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RESEARCH ARTICLE

Clinical, Pathological, Immunohistochemical and Ultrastructural Observations on Enzootic Nasal Adenocarcinoma in Five Goats

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Summary

The objectives of this study were to evaluate clinicopathological, immunohistochemical and electron microscopical findings of Enzootic Nasal Adenocarcinoma (ENA) in five goats and examine the immunoreactivity of these tumors with different markers. At the histopathological examination, tubular, papillary and acinar subtypes were diagnosed in this study. Tumor interstitial cells strongly expressed S100, Neuron Specific Enolase (NSE), Smooth Muscle Actin (SMA) and Vimentin, epithelial cells were positive for Pancytokeratin (PCK), Carcinoembryonic Antigen (CEA) and Gross Cystic Disease Fluid Protein (GCDFP). Both interstitial and epithelial cells strongly expressed Caspase-3, Ki-67 and proliferating cell nuclear antigen (PCNA); both interstitial and epithelial cells were negative for Alpha Fetoprotein (AFP). Immunohistochemical observations revealed that ENA consisted of both epithelial and mesenchymal cell proliferations based on the increased PCNA and Ki-67 positive reaction of these cells. Electron microscopical studies revealed the presence of the retrovirus-like particles in the cytoplasm of neoplastic epithelial cells. At the same time, this study is the first report of ENA cases from Turkey in goats.

Keywords: *Goat, Enzootic nasal adenocarcinoma, Pathology, Immunohistochemistry, Electron microscopy*

Beş Keçide Gözlenen Enzootik Nazal Adenokarsinomda Klinik, Patolojik, İmmunohistokimyasal ve Elektron Mikroskobik İncelemeler

Özet

Bu çalışmanın amacı, 5 keçide Enzootic Nazal Adenokarsinomun klinik, patolojik, immunohistokimyasal ve elektron makroskobik olarak incelenmesi ve bu tümörlerin değişik markırlar ile immunoreaktivitesinin incelenmesidir. Bu çalışmada tümörlerin histopatolojik incelemesinde tubuler, papiller ve asiner subtipler teşhis edildi. Tümör interstiyel hücreleri S100, NSE, SMA ve Vimentin ile epitel hücreleri ise PCK, CEA ve GCDFP ile pozitif reaksiyon verdi. Caspase-3, Ki-67 ve PCNA ile hem epitel hemde intertisyel hücrelerde pozitif reaksiyon saptanırken, AFP bütün hücrelerde negatif reaksiyon gösterdi. Immunohistokimyasal incelemesi sonucunda ENA'da hem epitel hemde intertisyel hücrelerde artış olduğu PCNA ve Ki-67 artışı ile saptandı. Elektron makroskobik incelemede neoplastik epitel hücrelerinde retrovirus-benzeri yapılar saptandı. Bu çalışma aynı zamanda Türkiye'de keçilerde saptanan ilk ENA olgusudur.

Anahtar sözcükler: *Keçi, Enzootik nazal adenokarsinom, Patoloji, İmmunohistokimya, Elektron mikroskop*

INTRODUCTION

Enzootic nasal adenocarcinomas (ENA) belong to a unique group of nasal carcinomas of sheep, goats and cattle that arise from the ethmoidal conchae [1-5]. Enzootic nasal adenocarcinoma virus (ENAV) is a retrovirus, related to Jaagsiekte virus and that induces neoplastic growth of the mucosal glands from the ethmoidal area

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in the nasal cavity [6-8]. Definitive diagnosis of ENA is usually made based on histopathological examination of the tumor. It is classified as a low-grade adenocarcinoma and metastasis of this tumor to the other organs are rare [1,9,10]. The tumors originate from Bowman's glands in the ethmoid mucosa. In goats, serous glands of the nasal cavity also proliferate in ENA cases. This tumor has been reported from numerous countries [11].

