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Research Article/Özgün Araştırma

Are fungi and EBV effective in cholesteatoma etiology?

Kolesteatom etyolojisinde mantar ve EBV etken mi?

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Abstract

Aim: Cholesteatoma is a commonly seen disease whose pathogenesis remains unknown. Although not a neoplastic process, it may progress to a fatal condition with local bone destruction. In this study, we aimed to present new insights concerning the etiology of cholesteatoma triggered by an inflammatory process.

Materials and Methods: The study included 34 patients diagnosed with cholesteatoma upon mastoidectomy performed between 2011-2019. Due to a provisional diagnosis of cholesteatoma. The cases were investigated for the latent membrane protein (LMP-1) encoded by the Epstein-Barr Virus (EBV) using the immunohistochemical method and for the presence of fungi using Grocott's methenamine silver (GMSII) stain.

Results: No fungi was detected in any of the 34 patients by GMSII staining. Thirty-two of the 34 patients were negative with but a suspicious result was seen in 2 patients with the immunohistochemical EBV antibody. EBV-encoded RNA (EBER) analysis was applied to these 2 cases with the silver in situ hybridization method and no reaction was observed.

Conclusion: In our study, we investigated the presence of fungi and EBV, which can trigger the inflammatory process. However, no EBV or fungi was detected in the tissues. Our study is the first to investigate the presence of EBV and fungi in formalin-fixed tissue in cases of aggressive cholesteatoma.

Keywords: Cholesteatoma; Etiology; Fungi; EBV.

Öz

Amaç: Kolesteatom, patogenezi bilinmeyen, toplumda sık görülen bir hastalıktır. Neoplastik bir süreç olmamasına rağmen lokal kemik destrüksiyonu ile mortal hastalık olabilir. Çalışmamızda inflamatuvar süreç ile tetiklenen kolesteatometyolojisine yönelik yeni bilgiler sunmayı hedefledik.

Gereç ve Yöntem: Çalışmaya 2011-2019 yılları arasında mastoidektomi yapılankolesteatom tanılı 34 hasta dahil edildi. Olgular, immünohistokimyasal yöntem kullanılarak Epstein-Barr virüsü (EBV)'nın kodladığı gizli membran proteini (LMP-1) ve Grocott's methenamine silver boyası ile mantar varlığı araştırıldı.

Bulgular: 34 hastada GMSII boyama ile mantar tespit edilmedi. Bu 34 hastanın 32'si immunohistokimyasal EBV antikoru ile negatif, ancak 2'si şüpheli değerlendirildi. Bu 2 olguya silver in situ hibriditasyon yöntemiyle EBER uygulandı ve reaksiyon elde edilmedi.

Sonuç: Araştırmamızda inflamatuvar süreci tetikleyebilecek mantar, EBV varlığını araştırdık. Ancak dokularda EBV, mantar tespit edilmedi. Çalışmamız agresif kolesteatom olgularında formalin fikse dokuda EBV ve mantar varlığını araştıran ilk araştırmadır.

Anahtar Kelimeler: Kolesteatom; Etyoloji; Mantar; EBV.

