelic fraction >5% were considered positive. Detected variants were named according to the Human Genome Variation Society (HGVS) Sequence Variant Nomenclature guidelines [14].

Variant classification

For the classification of the detected variants, the American Medical Genetics and Genomics Society (ACMG) and Association of Molecular Pathology (AMP)'s guideline for pathogenicity classification of sequence variants were used [15]. The population frequencies of the identified variants were evaluated using the gnomAD [16], TOPMed Bravo, GME Variome [17] population databases. In addition, previous reports about the detected variants were searched on Clinvar (https://www.ncbi.nlm.nih.gov/clinvar) and COSMIC (https://cancer.sanger.ac.uk/cosmic) databases. For variants without any clinical or functional information in these databases, variant-specific literature query was conducted for possible clinical and/or functional publications. Furthermore, the variants were also evaluated using in-silico analysis algorithms. For missense changes: SIFT [18], PolyPhen2 [19], FATHMM [20], DANN [21], REVEL [22]; and for splicing variants: SpliceAI [23], and dbscSNV [24] were used. Then, variants were classified as 'benign', 'likely benign', 'variant of unknown significance (VUS)', 'likely pathogenic' or 'pathogenic' in accordance with the standards set by ACMG/AMP [15].

Results

15 different variants, 3 somatic and 12 germline, were detected in 10 of 15 patients. Of the 12 germline variants, one was classified as likely pathogenic, 9 as VUS, and 2 as likely benign. Of the 3 somatic variants, one was evaluated as likely pathogenic and two as VUS.

Somatic variants

In the cholesteatoma tissue of P-6, JAK3 c.2164G>A (ENST00000458235.1)/p.Val722Ile (ENSP00000391676.1) was detected with an allele fraction of 8% (Figure 1). But was not detected in the blood sample. Therefore, this variant categorized as somatic (Table 1).

In P-8, a frameshift variant in TP53 (c.284delC (ENST00000269305.4)/p.Ser95PhefsTer28 (ENSP00000269305.4)) was detected with an allelic fraction of 10% (Figure 1) in the cholesteatoma tissue. Additionally, KRAS gene c.377A>T (ENST00000256078.4)/p.Asp126Val (ENSP00000256078.4) variant was detected with an allelic fraction of 5% in cholesteatoma tissue of the same patient (Figure-1) and classified as a variant of unknown significance. Both variants were not detected in the blood sample and categorized as somatic (Table 1).