The objectives of this study were to examine clinico-pathological and ultrastructural findings of ENA; to measure the proliferation indices in this tumor using immunohistochemical detection of Ki-67 and proliferating cell nuclear antigen (PCNA), and determine the relationship of these antigens to clinical and pathologic variables; to examine the immunoreactivity of these tumors with different markers; and to evaluate the origin of the proliferated cells. At the same time, this study is the first report of the pathomorphologic and electron microscopic findings of five ENA cases from Turkey.

MATERIAL and METHODS

This study was carried out on a total of five male goats from a herd numbering 350 goats with a 10-year history of respiratory nasal problems and death with respiratory distress. They were goats *(Capra hircus)* ranging in age from 8-20 months and kept in a small shelter together with different ages animals. Clinically, signs of dyspnea and mucous nasal discharge were manifested in all animals. Due to the inappatence, mild to severe emaciation was noted in all affected animals. Owner stated that more than 100 goats died from same illness with dyspnea and in some cases nasal bone perforation in 10 years period. Five animals were suffering from the disease the time of the study, two had died and three were euthanatized because of poor prognosis during a one month period. Before necropsy, blood samples in EDTA tubes were taken from the jugular vein for hematological analysis from three live goats. Five normal goats blood were used as control. MS9 blood counting equipment was used for complete blood count. At necropsy, unilateral intranasal tumors were found in all animals. Tissue samples were taken from tumors and the visceral organs for histopathological examinations. Samples were fixed in 10% neutral formalin. Using standard methods, tissues were stained with Hematoxylin-Eosin (HE), and examined microscopically.

Selected tumor sections were stained immunohistochemically in order to demonstrate Ki-67 [Neomarker-Westinghouse, California (Ki-67- Proliferation Marker) Ab-3, Rabbit Polyclonal Antibody, (1/50 dilution)]; NSE [Neomarker, (NSE Ab-1 (Clone E27) Mouse Monoclonal Antibody, (1/100 dilution)]; AFP [DAKO- Glostrup, Denmark, Polyclonal Rabbit Anti-Human Alpha-1-Fetoprotein, A 0008 (1/100 dilution)]; PCK [Neomarker, (Keratin, Pan Ab-1, Clone AE1/AE3, Mouse Monoclonal Antibody (1/100 dilution)]; PCNA [DAKO, Monoclonal Mouse Anti-PCNA, Clone: PC10, (1/100 dilution)]; CEA Protein [Neomarker, (CEA/CD66e Ab-2, Rabbit Polyclonal Antibody (1/100 dilution)], S100 protein [Neomarker, (S100 Protein Ab-2, Rabbit Polyclonal Antibody (1/100 dilution)]; SMA [(DAKO, Monoclonal Mouse Anti-Human, Clone: 1A4), (1/100 dilution)], Vimentin [DAKO, Monoclonal Mouse Anti- Vimentin Clone: V9, (1/100 dilution)]; GCDFP [Neomarker, (GCDFP-15) Ab-1 Clone23A3, Mouse monoclonal Antibody (1/50 dilution)] and Caspase [Neomarker, (Caspase 3 (CPP32) Ab-4, Rabbit Polyclonal Antibody (1/100 dilution)] using a routine streptavidine-biotin peroxidase technique. For immunohistochemical examination sections were routinely processed according the manufacturer's instructions.

Samples from tumors were also processed for transmission electron microscopy (TEM). They were fixed in 2.2% Glutaraldehyde and post fixed in 1% Osmium tetroxide (OsO_4) prepared in 0.1M phosphate buffer solution. Then, the tumor samples were dehydrated in graded alcohol series and embedded in Araldite CY212. Ultrathin sections were taken from plastic blocks and stained with uranyl acetat/lead citrate.

The proliferation index was determined for the 5 tumors by means of immunohistochemistry for Ki-67 and PCNA antigen. Proliferation indices were determined as the percentage of positive cells among 1.000 tumor cells in multiple microscopic high powered fields under the 40x objective. The number of positively stained nuclei was divided by the total number of tumor nuclei and expressed as a percentage. Fields were selected from areas with the greatest proportion of cells staining positive which gives higher values than randomly selected fields.

