

EXPRESSION OF NECTIN-4 AS A POTENTIAL BIOMARKER IN ENZOOTIC NASAL ADENOCARCINOMA OF GOATS

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(Received 5 December 2014; accepted 28 October 2015)

The aim of this study was to test nectin-4 by immunohistochemistry as a potential biomarker in enzootic nasal adenocarcinoma (ENA) of goats. Twenty-four archival ENA case samples [from 14 male and 10 female hair goats (*Capra hircus*)] were used. The samples were stained with haematoxylin and eosin (HE). Nectin-4 expression was studied by immunohistochemistry. By microscopy, tubular, papillary, and mixed patterns of ENA were diagnosed in the cases. Immunohistochemically, the tumours showed moderate nectin-4 expression (++) in 14 cases (58.3%), strong expression (+++) in five cases (20.8%), and weak expression (+) in three cases (12.5%), while two cases (8.3%) were negative. Normal nasal tissues were not stained with nectin-4. The results suggest that nectin-4 may be used as a valuable biomarker of ENA.

Key words: Goat, nectin-4, enzootic nasal adenocarcinoma, pathology, immunohistochemistry

Enzootic nasal adenocarcinoma (ENA) is a contagious tumour of the nasal mucosal glands in sheep and goats. The disease naturally occurs in many countries around the globe, such as India, Turkey, and Slovenia (Rajan et al., 1980; De las Heras et al., 2003; Svava et al., 2006; Ozmen et al., 2010). The clinical signs of ENA consist of profuse seromucous nasal exudate, dyspnoea, and weight loss (De las Heras et al., 1991; Svava et al., 2006; Ozmen et al., 2010). Respiratory diseases of sheep and goats such as parainfluenza, ovine pulmonary adenomatosis and various tumour-like nasal polyps (adenopapillomas) should be considered in the differential diagnosis of ENA (Chakraborty et al., 2014). Histologically, ENA is classified as a low-grade adenocarcinoma according to the latest classification system, and its diagnosis is confirmed by histopathological examination and immunohistochemical analysis (De las Heras et al., 1998; Svava et al., 2006; Ozmen et al., 2010; Aydogan et al., 2013). Enzootic nasal tumour virus (ENTV) is a simple retrovirus, which causes unilateral or bilateral tumour

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growth of the mucosal glands in the ethmoidal segment of the nasal cavity (De las Heras et al., 1993, 2003).

Nectin was first described in 1960 (Abrams et al., 1960), and it is required for the attachment of the membrane enzyme, ATPase (Baron and Abrams, 1971). The nectin family belongs to the Ca^{2+} -independent immunoglobulin-like molecules and is comprised of four members named nectin-1, -2, -3, and -4. This family plays a role in cell-cell adhesion that regulates the formation of adherent junctions and tight junctions by epithelial cells in a homophilic and a heterophilic manner (Fabre-Lafay et al., 2007; Takai et al., 2008). The cytoplasmic region of nectins binds afadin, which directly connects to the actin cytoskeleton (Takai et al., 2003). Nectin-4 is a type I transmembrane glycoprotein which has recently proven to be overexpressed in human breast carcinoma and is thus considered a specific tumour-associated marker, as the normal mammary gland lacks nectin-4 expression (Fabre-Lafay et al., 2007). In non-small cell lung cancer, a high level of nectin-4 expression has been shown to be associated with a poor prognosis (Takano et al., 2009).

In spite of recent studies on nectin-4 expression in cancer, the biological and clinical importance of nectin-4 is not fully understood in different types of cancer in humans and animals. The aim of the present study was to evaluate nectin-4 immunoreactivity as a potential tumour marker in ENA tissues. To the best of our knowledge, the expression of nectin-4 has not been studied and reported in ENA so far.

Materials and methods

Tissue samples

The material of this study consisted of 24 formalin-fixed, paraffin-embedded archival ENA case samples [14 male and 10 female hair goats (*Capra hircus*)]. Fixation time in 10% neutral formalin solution did not exceed 48 h. The age of the goats was between one and eight years.

