Immunohistochemistry of poorly differentiated squamous cell carcinoma in a Slovak Warmblood gelding – a case report

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Received October 22, 2022
Accepted December 12, 2024

Abstract

This case report describes the immunohistochemical examination of poorly differentiated squamous cell carcinoma (SCC) in a sixteen-year-old Slovak Warmblood gelding. Clinical examination detected a dull, painless percussion in frontal and maxillary sinuses. The left arch of the mandible had an irregular surface with a painful tap. The submandibular lymph nodes were bilaterally asymmetric with left sided hypertrophy, smooth to palpation, hard and painless. The tumorous mass was overgrown through the laryngeal mucosa, not observed in the oral cavity. The patient was in a poor clinical condition and was euthanized six months after the first clinical signs were observed. Histological examination of the samples taken from the tumorous mass showed the presence of proliferated cells of epithelial origin. The histological finding of the mandible showed focal osteolysis with multinuclear cells, which were bordered by mesenchymal tissue and a moderately dense collagen. Immunohistochemically, tumorous cells were positive for p63 and cytokeratin AE1/AE3. Protein p63 confirmed the metastatic spread of the tumour and was demonstrated to be a suitable marker for poorly differentiated SCC.

Equine, histology, immunohistochemistry, p63

Squamous cell carcinoma (SCC) is one of the most common skin tumours in horses and other equids (Armando et al. 2020). The incidence of SCC is within 7–37% of skin lesions (Sykora and Brandt 2017). A tumour of epithelial origin primarily arises on the skin or mucous membrane. Predisposing sites for SCC are nonpigmented skin or mucocutaneous junctions (Van den Top et al. 2008). Recently, the larynx and pharynx were demonstrated as SCC affected localities with progression in horses (Armando et al. 2020). Equine SCC is known to be invasive with metastasing to the regional lymph nodes in up to 30% of the cases (Jones 1994). Immunohistochemical analysis of tumours is helpful mainly in diagnosing the low-grade ones.

This case report describes the histological and immunohistochemical evaluation of cells of laryngeal low-differentiated SCC in a Slovak Warmblood gelding.

Case presentation

Clinical examination of a sixteen-year-old Slovak Warmblood gelding detected a dull, painless percussion in frontal and maxillary sinuses. The left arch of the mandible had an irregular surface with a painful tap. The submandibular lymph nodes were bilaterally asymmetric with left sided hypertrophy, smooth to palpation, hard and painless. The patient was in poor clinical condition. After receiving an unfavourable prognosis, the owner declined treatment for the horse and opted for euthanasia. The horse was euthanized six months after the finding of first clinical signs. A complete diagnostic autopsy was not performed. The tumorous mass was overgrown through the laryngeal mucosa, not observed in the oral cavity.

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Histology and immunohistochemistry

Tissue samples for histological and immunohistochemical examination were taken from the affected tissue: dorsal mass of the larynx, retropharyngeal lymph node, a mass reaching the epiglottis, thyroid/salivary gland, and the left branch of the osteolytic mandible.

Excised samples were fixed in 10% neutral buffered formaline and paraffin embedded. Samples were routinely processed, and 2-μm thick sections were stained with haematoxylin-eosin. For immunohistochemistry the following antibodies were used: vimentin, SOX10, anti-alpha smooth muscle actin antibody (SMA), CD163, pancytokeratin AE1/AE3, and p63 (Table 1).

Table 1. Antibodies used for immunostaining and their analysis.

Antibody	Clone	Dilution	Reaction +/−
Vimentin mouse MoAb	V9	Ready to use	−
SOX 10 rabbit MoAb	EP 268	1:50	−
SMA mouse MoAb	1A4	Ready to use	−
CD 163 mouse MoAb	MRQ-26	Ready to use	−
Pancytoceratin AE1/AE3 mouse MoAb	AE1/AE3	1:100	+
p63 mouse MoAb	DAK-p63	1:100	+

Vimentin: The samples were pretreated with Ultra cell conditioning 1 (Ultra CC1) for 20 min in Ventana Benchmark Ultra. The samples were stained and incubated for 8 min with Vimentin (V9) mouse monoclonal antibody ((Roche Ventana, Arizona, USA). Ultraview DAB IHC Detection Kit was used to visualize the staining of primary antibodies in tissue sections. Counterstaining was performed with haematoxylin II.

SOX 10: The samples were pretreated with Ultra cell conditioning 1 (Ultra CC1) for 36 min in Ventana Benchmark Ultra. The samples were stained and incubated for 16 min with SOX10 (EP268) rabbit monoclonal antibody (Dilution 1:50, Cell Marque, Millipore Sigma, USA). Ultraview DAB IHC Detection Kit was used to visualize the staining of primary antibodies in tissue sections. Counterstaining was performed with haematoxylin II.

SMA: The samples were pretreated in PT-Link using TRS High pH (20 min at 95–97 °C). The samples were stained in Dako Autostainer Link and incubated for 20 min with SMA (1A4) mouse monoclonal antibody (Agilent Dako, Santa Clara, USA). EnVision FLEX (H2O2, HRP, DAB+chromgen) was used to visualize the staining of primary antibodies in tissue sections. Counterstaining was performed with FLEX haematoxylin.