RESULTS

Clinically all of the five goats exhibited severe respiratory distress for more than 5 weeks. Seromucous exudates were observed around the nares. Inappatence and cachexia were the other findings. Body temperature was normal or below normal. Hematological analyses of blood was taken before the death identified lymphocytosis ($12\text{-}15x10^3/\mu L$) in all three animals compared to normal goats ($8\text{-}10x10^3/\mu L$) indicating chronic disease. The nasal tumors were observed as polypoid (1-2.5cm in length) and sessile (0.5-3cm in diameter) masses in the ethmoidal area and completely filled the nasal cavity *(Fig. 1).* The tumors were unilateral in all cases. Four of

the tumors were located on the right side and only one located on the left. The surface of the tumoral masses was covered by seromucous exudate. Surface and cut surface were pink-white in color. The tumors were irregular in shape, soft to firm, and friable. Although the owner stated that there was softening and perforation of the nasal bones in some previous cases, but there was no bone deformity in our five cases. Metastases were not detected in any organ (lungs, lymph nodes, heart, liver, kidney, central nervous system and gastrointestinal system) both on gross and microscopical examination.

At the histopathological examination, similar findings were observed in all cases which were characteristic for ENA. The tumoral cells were generally uniform and no cellular atypia was observed. In HE stained slides, tubular, papillary, acinar and in some areas mixed structures were observed in the neoplastic tissue *(Fig. 2-3)*. The tumoral cells were generally cuboidal with large round nuclei. No indication of invasion in any of the cases.

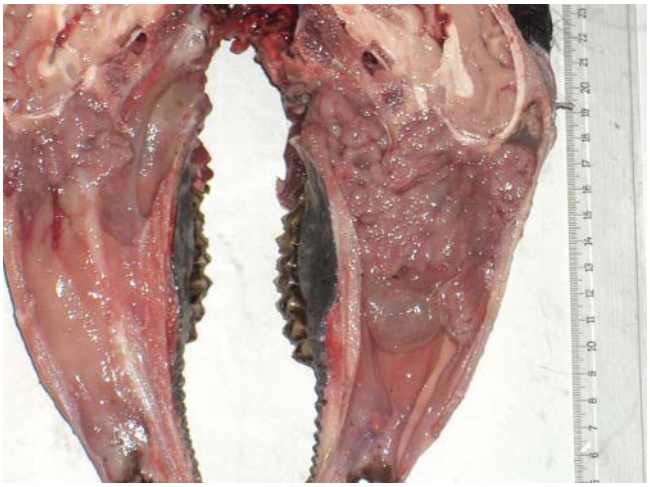

Fig 1. Gross appearance of the tumor, totally filled the nasal cavity

Şekil 1. Burun boşluğunu doldurmuş olan tümörün görünümü

The tumoral tissue was well vascular and mitoses were very rare in the tumoral cells. Predominantly lymphocytic infiltrations were seen in connective tissue stroma. Neutrophils, plasma cells and macrophages also infiltrated the tumoral tissue. Additionally small necrotic areas in the tumor and fibrinopurulent exudate on the tumor were present.

Immunohistochemical examination of tumors revealed mesenchymal cells were positive for S100, NSE, SMA and vimentin, epithelial cells were positive for PCK, CEA and GCDFP, both mesenchymal and epithelial cells were positive for Caspase-3, Ki-67 and PCNA, and both mesenchymal and epithelial cells were negative for AFP. Immunohistochemical results showed that epithelial cell proliferation more prominent than mesenchymal cell proliferation. In the tumor tissue, intensity of Ki-67, PCNA, NSE, pancytokeratin, S100 protein, SMA, vimentin, and caspase-3 expression were strong, GCDFP and CEA were slight *(Fig. 4)*. In general Ki-67 and PCNA immunostaining was uniform. High proliferation indices were observed in both mesenchymal and epithelial cells. While Ki-67 indices varied between 15-20%, PCNA indices were between 10-15% at the end stage of the tumor. These results showed that proliferation was occurring in both the epithelial and interstitial cells of the tumor. Our results also revealed that this tumor has potentially malign behavior.

