

The expression of caspase-3, caspase-7, caspase-9 and cytokeratin AE1/AE3 in goats with enzootic nasal adenocarcinoma: an immunohistochemical study

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ABSTRACT: The aim of this study was to examine the expression of caspase-3, caspase-7, caspase-9 and cytokeratin AE-1/AE-3 using the avidin-biotin complex (ABC) immunoperoxidase technique in 20 goats with enzootic nasal adenocarcinoma (ENA). Clinically, dyspnoea and nasal discharge were observed in all cases. Macroscopically, polypoid and sessile masses were seen in the ethmoidal area. At the histopathological examination, tubular, papillary and mixed patterns of ENA were diagnosed. Immunohistochemically, strong positive reactions were generally seen for caspase-3, while strong to moderate and slight reactions were observed for caspase-7 and caspase-9 in the cytoplasm of the tumour cells. Positive reactions for cytokeratin AE-1/AE-3 were only seen in epithelial cells. In addition, the causative agent of ENA, retrovirus, was detected immunohistochemically in tumour cells.

Keywords: enzootic nasal adenocarcinoma; caspase-3; caspase-7; caspase-9; cytokeratin AE-1/AE-3; goat

Enzootic nasal adenocarcinoma (ENA) is a chronic and contagious disease, characterised by neoplastic proliferation of secretory epithelial cells in the ethmoidal region of the respiratory tract and it have been reported in sheep, goats, cattle, pigs, horses and buffaloes (Wilson and Dungworth 2002; De las Heras et al. 2003). Genetic, breed and sex predispositions were not reported in any published study (McKinnon et al. 1982; De las Heras et al. 1998). The causative agent of ENA is a simple retrovirus and it induces unilateral or bilateral neoplastic growth in the mucosal nasal glands of the ethmoidal area (De las Heras et al. 1993, 2003).

Caspases are interleukin-1 β -converting enzyme family proteases and play a vital role in the induction of apoptosis. While some caspases are initiators (caspase-1, -2, -4, -5, -8, -9 and -10), others are effectors (caspase-3, -6 and -7) (Harvey and Kumar 1998; Stegh and Peter 2001). In mammals, apoptosis is mediated by the extrinsic pathway or intrinsic pathway. The extrinsic pathway is initiated by ligation of death receptors leading to activation of caspase-8, while the intrinsic pathway is activated by cellular stress and leads to activation of caspase-9. Caspase-3 is a downstream effector

protease in the each of these pathways (Salvesen and Dixit 1997; Thornberry and Lazebnik 1998; Denault and Salvesen 2002). Overexpression and loss of expression of different caspases has been reported in some human tumours such as hepatocellular carcinomas and prostate cancers (Winter et al. 2001; Persad et al. 2004; Yoo et al. 2010). In addition, caspase-3 activity indicative of apoptosis has been reported in tumour cells of enzootic nasal adenocarcinoma (Ozmen et al. 2010). However, caspase expression is not fully characterised in animal tumors, especially in ENA. In this study, retrovirus antigen, caspase-3, caspase-7, caspase-9 expression, and cytokeratin AE-1/AE-3 reaction were immunohistochemically examined in twenty goats naturally affected with enzootic nasal adenocarcinoma in Turkey.

MATERIAL AND METHODS

Tissue Samples and histopathology. Samples from ENA cases (12 male and eight female hair goats (*Capra hircus*)) were taken from the archive of the Department of Pathology, Faculty of

Veterinary Medicine, University of Mehmet Akif Ersoy, Burdur, Turkey. The ages of the goats were between one and seven years. Macroscopically, unilateral (13 cases) and bilateral (seven cases) tumour masses were found in the nasal cavity of the goats. For histopathological examination, tumour samples were fixed in 10% neutral buffered formalin. After routine procedures, sections were stained with Haematoxylin-Eosin (H&E), and examined microscopically.

Immunohistochemistry. The routine streptavidin biotin technique was used for the detection of Retrovirus antibody [Retrovirus, Idexx, Maine USA (ready for use)]; Caspase-3 [Neomarker, Caspase-3 (CPP32) Ab-4, Rabbit Polyclonal Antibody (1/100 dilution)]; Caspase-7 [Santa Cruz, Caspase-7, sc-8512, Goat Polyclonal Antibody (200 µg/ml)]; Caspase-9 [Abcam, Anti-Caspase-9 Rabbit Polyclonal Antibody, ab52298, (1/100 dilution)]; Cytokeratin AE-1/AE-3 [Neomarker, Keratin, Pan Ab-1, Clone AE1/AE3, Mouse Monoclonal Antibody (1/100 dilution)] using immunohistochemistry. For the immunohistochemical staining, tumour sections were routinely processed according to the manufacturer's recommendations. These sections were counterstained with Harris haematoxylin. Non-tumour ethmoidal tissues provided from five healthy goats were used as control. To evaluate the degree of the immunohistochemical reaction of the tumour cells semiquantitative analysis was performed using an arbitrary visual scale with a grading score ranging from (–) to (+++) as follows: (–) = negative, (+) = weak staining, (++) = moderate staining, (+++) = strong staining.