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Introduction

Cholesteatoma is an inflammatory lesion that often occurs in the middle ear or the mastoid, or rarely in both, and may also be seen in the temporal bone. Although not neoplastic in nature, these lesions mostly occur as a destructive process that is locally invasive in the form of a unilateral lesion. The histopathology of cholesteatoma is characterized by connective tissue accompanied by proliferated squamous epithelium and inflammatory cells surrounding desquamative keratin bundles in a cystic structure. In 1838, Johannes Müller coined the term, "cholesteatoma" considering this lesion as a tumor of adipose tissue with "chole" for cholesterol, "steat" for fat and "oma" representing tumor formation, resulting in misnomer of these lesions.[1] Annual incidence of cholesteatoma is 3/100,000 in children and 9.2/100,000 in adults. The incidence appears to be higher in males compared to females (M/F: 1.4/1). Cholesteatoma located in the middle ear is more common in those younger than 50 years of age.[1] Cholesteatoma is divided into three categories; the congenital form seen in children, the acquired type that may affect both adults and children, and the unspecified type. Congenital cholesteatoma is typically a cystic mass of keratinized squamous epithelium that extends towards the intact tympanic membrane. The most widely accepted hypothesis of congenital cholesteatoma etiology is the 'epithelial rest' theory. Epithelial cells referred to as the epibranchial placode are located behind the intact tympanic membrane and are normally absorbed by involution at 33 weeks of gestation. In the event of involution failure, congenital cholesteatoma may develop due to the damage in the surrounding tissue. According to another theory, i.e. the invagination theory, squamous epithelium migrates from the tympanic membrane to the middle ear, ending up in the external canal and resulting in congenital cholesteatoma. Acquired type cholesteatoma is assumed to develop due to Eustachian tube dysfunction following a previous disease of the middle ear. However, there is not yet an accepted mechanism that fully describes the development of acquired cholesteatoma despite the presence of multiple pathophysiological theories suggested thus far. Acquired type cholesteatoma is further divided into subclasses referred to as the retraction pocket variant and non-retraction pocket variant. The retraction pocket variants of cholesteatoma usually occur in secondary fashion in patients with acute otitis media. There are three different theories concerning the development mechanism of these lesions, namely the epithelial migration theory, the squamous metaplasia theory and the basal cell hyperplasia theory.[1,2] It remains unknown why the benign epithelium covering the external ear canal leads to erosion of bony structures after migration to the middle ear. In histological terms, papillomatous growth and koilocyte clusters are typical features of bone destructive sites seen in aggressive cholesteatoma.[3] The effect of cell debris and keratinocytes that accumulate in the retraction pocket interfere with self-clearance mechanisms, which is accompanied by local infection consisting of Langerhans and T-cells as well as macrophages. There is a vicious cycle between epithelial proliferation, keratinocyte differentiation and maturation, prolonged apoptosis and disruption of the self-clearance mechanisms. The inflammatory stimulus induces epithelial proliferation together with the expression of lytic enzymes and cytokines. Bacteria that may be colonized in the retraction pocket produce certain antigens that subsequently activate different cytokines and lytic enzymes. These cytokines lead to disruption of the extracellular bone matrix, proliferation, bone erosion and finally, progression of disease, thereby resulting in activation and maturation of osteoclasts. Currently, the etiological role of bacterial infection is under investigation upon the identification of biofilms.[1]

Cholesteatomas often remain asymptomatic for years without causing any potential harm. In general, these lesions are left untreated until the rapid progression phase and the potential risk of invasion in intratemporal structures. Several patients suffering from cholesteatomas describe a frequently recurring and foul-smelling otorrhea characterized by purulent discharge. Otalgia, headache, vomiting and

fever are atypical presentations of cholesteatoma; however, the occurrence of these symptoms may indicate the likelihood of intratemporal or intracranial complications. In this regard, prevention of fatal outcomes requires immediate assessment and timely treatment. Despite robust research on the treatment of acquired cholesteatoma, effective non-surgical treatment remains an unmet need. The surgical treatment in question primarily aims to control the condition, in other words, achieve a dry, unproblematic state in the ear without recurrence.[4,5,6] The aim of our study is to investigate the role of fungi and/or EBV in the etiology of aggressive cholesteatoma patients undergoing Canal Wall Up (CWU) and Canal Wall Down (CWD) mastoidectomy and provide insight on the clinical prognosis and treatment of such cases.