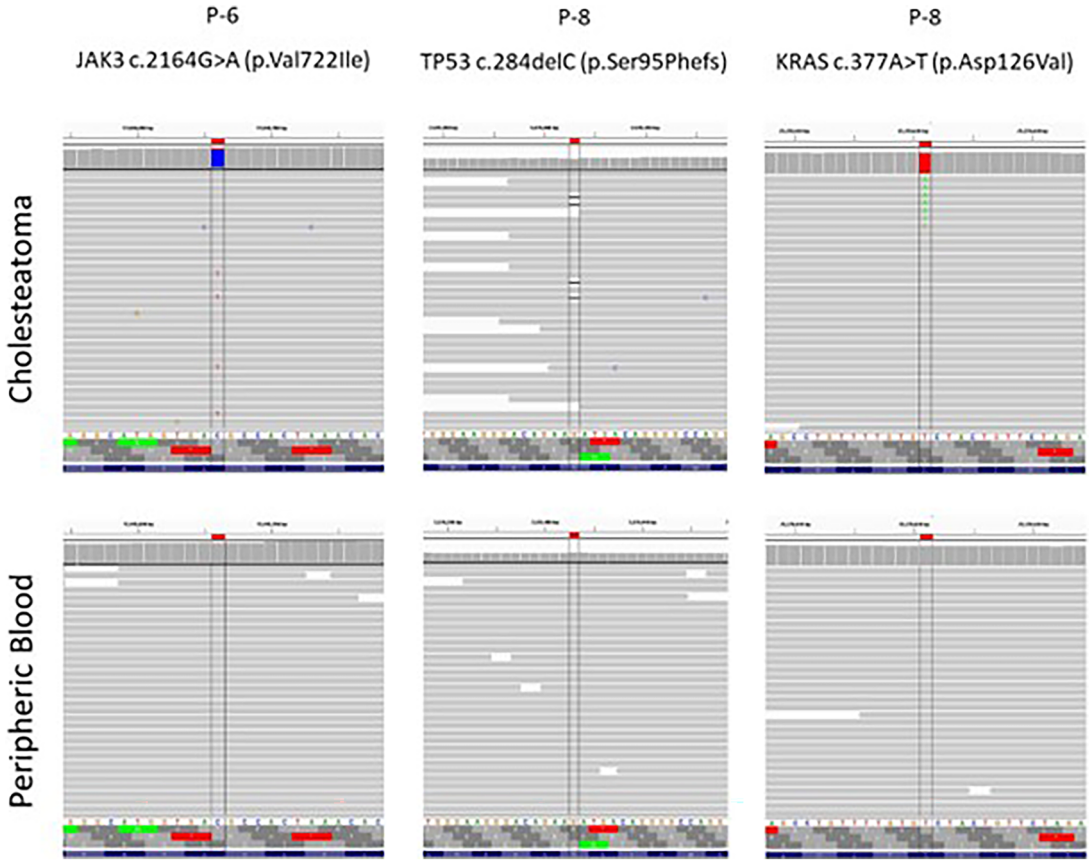

Figure 1. Reads of the somatic variants in integrative genomics viewer (IGV) 2.9.4. Note the absence of the related variants in the peripheric blood samples of P-6 and P-8.

Table 1. Detected somatic variants in cholesteatoma tissues of the patients.

Patient No	Gene	Variant coordinate	Protein coordinate	Allelic fraction	dbSNP ID	Variant type	Variant classification
P-6	JAK3	ENST00000458235.1 c.2164G > A	ENSP00000391676.1 p.Val722Ile	%8	rs3213409	Missense	VUS
P-8	TP53	ENST00000269305.4 c.284delC	ENSP00000269305.4 p.Ser95PhefsTer28	%10	–	Frameshift	Likely Pathogenic
P-8	KRAS	ENST00000256078.4 c.377A > T	ENSP00000256078.4 p.Asp126Val	%5	–	Missense	VUS

Germline variants

The variants with similar allele fractions within both cholesteatoma tissue and blood of a patient in either heterozygous or homozygous state were categorized as germline.

Benign and likely benign variants were excluded from our study. Only MYCN c.1093C > G in P-3 and c.6131G > C in P-12 were included, considering their possible clinical significance, despite they were classified as likely benign. The complete list of germline variants with likely clinical significance has been given in Supplementary Table 3.

The analyzes of P-1, P-5, P-7, P-11, and P-13 revealed no pathogenic, likely pathogenic, or VUS variant.

Discussion

The pathogenesis of cholesteatoma is still unclear despite dozens of studies over decades. Features such as invasion, migration, uncontrolled proliferation, and lack of differentiation are observed in both cholesteatoma and neoplasia. The present study investigated genetic variants in 60 different known proto-oncogene and tumor suppressor genes in 15 patients (see Supplementary Table-2). As a result, three somatic alterations were detected in cholesteatoma tissue of two different patients. These are: JAK3 c.2164G > A (p.Val722Ile), TP53 c.284delC (p.Ser95PhefsTer28), and KRAS c.377A > T (p.Asp126Val).

JAK3 c.2164G > A (p.Val722Ile)

JAK3 is a tyrosine kinase-acting proto-oncogene of the JAK-STAT pathway. Pathogenic changes in this gene have been demonstrated in various neoplasms, mostly hematological [25]. The JAK3 c.2164G > A is a missense variation that leads to the substitution of 722nd amino acid of the protein, which is in the tyrosine kinase domain of JAK3, from valine to isoleucine.

The allelic fraction of the variant in cholesteatoma tissue was 8% and the variant was not detected in blood sample of the same patient. Therefore, this variant considered somatic. The frequency of the variant in the GnomAD database is 0.008382 and 21 homozygotes has been observed. Its frequency in the TOPMed Bravo database is 0.007109, and it was detected in two individuals homozygously. In GME Variome database it was reported at a rate of 0.01712 and without any homozygotes. There were two somatic and ten germline report for this variant in the Clinvar database (last curated in 25.09.2024). Six of the germline reports were benign and four were likely benign. However, both somatic reports were likely pathogenic. Additionally, in COSMIC database, there were 57 entries of the same variant in several different cancer types; 6 of which were confirmed somatic alterations (last curated in 25.09.2024). Two in-silico analyzes for this variant showed pathogenicity (SIFT:0.03, DANN:0.98), and three favored benignity (FATHMM:0.06, REVEL:0.16, PolyPhen2:0.01). The high population frequency, the presence of benign and likely benign germline reports in Clinvar, and the benign results of some of the in-silico analyzes are findings pointing to benignity for this variant. But, it is noteworthy that the JAK3 c.2164G>A alteration was detected somatically in several different tumor samples in COSMIC. Furthermore, in our study the variant was observed only in cholesteatoma tissue and not in peripheric blood of the same patient.