Ultrastructurally, neoplastic proliferations were predominantly composed of cuboidal cell. Nuclei were round or oval and euchromatic with heavily stained heterochromatin regions located close to the nuclear envelope.

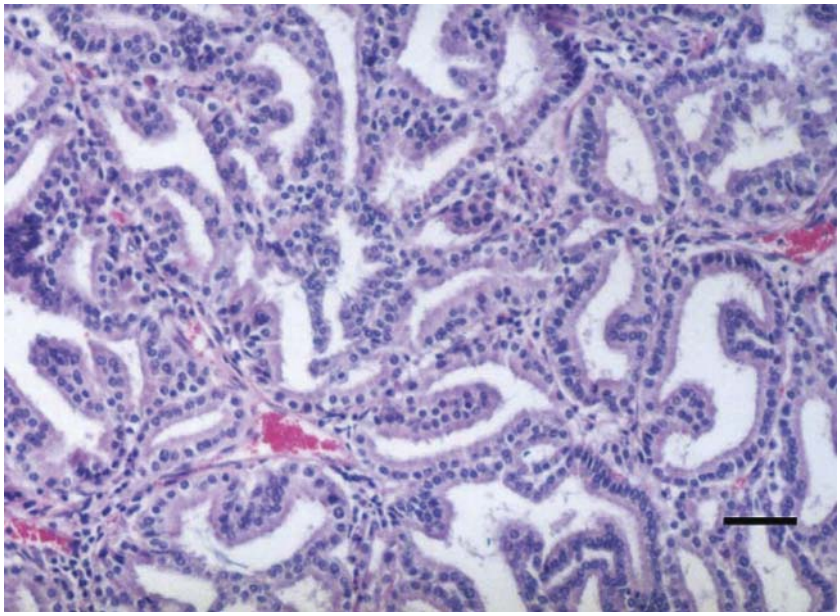

Fig 2. Histopathological appearance of the tumor, papillary type, HE; Bar = 100 µm

Şekil 2. Tümörün histopatolojik görünümü, papiller tip, HE; Bar = 100 µm

The cytoplasm of the cells had an electron lucent matrix containing numerous secretory granules and membrane coated structures. Numerous electron dense secretory granules were distributed throughout the cytoplasm. Examination for viral particles revealed numerous retrovirus-like particles in the cytoplasm supporting the viral etiology of the disease *(Fig. 5)*. The viral agents were 95-135 nm in diameter and spherical in shape.