Histopathology and immunohistochemistry

For histopathological examination, tissue samples were processed routinely for light microscopy. Five- μm sections were taken from paraffin-embedded tissues and stained with haematoxylin and eosin (HE).

Selected ENA sections were stained immunohistochemically in order to elucidate the expression of nectin-4 [Millipore, Anti-Nectin-4/PVRL4, clone N4.61, Monoclonal Antibody (1:200 dilution)]; cytokeratin AE1/AE3 [Neomarker, Keratin, Pan Ab-1, Clone AE1/AE3, Mouse Monoclonal Antibody (1:100 dilution)]; retrovirus antibody [Retrovirus, Idexx, Maine, USA (ready for use)] using a routine streptavidin–biotin–peroxidase technique according to the manufac-

turer's recommendations [Abcam, mouse and rabbit specific HRP (ABC) detection IHC kit (Ab93677)]. The colour reaction was performed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit [Abcam (ab64238)]-H₂O₂. All sections were counterstained with Harris' haematoxylin, washed in water, and cover slips were applied with mounting media. Immunohistochemical reactions were controlled to evaluate the specificity of the labels. Haematoxylin and eosin staining was performed and used as a reference of tissue cytoarchitecture. On each section, different areas were examined and immunopositive reactions were demonstrated by the presence of brown cytoplasmic staining. The primary antibody was omitted for negative controls. For positive control, human placental tissue constitutively expressing nectin-4 was used. Semiquantitative data analysis was performed with scores for nectin-4 ranging as '-' for negative staining or when none of the cells were stained; '+' for weak staining or when less than 20% of the cells were positive; '++' for moderate staining or when 20–59% of the cells were positive; and '+++' for strong staining or when more than 60% of the cells were positive.

Results

Clinically and macroscopically, cachexia, dyspnoea, and nasal discharge (Fig. 1A) were seen. The tumours located in the ethmoidal area of the nasal cavity were irregular and oval in shape, firm to the touch, polypoid (1 to 3.25 cm in length), sessile (0.3–3.5 cm in diameter), and covered with seromucous exudate (Fig. 1B). The surface and cut surface of the tumour masses were homogeneous or granular, and greyish or pinkish-white in colour. The expansion of tumours in the nasal cavity was unilateral in 13 animals and bilateral in 11 animals. No sign of secondary sinusitis was seen in the goats. Macroscopic and microscopic examination did not show metastasis to any organ in any of the cases.

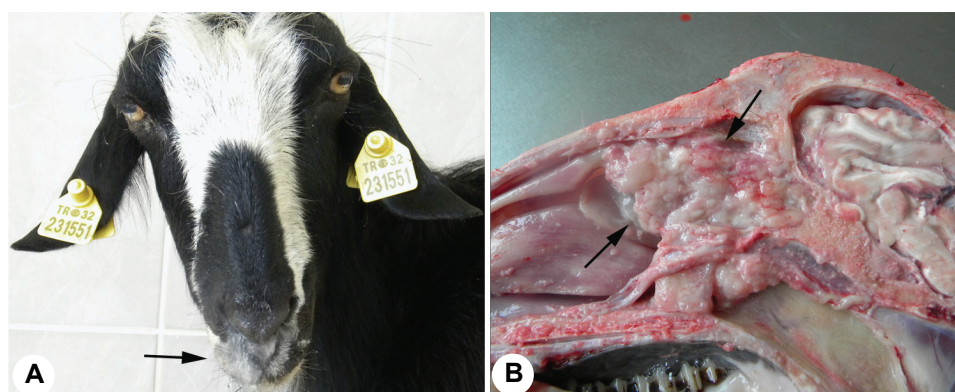


Fig. 1A–B. Gross appearance of enzootic nasal adenocarcinoma (ENA). A. Goat with nasal discharge (arrow); B. Tumour mass in the nasal cavity (arrows)