Pathogenic germline JAK3 variants cause autosomal recessive severe combined immunodeficiency and reported pathogenic variants cause loss of function of the protein. However, variants with oncogenic activity in the JAK3 gene are characterized by gain-of-function. Most of the changes in this gene that cause gain of function are localized in the protein pseudokinase region of the gene [26]. MacConaill et al. studied 41 cancer-related genes in tumor tissues of 5000 cancer patients, the c.2164G>A (p.Val722Ile) variant was detected in 2.67% of the patients [27]. Moreover, in another study, this change was observed in 11 of 91 pediatric Acute Lymphoblastic Leukemia (ALL) cases [28]. However, since there was no normal control tissue data for comparative analysis in these studies, somatic/germline differentiation of the variant couldn't be assessed.

In addition, there were some in-vitro experiments considering p.Val722Ile in the literature. Walters et al. showed that the change of JAK3 p.Val722Ile caused factor-independent proliferation in the hematopoietic cell line [29]. In another study, factor-independent activation was detected in the p.Val722Ile mutant pro-B Ba/F3 cell line, and a return to factor-dependent growth was observed as a result of the application of a JAK3 inhibitor: CP-690 550 [28]. Moreover, there is an interesting study regarding the response to p.Val722Ile and PD-L1 treatment. In this study, Van Allen et al. detected germline heterozygous p.Val722Ile change in a case with lung adenocarcinoma responsive to anti-PD-L1 therapy and showed loss of heterozygosity of this variant in metastatic cancer tissue of the patient [30].

Loss of heterozygosity is an important mechanism by which many cancer-associated genes cause neoplasia, especially in hereditary cancers. Demonstration of loss of heterozygosity of a heterozygous variant in tumor tissue is an important finding for the pathogenicity of the variant. Additionally, in the same study, it was shown that p.Val722Ile caused autophosphorylation and autoactivation in JAK3 protein and increased PD-L1 expression [30]. These findings are also promising in terms of possible pharmacotherapeutic solutions to cholesteatoma, considering the effectiveness of anti-PD-L1 agents in cancer treatment.

In conclusion, the fact that the JAK3 p.Val722Ile variant is located in the region of the JAK3 protein showing enzymatic activity and has been previously reported in different neoplasms strengthens the possibility of pathogenicity. However, studies with larger samples are needed to clarify the relationship of this variant with cholesteatoma.

TP53 c.284delC (p.Ser95PhefsTer28)

TP53, known as the 'guardian of the genome', is a crucial tumor suppressor gene in the pathogenesis of cancer, whose pathogenic alterations have been detected in many cancer types [31]. The TP53 c.284delC variant detected in P-8 is a frameshift change, leading to premature termination of translation due to the stop codon formed 28 codons ahead. The variant had 10% allelic fraction and was considered as somatic. This variation has never been reported in the GnomAD, TOPMed, and GME Variome databases. There was no report about this variant in the Clinvar database (last curated in 25.09.2024). There was no entry in COSMIC database about this variant (last curated in 25.09.2024), but when we browsed with a broadened range, we found a report of a somatic two nucleotide deletion in the same region (TP53 c.284_285del) in a gallbladder adenocarcinoma [32]. Because the in silico alghorithms we used were not optimised for truncating variations, no in silico prediction was possible.

Increased TP53 expression has been shown in cholesteatoma tissue in more than one study [7,33]. However, these studies were based on protein expression and genetic alterations of TP53 were not investigated thoroughly. In addition, inferences that TP53 upregulation occurs because of hyperproliferation in cholesteatoma tissue are discussed in different publications [7,33,34]. Therefore, it is important to evaluate TP53 in terms of gene-based changes.

TP53 is the most frequently mutated gene in cancer [31]. From this point of view, the detection of TP53 loss of function in cholesteatoma is significant for the elucidation of the pathogenesis of cholesteatoma. The report of this variant is a first in terms of demonstrating the possible loss of function of the TP53 gene in cholesteatoma.