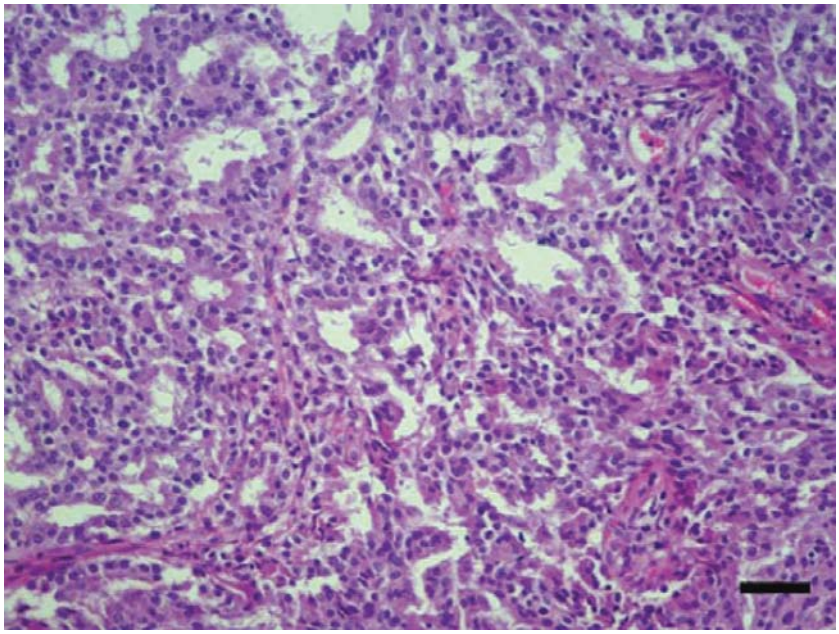

Fig 3. Histopathological appearance of the mixed type of the ENA, HE; Bar = 100 µm

Şekil 3. Miks tip ENA'nın histopatolojik görünümü, HE; Bar = 100 µm

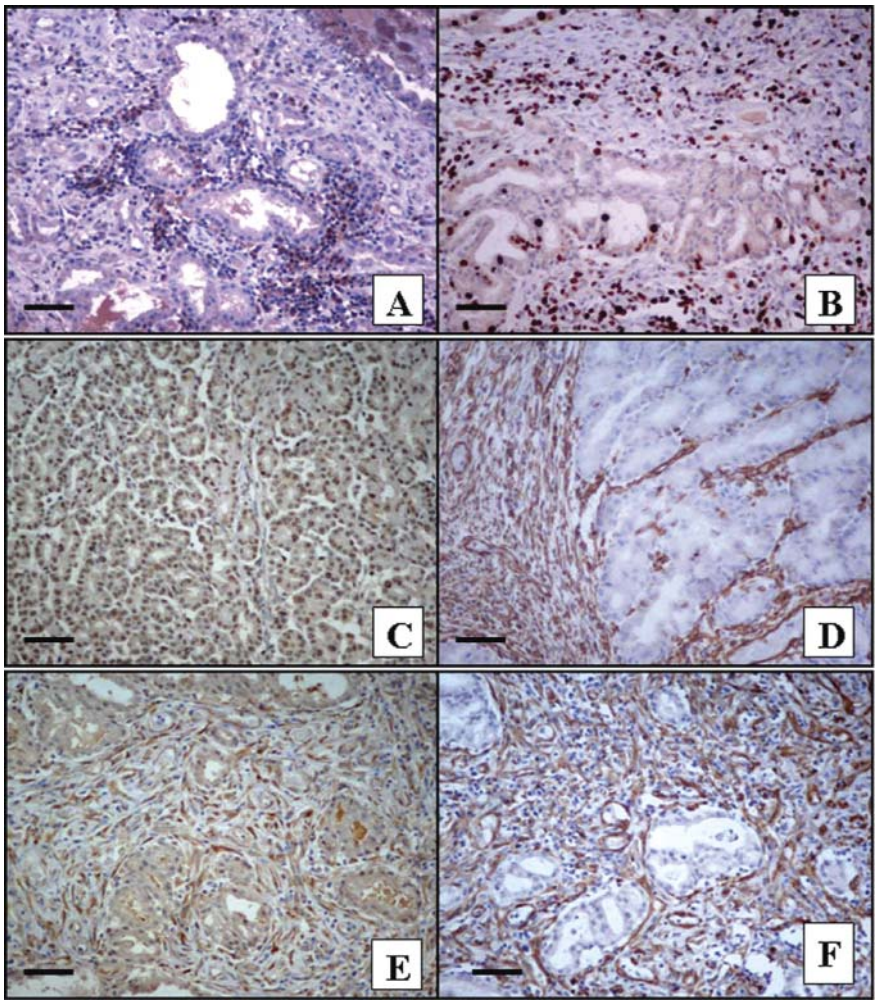

Fig 4. Nasal tumor tissue, **A)** Caspase positive immunoreaction of the ENA; **B)** Ki-67 positive immunoreaction of the ENA; **C)** PCNA positivity of the ENA; **D)** Vimentin expression of the ENA **E)** NSE expression of ENA; **F)** SMA positive reaction of ENA, ABC method, with DAB, Harris hematoxylin counter stain, Bars = 100 µm