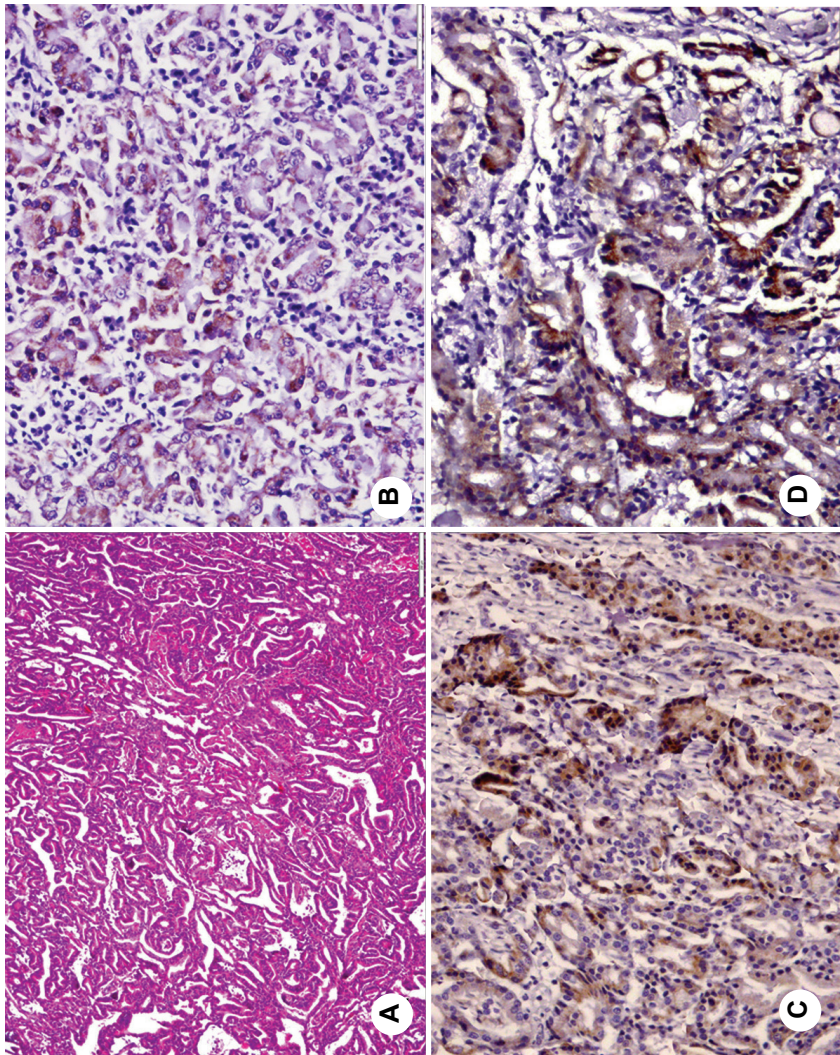


Fig. 2A–D. Histopathological appearance and staining patterns of nectin-4 in ENA. *A.* Haematoxylin and eosin stained section of mixed structure in ENA, Bar = 200 μ m; *B.* Multifocal weak cytoplasmic expressions of nectin-4, Bar = 50 μ m; *C.* Multifocal moderate cytoplasmic expressions of nectin-4, Bar = 50 μ m; *D.* Multifocal strong cytoplasmic expressions of nectin-4 in the nasal mucosal glands, Bar = 50 μ m. Streptavidin–biotin method, Harris' haematoxylin counterstain

The histopathological findings were characteristic of ENA and were similar in all cases examined. Tubular, papillary and mixed structures (Fig. 2A) were seen in the cases. Tubular structure was seen in nine cases, papillary structure was noted in 10 cases, while mixed structure was observed in five cases. The tumour cells were mostly uniform and cuboidal in shape with round or oval, hyperchromatic nuclei. Mitotic figures were uncommon, and the mitotic index was 2 per 10 high power fields. In seven cases, infiltrations by numerous lymphocytes, plasma cells and macrophages were noted in the stroma. In four cases, areas of coagulation necrosis were observed. There was no histopathological indication of secondary bacterial infection, vascularisation or intratumoural bleeding.