RESULTS

Clinically, respiratory distress, seromucous exudates around the nares and cachexia were seen in all of the effected goats. Macroscopically, tumour masses were found in the ethmoidal area of the nasal cavity (Figure 1). These masses were irregular in shape, soft to firm, polypoid (1–2.5 cm in length), and sessile (0.5–3 cm in diameter) and covered with seromucous exudate. The surface and cut surface of the masses were homogeneous and pinkish-white in colour. The gross and microscopic examination did not reveal metastasis in any organ.

Microscopically, enzootic nasal adenocarcinoma was diagnosed according to the characteristic microscopic features. Case findings were similar

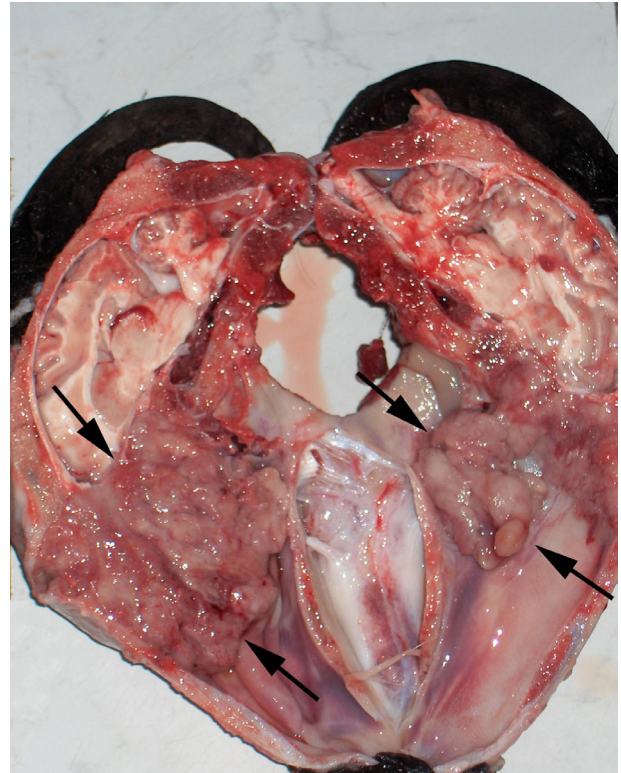


Figure 1. Gross appearance of ENA. Tumour mass fills the nasal cavity (arrows)

in all examined tumours. The tumour cells were arranged in tubular, papillary and mixed patterns (Figure 2). The tubular pattern was seen in nine cases; the papillary pattern was noted in 8 cases; mixed pattern was observed in three cases. The tumour masses consisted of uniform tumour cells and were well vascularised. Marked atypia in the tumour cells was seen but mitotic figures were not common. The tumour cells had a cuboidal shape

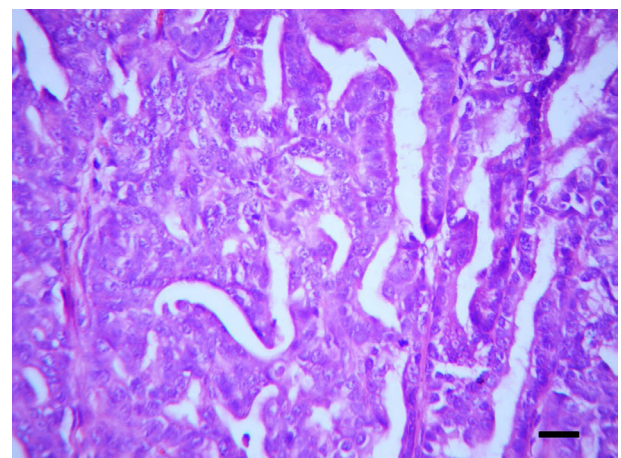


Figure 2. Histopathological appearance of the mixed type of ENA. Haematoxylin and eosin; bar = 100 µ