Materials and Methods

The type and sample of the research

This retrospective study included a total of 34 patients diagnosed in 2011-2019 with cholesteatoma. The slides of these 34 patients were retrieved from the archive and reassessed by two pathologists under an Olympus BX46 light microscope. Age and gender of the patients were recorded. Using the paraffin-embedded tissues of these cases, 4-micron sections were obtained, and the sections were then transferred onto positively charged slides. The sections were allowed in an incubator at 60°C for an hour and deparaffinized with xylene for 15 minutes. The samples were hydrated through descending-grade series of alcohol and washed in distilled water. They were then placed into a BenchMark XT device (Cell Marque EBV–RTU) for the antibody analysis and slides were placed in a BenchMark Special Stains histochemistry device to undergo GMSII staining. The samples stained in the automated staining device were covered using fluid-based covering material. These slides were examined under a BX46 Olympus light microscope at x40 magnification for fungal hyphae and spores with GMSII stain. The anti-EBV antibody for EBV targets the latent membrane protein (LMP-1) encoded by the BNLF1 gene and produces brown-colored membranous staining in positive cells.[6]

Analysis of data

To evaluate EBV, cytoplasmic and membranous staining pattern was scored on a scale of 0-1 as follows; 0: no staining, 1: membranous staining in squamous epithelial cells. To evaluate GMSII, the extracellular staining pattern was scored on a scale of 0-1 as follows; 0: no staining, 1: staining present.

Statistical method

Patient demographics were analyzed using the SPSS 24 program.

Ethics committee approval

The protocol was approved by the Ethics Committee of Tekirdağ Namık Kemal University' Faculty of Medicine (Approval date and number: 28.05.2020/2020.102.05.03). The research has been prepared in accordance with the Declaration of Helsinki Principles.

Results

Of the 34 patients who underwent surgery due to mass in the middle ear, 15 (44.1%) were female and 19 (55.9%) were male. The Male-Female ratio was 1.26. Mean age was 37.6 years (min: 10, max: 68). The main clinical presentation was loss of hearing and chronic ear discharge. HRCT was used for the radiological evaluation in all patients. Contrast-enhanced technique was utilized for those with suspected intracranial lesion. Identified in 44% of the patients, combined pars flaccida and pars tensa cholesteatoma were the most common type (Table 1). Acquired cholesteatoma extending to mastoid antrum was the most common localization with 67% of the patients, followed by mesotympanum cholesteatoma in 17% of the patients (Table 2). Scutum and lateral attic wall erosion was the most common finding seen in 76% of the patients, followed by eroded superior and posterior meatal wall in 73% of the cases (Table 3). One the patients died due to brain abscess as an intracranial complication of the cholesteatoma.

Immunohistochemical analysis for the EBV antibody yielded negative results in 32 patients while 2 were deemed as suspicious and further examined by silver in situ hybridization

(SISH) for EBER. No reaction was observed in these two cases, therefore all patients included in the study were deemed as EBV-negative. GMSII histochemical analysis did not reveal fungal hyphae or spores in any of the tissues.EBV and GMS analysis of the cases by gender is presented in Table 4. Figure 1 shows samples of Hematoxylin and Eosin (H&E), EBV and GMSII staining of one of our patients. Figure 2 and 3 shows samples of GMS and EBV staining of control positives.

Table 1. Presents the type of cholesteatomas.

Type of cholesteatoma	No. of patients	%
Combined cholesteatoma	15	4
Pars tensa cholesteatoma	9	6
Parsflaccidacholesteatoma	10	0

Table 2. Localization of cholesteatomas.

Localization and extension	No. of patients	%
Atticoantral	5	14
Mesotympanum	6	17
Extended to mastoid antrum	23	67

Table 3. Shows the erosion in middle ear cavity.

Middle ear bony wall erosion	No. of patients	%
Eroded scutum and lateral attic wall	26	76
Eroded tegmen	4	11
Thinning of the tegmen	8	23
Eroded superior and posterior meatal wall	25	73
Eroded lateral semicircular canal	4	11
Eroded Koerner's septum	17	50

Table 4. EBV and GMSII staining distribution ofthe cases by gender.