Şekil 4. Nazal tümör dokusu, **A)** ENA'nın Caspase pozitif immuno reaksiyononu; **B)** ENA'nın Ki-67 pozitif immuno-reaksiyonu; **C)** ENA'nın PCNA pozitifliği **D)** ENA'nın Vimentin reaksiyonu **E)** ENA'nın NSE reaksiyonu; **F)** ENA'nın SMA pozitif reaksiyonu, ABC metodu, DAB, Harris hematoksilen karşıt boyaması, Barlar = 100 µm

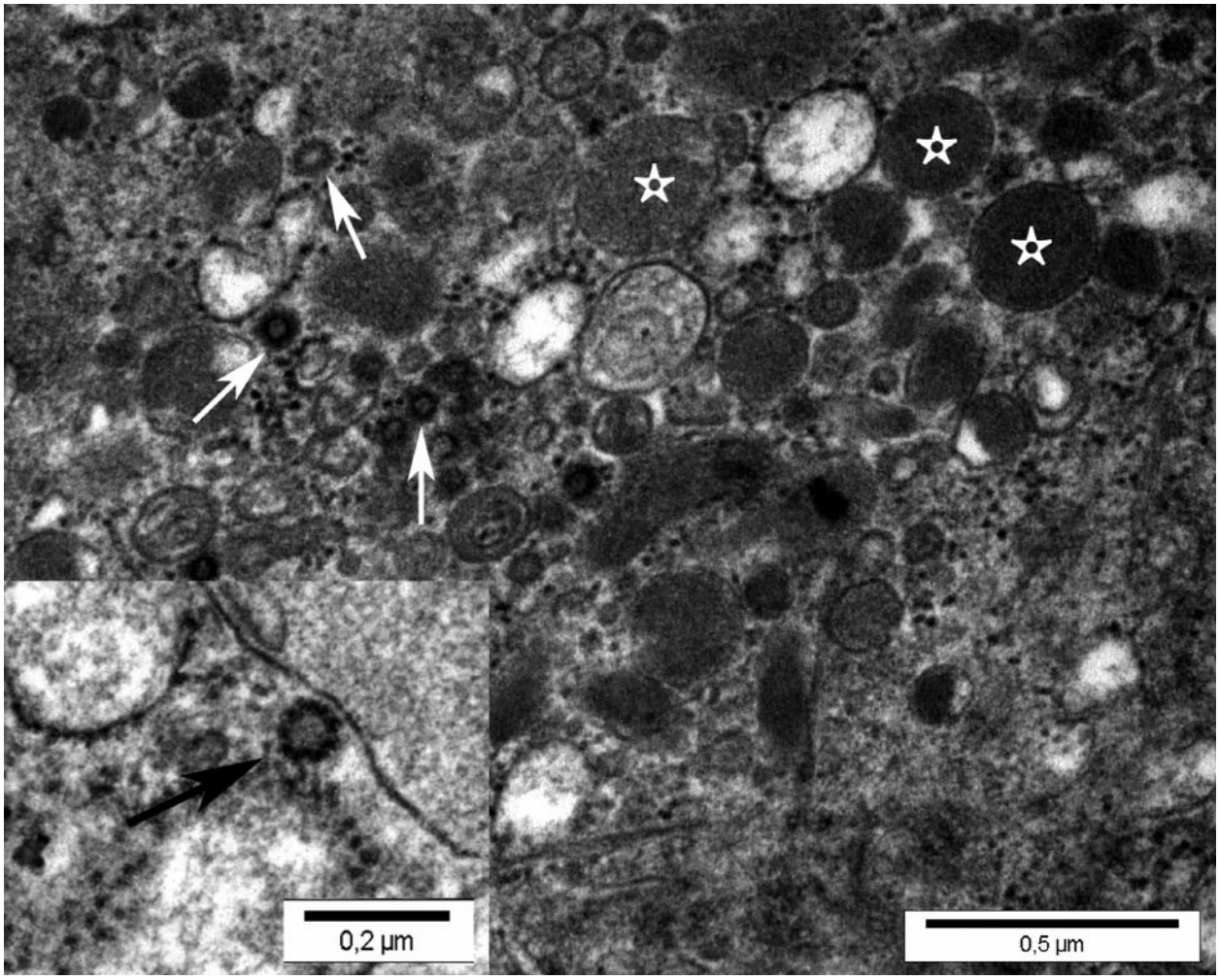

Fig 5. Ultrastructural appearance of the viral particles (arrows) and secretory granules (stars) in cytoplasm of the tumor cell. Inset: higher magnification of viral particle *(arrow)*

Şekil 5. Viral partiküllerin elektron mikroskobik görünümü, tümör hücrelerinin sitoplazmalarında viral partiküller (oklar) ve salgı granülleri (yıldızlar). İç resim: Viral partiküllerin büyük büyütmesi *(oklar)*

DISCUSSION

Clinically in ENA cases, nasal and respiratory signs predominate with affected animals having profuse seromucinous nasal exudate, dyspnea, stertorous breathing, and coughing. Especially severely affected animals have open-mouthed breathing, exophtalmos, and facial deformity are possible complications [9,10]. At necropsy, the tumors may be unilateral or bilateral. They present as polipoid to confluent masses originated from the ethmoid region with strong association with etmoidal mucosa. In most cases, they have a granular or papillary surface covered by mucus. In severely affected animals, tumoral masses cause local bone destruction, invade frontal or maxillary sinuses, and invade gingiva and orbit. Occasionally, tumor tissue can protrude through the anterior nares or into the nasopharynx [3,9,10]. Clinico-pathological manifestation of the disease was in all our cases similar to those described previously. But there were no bone deformities observed in this study. Some authors reported that the average age of animals at diagnosis was 4.5 years in sheep; but the animals were younger in our study in contrast to previous reports [10,12]. Possible cause of the effected animals with younger ages may be due to closed and crowded herd in present study and contagious behavior of the disease. Rearing of the different aged goats in the same shelter may be predisposing factor to introduce the disease in younger ages.