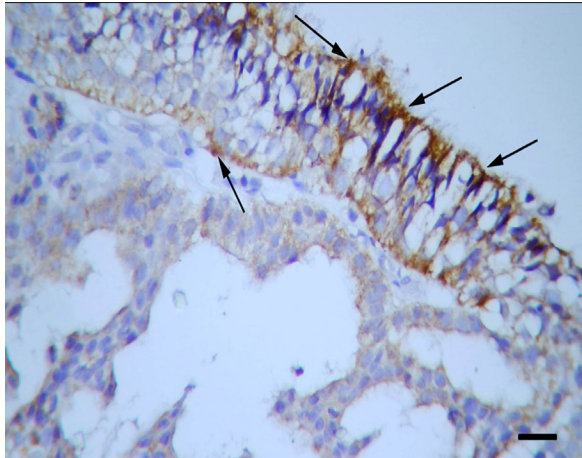


Figure 3. Immunolabeling of retrovirus-related antigen on the surface of secretory epithelial cells of the ethmoid region (arrows). Streptavidin biotin method. Harris haematoxylin counterstain; bar = 100 μ

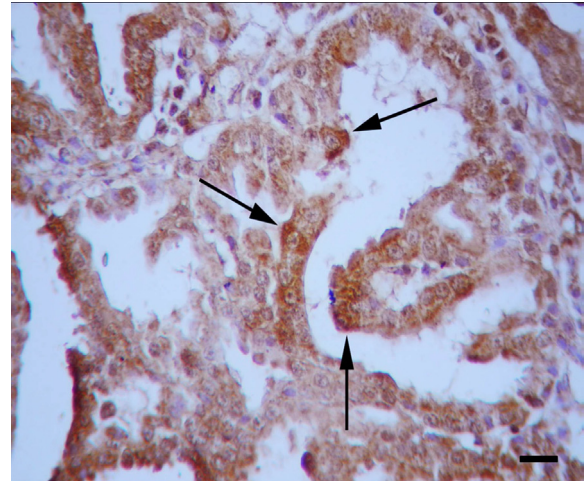


Figure 4. Caspase-3 expression in the cytoplasm of tumour cells (arrows). Streptavidin biotin method. Harris haematoxylin counterstain; bar = 100 μ

with large round nuclei. Metastasis and invasion were not detected. In the tumour stroma, lymphocytes, neutrophils, plasma cells and macrophage infiltrations were commonly seen. In some areas of the tumours, necrosis and fibrinopurulent exudate were also observed.

Immunohistochemically, strong positive reactions for retrovirus-related antigens were found especially on the surface of secretory epithelial cells of ethmoid region (Figure 3). Immunohistochemical staining for caspase-3 revealed strong (+++) staining in 16 cases (Figure 4), and moderate (++) staining in four cases in the cytoplasm of tumour cells. Immunostaining of caspase-7 gave a moderate signal (++) in seven cases (Figure 5) and was weak (+) in 13 cases.

Immunoreactivity of caspase-9 was strong (+++) in three cases, moderate (++) in 12 cases (Figure 6), and weak (+) in five cases in the cytoplasm of tumour cells. Immunostaining of cytokeratin AE-1/AE-3 was strong (+++) in eight cases and moderate in 12 cases in epithelial cells. Control sections showed negative or weak immunostaining confirming the specificity of the primary antibodies.

DISCUSSION

ENA arises from secretory epithelial cells in the ethmoid turbinate of the respiratory tract (De las Heras et al. 2003). Clinically, profuse seromucous

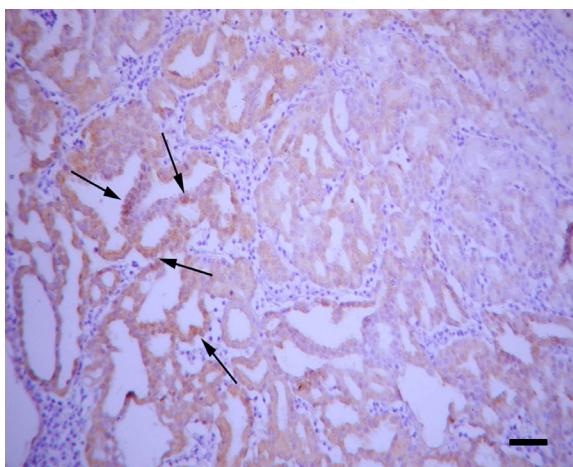


Figure 5. Caspase-7 immunoreaction in the cytoplasm of tumour cells (arrows). Streptavidin biotin method. Harris haematoxylin counterstain; bar = 200 μ