KRAS c.377A>T (p.Asp126Val)

KRAS is an important oncogene acting on the mitogen-activated protein kinase (MAPK) pathway. Pathogenic changes in this gene have been reported in many neoplasms such as bladder, breast, pancreatic, colorectal and lung cancer [35]. In addition to the TP53 c.284delC variant in H-8, the missense c.377A>T (p.Asp126Val) substitution leading to the conversion of aspartic acid to valine in the KRAS gene was also detected. This

variant, which had an allelic fraction of 5% in cholesteatoma tissue and was not detected in normal tissue, was considered somatic. When the GnomAD, TOPMed, and GME Variome databases were scanned, no data about the change was found. Likewise, there were no reports of this variant in the Clinvar database (last curated on 25.09.2024). As a result of the literature review, it is seen that this variant was reported somatically in only one breast cancer patient [36]. In COSMIC, there was only one entry for this variant (last curated in 25.09.2024) which was reported first in the aforementioned publication [36]. Two of the in-silico analyzes were in favor of pathogenicity (SIFT: 0.01, DANN: 0.98,), and three were in favor of benignity (FATHMM: −1.07, REVEL: 0.61, PolyPhen2: 0.004).

MAPK pathway activation in cholesteatoma has been reported in different publications before [37]. However, the relationship of genetic alterations of KRAS in cholesteatoma tissue has not been studied thoroughly so far. The role of gain of function variants in the KRAS gene is known in multiple cancer types. From this point of view, the somatic KRAS change we detected in cholesteatoma tissue gains extra importance. It is noteworthy that the KRAS p.Asp126Val variant was reported as somatic in a breast cancer case in the literature [36]. Considering the effectiveness of drugs targeting the MAPK pathway in cancer treatment, the importance of new studies focused on KRAS and MAPK pathway becomes even more prominent.

In a recent study, Satoh et al. found somatic alterations of MYC and NOTCH1 genes in two and five samples from 22 cholesteatoma patients respectively [38]. Nevertheless, our study showed no somatic variant in either of these two genes.

One of the two patients with a somatic variant was 36 years old female (P-6) and the other one was 38 years old male (P-8). However, the sample size was insufficient to make a statistical analysis between the demographic features of the patients and somatic genetic variations.

Overall, our study showed 3 somatic genetic variants in 2 of 15 cholesteatoma patients. The discussion of the 12 germline variants detected in 10 patients was not possible in this publication. The information about these variants can be viewed in Supplementary Table 3.

Despite the somatic variants, no known pathogenic genetic alterations were detected in most of the samples. The absence of a well-established pathogenic variant in the analysis may be due to the nature of the pathogenesis of cholesteatoma; because cancer-associated variants classified as definitively pathogenic in the literature, are mostly null variants, variants causing complete disruption of protein function, or gain-of-function variants with known oncogenic activity. The fact that cholesteatoma is more benign both histopathologically and clinically compared to cancer, may suggest that variants with subtle pathogenic effects may be involved in this regard.

Although the genes evaluated in our research have clearly known effects on the pathogenesis of cancer, further studies involving more genes may reveal new knowledge about the etiopathogenesis of cholesteatoma. In this context, Genome-Wide Association Studies (GWAS) using whole-exome or whole-genome sequencing techniques can be beneficial.

The Next-Generation Sequencing (NGS) technology we utilized in our study is advantageous in that it enables many regions of many genes to be sequenced at very high depths at the same time. However, this method is disadvantageous in detecting large deletions, duplications and other structural variants. In the future, methods such as Fluorescence *In Situ* Hybridization (FISH), Multiplex Ligation-Dependent Probe Amplification (MLPA), and array Comperative Genomic Hybridisation (aCGH), which can detect large deletions, duplications and structural alterations, can be used to overcome this disadvantage. Considering that various aneuploidies and oncogene amplifications have been detected cytogenetically in cholesteatoma tissue in previous studies [39–40], research based on detecting structural genetic variants will be complementary to our study.

Likewise, the next-generation sequencing method used in our study cannot detect epigenetic changes in the genome. New studies on DNA methylation, histone modification and chromatin remodeling in cholesteatoma have the potential to reveal significant data, especially for the effects of environmental factors.

Conclusion

Although the etiopathogenesis of cholesteatoma has not been fully elucidated, accumulating evidence points to a complex network of different factors. Whether due to genetic, epigenetic, or environmental factors, cholesteatoma is a complex disease in which various genetic mechanisms play a role in its pathogenesis. In literature, there is a limited number of publications in which somatic analysis of cancer-related genes in cholesteatoma tissue was compared with normal tissue. We hope that the genetic alterations emerged in this study, will be beneficial in guiding future research in this field.

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Ethical approval and consent to participate

Our research has ethics approval from Necmettin Erbakan University Meram Medical School Ethics Committee and informed written consent for participation and publication have been obtained from the patients (protocol no: 2020/2581).

Disclosure statement

No potential conflict of interest was reported by the author(s).

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