Numerous authors reported that the tumors have been called adenomas or adenocarcinomas at the histopathological examination. The most common subtype is papillary type. In addition, mucinous, tubular, and acinar patterns are seen. The tumors in goats are interpreted to be well-differentiated (low grade) carcinomas, also

with papillary, tubular, or acinar patterns [5,11,12]. Although the histopathological appearance of the tumor suggests a benign behavior, in some cases, the progressive infiltrative and destructive growth indicative of a well-differentiated adenocarcinoma may be seen [5,11]. ENA is typically invasive, but does rarely metastasize [3,5,10]. Although the general histopatological appearance of the tumors resembles the benign behavior, proliferation indices throughout the tumor tissue may have malignant potential in this study.

Breed and sex or genetic predisposition was not reported in any previous study both in sheep and goats [8,11,13,14]. In our study all of the animals were goat *(Capra hircus)* and the contagious nature of the disease was more important than genetic predisposition because of the crowded herd and improper management. The owner stated a more than 10-year history of the disease in the flock, therefore it was impossible to find out how ENA was introduced into the flock. Severe clinical signs and death were reported in previous studies [8,13,14]. Similar progressive respiratory clinical signs were described for of the animals in our study, an observation that is most likely because of the large size of the tumors. Because of the inappatence, severe emaciation was seen in affected animals. The finding of unilateral tumors resembled those of previous reports that the occurrence of unilaterally arising tumors prevail over bilateral ones [8,9,16]. There is no information regarding the preference of localization of the tumors for the right or left nasal cavity. Clinical and pathological findings were very similar in our study compared with the previous reports [12,14,16]. No previous reports are available for hematological data before this study [9,12-15]. Lymphocytosis was the marked changes in the hematological analysis; this result attributed the chronic behavior and viral etiology of the disease.