Immunohistochemically, normal nasal tissues were not stained with nectin-4. In contrast, in the tumours the immunohistochemical results of nectin-4 showed that expression was weak (+) in three cases (12.5%) (Fig. 2B), moderate (++) in 14 cases (58.3%) (Fig. 2C), and strong (+++) in five cases (20.8%) (Fig. 2D); there was no expression (–) in two cases (8.3%). Nectin-4 immunoreactions were heterogeneous and multifocal in the tumour areas. Positive immunoreactions of nectin-4 were seen in the cytoplasm of tumour cells and secretory epithelial cells of the ethmoid region. In all cases, cytokeratin AE1/AE3 gave a positive immunoreaction in tumour cells and secretory epithelial cells. In addition, retrovirus-related antigen was expressed in the cytoplasm of tumour cells and on the surface of secretory epithelial cells. Marked nectin-4 immunoreactions were more frequently observed in tumours of big size than in small ones.

Negative immunostaining was seen in the negative control sections confirming the specificity of the primary antibodies.

Discussion

Enzootic nasal adenocarcinomas of sheep and goats originate from the ethmoid turbinates and show a rising prevalence worldwide (De las Heras et al., 2003; Fox et al., 2011). This tumour is invasive, mainly of low grade, and it rarely forms metastases (De las Heras et al., 1991, 2003). For a suspect case of ENA on cytopathology or histopathology, additional diagnostic methods such as immunohistochemistry and/or polymerase chain reaction (PCR) are required for a precise diagnosis (Stowe et al., 2012). However, different markers have not been fully studied immunohistochemically to prove their usefulness for establishing the precise diagnosis and prognosis of ENA. In this study, nectin-4 expression was evaluated in nine tubular, 10 papillary and five mixed ENA tissues immunohistochemically. In addition, in the cases of this study, the causative agent of ENA was demonstrated by retrovirus-related antigen, and the epithelial origin of the tumour was immunohistochemically confirmed using an epithelial marker named cytokeratin AE1/AE3.

Nectins are a newly characterised family of cell adhesion molecules homologous to PVR/CD155 (the poliovirus receptor) (Lopez et al., 1995; Reymond et al., 2001). The nectin family, forming adherent junctions in epithelial cells, consists of four members (nectin-1, nectin-2, nectin-3, and nectin-4) (Ikeda et al., 1999; Takai et al., 2008). Normally, nectins 1, 2 and 3 show abundant expression in adult tissues, but nectin-4 is primarily expressed in the embryo and the placenta (Reymond et al., 2001; Fabre-Lafay et al., 2007). Nectin-4 has been shown to be a specific tumour-associated marker for ductal mammary carcinoma, lung adenocarcinoma and ovarian carcinoma in humans (Fabre-Lafay et al., 2007; Takano et al., 2009; Derycke et al., 2010). Studies in humans and experimental animal models have shown that nectin-4 is a valuable biomarker for evaluating tumour progression and the metastatic status of carcinomas (Fabre-Lafay et al., 2007; Oshima et al., 2013). In this study, we used nectin-4 in ENA, which is a type of carcinoma. In spite of a lack of expression of nectin-4 in normal ethmoidal tissues of goats, there was a strong cytoplasmic nectin-4 immunoreaction in five cases, a moderate cytoplasmic immunoreaction in 14 cases, and a weak cytoplasmic immunoreaction in three cases of ENA. In accordance with previous reports in the literature, in this study nectin-4 showed positive immunoreaction in a type of carcinoma (ENA) and in adult tissues. However, immunohistochemical tests for nectin-4 were negative in the tissues of normal adult goats. This study showed that marked nectin-4 reactions were more frequently observed in big-size tumours than in small ones.

In conclusion, this study provides the first results suggesting that nectin-4 can be used as a valuable biomarker in ENA. These preliminary results have revealed that there is a need for further nectin-4 studies in ENA for establishing the diagnosis and prognosis of this tumour, and nectin-4 could also serve as a potential target for anti-tumour therapy.

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