Sex	Male	Female
EBV-negative	19	15
EBV-positive	0	0
GMSII-negative	19	15
GMSII-positive	0	0

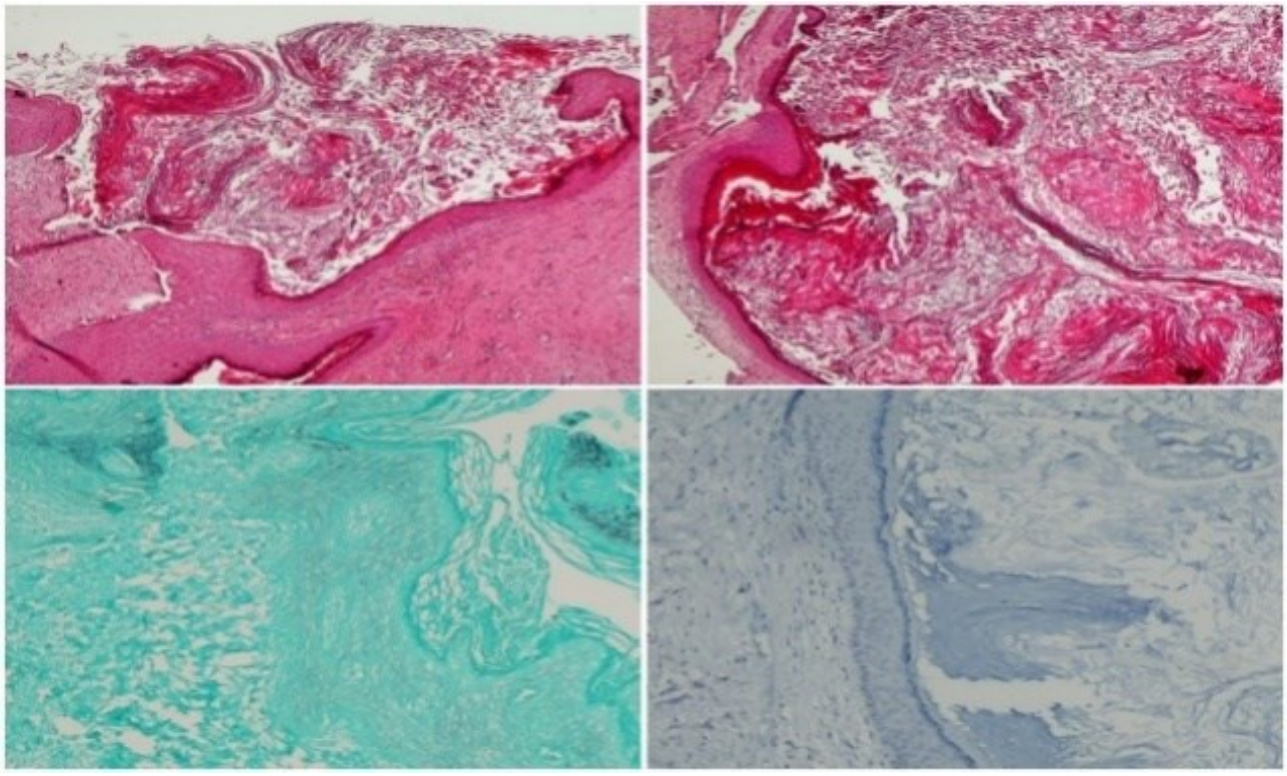

Figure 1. Cholesteatoma A) H&E x40 B) H&E x100 C) GMSII x100 D) Anti-EBV x100

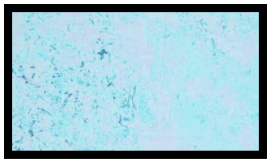

Figure 2. GMSII positive control x40

Figure 3. Anti-EBV positive control x100.

Discussion

Cholesteatomas often exhibit variable clinical courses. They may remain silent and progress slowly over years or spread rapidly and progress with mortal complications such as meningitis and destructive damage in local bone structures.[3,6,7,8] The clinical differences are thought to result from the microorganisms that may colonize in the proliferated squamous epithelium in the retraction pocket during the development of acquired cholesteatoma and the damage caused by T-lymphocytes, histiocytes, monocytes as well as the interleukins and cytokines these cells release.[2,3,9,10] Based on this hypothesis, the present study aims to investigate fungi in paraffin-embedded block tissue sections of relevant cases. For this purpose we used GMSII, a dye produced with a specific silvering technique that is highly effective in showing fungal hyphae and spores. However, we did not detect any fungal spores in the light microscopic evaluation of paraffin block sections.