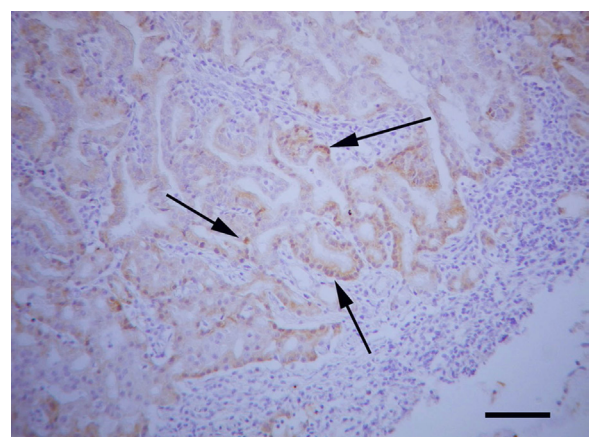


Figure 6. Caspase-9 immunostaining in the cytoplasm of tumour cells (arrows). Streptavidin biotin method. Harris haematoxylin counterstain; bar = 200 μ

nasal exudates, dyspnoea and stertorous breathing have been noted in affected animals which lose weight and eventually die (De las Heras et al. 1991a). In this study, the clinical findings included respiratory distress, seromucous exudates around the nares and cachexia. These findings are similar to those reported in the veterinary literature (De las Heras et al. 1991a; Svava et al. 2006; Ozmen et al. 2010). Histopathologically, ENA is classified as having papillary, mucinous, tubular and acinar patterns, but in goats carcinomas with papillary, tubular or acinar patterns are also interpreted to be well differentiated (Wilson and Dungworth 2002). In this study, histopathologically, ENA was classified as having tubular, papillary and mixed patterns. According to the histopathological findings, the tumour was classified as a low grade adenocarcinoma. No metastases were detected in regional lymph nodes, brain or other organs and tissues.

In recent years, the aetiology of ENA has been reported widely. According to these reports, viral-like particles, compatible with a type-D retrovirus, are the cause of ENA. For detection of the causative agent of ENA, different techniques such as electron microscopy, immunohistochemistry, polymerase chain reaction (PCR), reverse transcriptase-PCR and western blotting have been used (De las Heras et al. 1991a,b, 1998, 2003; Cousens et al. 1996, 1999; Ozmen et al. 2010). In the present study, Retrovirus-related antigens were detected using the streptavidin-biotin complex peroxidase technique on the surface of secretory epithelial cells of the ethmoid region.

Cytokeratin is expressed in epithelial cells and constituted the cytoskeleton of these cells (Chu and Weiss 2002). In the present study, in all cases a positive immunoreaction was observed for cytokeratin AE1/AE3 immunostaining. This indicates an epithelial origin of the tumour cells in ENA.

Apoptosis is mediated by a family of intracellular cysteine proteases named caspases (Ghavami et al. 2009). Caspases are normally present in healthy cells as inactive precursor enzymes (Taylor et al. 2008), and are divided into initiator caspases (caspase-1, -2, -4, -5, -8, -9 and -10) that are responsible for triggering caspase activation; and effector caspases (-3, -6, -7) that are responsible for the breakdown of the cell in the later stages of apoptosis (Harvey and Kumar 1998; Stegh and Peter 2001; Logue and Martin 2008). In mammals, there are two main pathways for the induction of apoptosis, extrinsic and intrinsic (Salvesen and Dixit 1997; Denault and Salvesen 2002). The extrinsic pathway is induced

by death ligands and death receptors such as Fas and results in activation of caspase-8. By contrast, the intrinsic pathway is regulated by the Bcl-2 family which causes an alteration in the mitochondrial membrane potential leading to mitochondrial membrane permeabilisation and downstream activation of caspase-9 (Reed 2000). A downstream effector of apoptosis in both pathways is caspase-3 (Salvesen and Dixit 1997; Denault and Salvesen 2002). Previous immunohistochemical studies on human cancers, like breast cancers and gastric adenocarcinomas, have shown that expression of caspase-3 and caspase-9 in tumour cells is higher than in normal cells (Yoo et al. 2002). According to a previous study, expression of caspase-3 was found in more than 50% of tumour cells and caspase-9 in more than 10% of tumour cells in human anaplastic astrocytomas and glioblastomas (Bodey et al. 2004). Similarly, in stomach cancer both initiator and effector caspases are highly expressed (Yoo et al. 2002). In this study, strong immunostaining for caspase-3 was found in 16 cases and moderate immunostaining was seen in four cases. Caspase-9 expression was strong in three cases, moderate in 12 cases and weak in five cases. These findings indicate that apoptotic molecules of the mitochondria-dependent death pathway may play important roles in ENA.

Caspase-3 and caspase-7 are effector caspases and are highly homologous to each other (Degterev et al. 2003). However, in this study, the expression levels of caspase-3 and caspase-7 were not observed to be similar to each other. While caspase-3 gave strong immunoreactivity in 16 cases and moderate in four cases, immunoreactions of caspase-7 were moderate in seven cases and weak in 13 cases. Although caspase-3 and caspase-7 are highly homologous to each other, caspase-3 is more significant for apoptotic signaling in ENA compared to caspase-7.

In conclusion, to the best of our knowledge, this is the first immunohistochemical study to report the expression of caspase-7 and caspase-9 in goats with ENA.

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