Ki-67 is a nuclear antigen associated with cell proliferation and is present throughout the active cell cycle but absent in resting cells [16]. In this study, Ki-67 was strongly expressed in both epithelial and interstitial cells in ENA, indicating high proliferation of these cells. PCNA protein is one of the central molecules responsible for decisions of life and death of the cell [17]. We observed strong nuclear expression of PCNA in epithelial and interstitial cells of ENA indicating proliferation of both epithelial and mesenchymal components of the tumor. NSE is a glycolytic enzyme enolase and produced in central and peripheral neurons and malignant tumors of neuroectodermal origin [18]. The present study showed that NSE was strongly expressed in ENA indicating presence of neuronal component in ENA. AFP is a major plasma protein produced by the yolk sac and the liver during fetal life. AFP expression in adults is often associated with hepatoma or teratoma [19]. This study showed that, ENA was negative for AFP no indication was observed undifferentiated cell proliferation. The cytokeratins are a family of water-soluble proteins that form the cytoskeleton of epithelial cells [20]. PCK was strongly expressed by epithelial components of ENA in present study. CEA is one of the best characterized tumor-associated markers [21,22]. CEA was slightly expressed in ENA in this study indicating possible cell differentiation. S100 belongs to the family of calcium binding proteins such as calmodulin and troponin C it expressed in schwannomas, ependymomas, astrogliomas, and almost all benign and malignant melanomas and their metastases [23]. This study showed that it is expressed in ENA also. SMA shows no cross-reaction with actin from fibroblasts, striated muscle, and myocardium. Myo-epithelial cells in breast and salivary glands are also stained as they also contain this actin. This is useful for identifying tumors arising from smooth muscle and myoepithelial cells [24]. SMA was strongly expressed in ENA indicating the presence of myoepithelial cell proliferation in ENA. Vimentin is the main intermediate filament protein in mesenchymal cells, and therefore is of value in the differential diagnosis of undifferentiated neoplasms [25]. Because of the abundant mesenchymal cell proliferation, vimentin was abundant and strongly expressed in ENA in present study. GCDFP is a pathologic secretion from breast composed of several glycoporteins, including GCDFP-15. It is considered to be a marker of apocrine differentiation [26]. Slight positive reaction was seen with GCDFP in ENA in this study. In this study, caspase-3 was strongly expressed in ENA indicating apoptotic activity of this tumor's cells. All of this finding showed that ENA has both epithelial and mesenchymal cell proliferation. High apoptotic activity attributed the high proliferation rate in this tumor. At the same time this is the first immunohistochemical study about the origin of the proliferated cells in ENA. For to evaluating the origin of the cells and tumor behavior 11 different markers were used.

The proliferation index, determined by cell cycle-related markers such as Ki-67 and PCNA, has prognostic value in human and animal carcinomas [27]. Some researchers have noted the value of these nuclear antigens in the prediction of disease-free survival and overall survival as independent prognostic factors in multivariate analyses [28]. In our study, Ki-67 and PCNA were expressed in ENA, and a high proliferation index was observed but no metastasis was seen in our cases.

To examine the presence of viral particles, TEM analysis of tumor samples was performed. Numerous

retroviral particles were seen in the cytoplasm of the cells. The size, morphological appearance and the location of spherical retrovirus-like particles were also in agreement with previous reports [10,15].

This study showed that the ENA consists of both epithelial and mesenchymal cell proliferation. Electron microscopical study supported the viral etiology of the tumor. Because of the ENA is a contagious disease, it can cause high economical losses in goat economy.

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