In the literature, a prospective case series byEffat and Madany investigated the incidence and structure of fungal elements in keratin samples derived from cholesteatoma excised by primary mastoid surgery. Their study included 13 males and 5 females in the age group of 9-45 years and identified fungi in samples of 17 patients (89%).[10] We think that we could not detect fungi in our study, since we were investigating fungi in blocks embedded in paraffin, not in fresh tissue. However, in daily routine practice, we set up our study on paraffin-embedded tissues, as the tissues are embedded in paraffin for pathological examination, the tissues can be stored in this way for a long time and are suitable for other investigations.

In our study, minimum age was 10 and maximum age was 68 years, which is a distribution consistent with the literature.[2,11] Gender distribution was also consistent with the relevant literature, with a slightly higher incidence in females.[2,9,11] A prospective study by Singh et al.[12] investigating the prevalence of fungi in patients with cholesteatoma and suppurative otitis media enrolled a total of 46 patients. They analyzed fungal colonization in samples by means of microbiological methods.[12] While postoperative cholesteatoma was observed in 40 out of the 46 patients included in their study, fungal colonization was identified in 17 (42.5%) of these cases.[12] Additionally, the authors reported a statistically significant correlation between permanent otorrhea and fungal colonization of cholesteatoma.[12] Studies have shown that EBV in carrier form may be present up to 90% in the head and neck region.[13] EBV is frequently detected in malignancy samples from nasopharyngeal carcinoma and cervical lymph nodes by means of the polymerase chain reaction (PCR) method. Tsai et al. evaluated EBV results using PCR in nasopharyngeal swab samples and SISH in formalin-fixed paraffin-embedded block sections of nasopharyngeal biopsy samples. They compared EBER SISH versus PCR and recommended EBER SISH in that this method supports nasopharyngeal malignancy.[14] In this study, as in the study of Tsai et al.[14] we investigated the presence of EBV in formalin-fixed paraffin-embedded block sections by immunohistochemistry, and we used EBER SISH in two suspicious cases and negative results were found.

Reports on the expression of HPV 6 and 11 in cholesteatomas range from 3.1 to 27.3% based on PCR studies. HPV is also a potential etiological factor in cholesteatomas with aggressive growth. Koilocytic alterations and papillomatous growth suggest a possible viral etiology in histological terms that may be confirmed by PCR.[6,8] We investigated another viral agent in our study upon the detection of viral agents in the etiology of cholesteatoma.

In our study, the EBV agent could not be searched with the SISH method, which is more sensitive to the agent, due to the lack of budget.

Conclusion

In the literature, the presence of fungi and EBV has not been investigated in formalin-fixed paraffin block tissue sections in an attempt to understand the etiopathogenesis of cholesteatomas. Further studies with the collaboration of microbiology and pathology to explore the factors that trigger inflammation

are needed in order to elucidate the etiology of cholesteatomas.

EBV and fungi have not been investigated simultaneously in the etiopathogenesis of aggressively disseminated cholesteatomas.

Ethics Committee Approval

The protocol was approved by the Ethics Committee of Tekirdağ Namık Kemal University Faculty of Medicine (Approval date and number: 28.05.2020/2020.102.05.03). The research has been prepared in accordance with the Declaration of Helsinki Principles.

Informed Consent

Informed consent forms were obtained from all participants.

Authors' Contributions

Conception–A.İ.A.; Design–A.İ.A, T.E.; Supervision–A.İA., T.E.,S.K.; Materials–A.İA., S.K.; Data collection and/or processing–A.İ.A, S.K.; Analysis and/or Interpretation–A.İ.A., S.K.; Literature review A.İ.A, S.K.; Writer–A.İ.A,; Critical Review–A.İ.A, T.E,S.K.

Conflict of Interests

The authors declare that they have no conflict of interest.

Financial Disclosure

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Peer-review

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