CD163: The samples were pretreated with Ultra cell conditioning 1 (Ultra CC1) for 64 min in Ventana Benchmark ultra. The samples were stained and incubated for 32 min with CD163 (MRQ-26) mouse monoclonal antibody (Cell Marque). Ultraview DAB IHC Detection Kit was used to visualize the staining of primary antibodies in tissue sections. Counterstaining was performed with haematoxylin II.

Pancytokeratin AE1/AE3: The samples were pretreated with Ultra cell conditioning 1 (Ultra CC1) for 36 min in Ventana Benchmark ultra. The samples were stained and incubated for 24 min with pancytokeratin AE1/AE3 (AE1/AE3) mouse monoclonal antibody (dilution 1:100, Dako, Glostrup, Denmark). Ultraview DAB IHC Detection Kit was used to visualize the staining of primary antibodies in tissue sections. Counterstaining was performed with haematoxylin II.

p63: The samples were pretreated with Ultra cell conditioning 1 (Ultra CC1) for 76 min in Ventana Benchmark ultra. The samples were stained and incubated for 32 min with p63 (DAK-p63) mouse monoclonal antibody (dilution 1:100, Dako). Ultraview DAB IHC

Detection Kit was used to visualize the staining of primary antibodies in tissue sections. Counterstaining was performed with haematoxylin II.

Immunoreactivity of samples was evaluated using a light microscope.

Results

Histologically, an infiltrative neoplastic mass was found within the mucosa of larynx invading into the mandibular bone as well as the salivary gland, the thyroid and the oesophagus. The mass was composed of poorly differentiated epithelial structures (Plate IV, Fig. 1) forming nests and cords and containing individual keratinized cells. The histological findings of the mandible showed nests of cells, which were bordered by mesenchymal tissue with a moderately dense collagen and bone matrix (Plate IV, Fig. 2). Neoplastic cells were highly polymorphic, polygonal with vesicular chromatin, large nucleoli and frequent mitotic figures. However, spindle shaped cells appeared to be diffusely dominant. Numerous keratohyaline tonofilaments with modified keratin pearls, hypochromic nuclei and necrotic-haemorrhagic foci in the tissue were found (Plate V, Fig. 3).

Cells positive for protein p63 (Table 1) were found in the tumorous mass of the larynx, salivary gland (Plate V, Fig. 4) as well as in the tissue of the thyroid gland. A positive control was performed in skin with a melanocytic naevus (Plate VI, Fig. 5). Cytokeratin AE1/AE3 from the mandibular tumorous mass showed cytoplasmic positivity in polygonal epithelial cells (Plate VI, Fig. 6, Plate VII, Fig. 7). As positive external control, human skin melanocytic naevus was used, with positive keratinocytes and epithelium of adnexal structures (Plate VII, Fig. 8). Marker for CD163 showed a negative reaction in tissue taken from the mandible. The control sample was skin excision with nonspecific inflammatory repair changes, in which histiocytes presented cytoplasmic positivity. Nuclear transcription marker SOX10 was negative in the tumorous mass of mandible. Skin tissue with melanocytic naevus was used as control (nuclear positivity in melanocytes). Vimentin in tissue from tumorous mass of the larynx area presented a negative reaction. Schwannoma with its cytoplasmic positivity was used as control tissue.

Discussion

Cutaneous forms of SCC occur on the skin and mucosa, but preferentially develop on non-pigmented skin and mucocutaneous junctions, such as the ocular and oral regions, external genitalia, and perianal skin (van den Top et al. 2008). Squamous cells also form the mucosal surface that lines the lips, nasal cavities, and pharynx/larynx. Tumours affecting the oral cavity are less common. The most common sites of oral tumours in horses are the gums, dental alveoli, and lips (Laus et al. 2014). Mucosal forms of SCC often have an aggressive destructive nature and can present as large proliferative lesions with extensive destruction of the surrounding area (Schuh 1986; Laus et al. 2014). The patient in our case showed focal lysis of the jaw, basihyoid, and thyroid bones caused by the infiltrative growth of tumorous cells into these locations. These changes demonstrate the aggressive form of SCC in our case.

In horses, the occurrence of the mucosal form of SCC is accompanied by deterioration of health, weight loss, hypersalivation and, in the case of tongue involvement, its ulceration (Laus et al. 2014). The main clinical signs may include haemorrhagic to haemorrhagic-purulent nasal discharge, or bleeding from the oral cavity (Schuh 1986; Laus et al. 2014). Secondarily, the mucosal form of SCC can be accompanied by obstruction of the nasal mucosa, difficulties in swallowing and taking in food (dysphagia). However, early clinical signs may also not manifest clinically, as these tumours lead to proliferative facial deformities and swelling which is observed in the affected area (Howie et al. 1992).

Deteriorated health of the animal, difficult food intake, hypersalivation, and weight loss were observed in our examined patient. Examining the oral cavity in horses is quite difficult, as several common diseases have similar clinical manifestations.

Histological examination of the affected tissue in examined patient showed poorly differentiated SCC and its connection with lysis of the left branch of the mandible. Despite its invasiveness, SCC metastasize to regional lymph nodes only in 30% (Jones 1994).

For the immunohistochemical examination of the tumour, transcription factor p63 (Zeng et al. 2001) was chosen. This protein is a key regulator of epidermal keratinocyte proliferation and differentiation. In human medicine, p63 immunostaining has utility for head and neck SSCs (Shiran et al. 2007). Protein p63 is also helpful in distinguishing poorly differentiated SSC from small cell carcinoma or adenocarcinoma (Zhang et al. 2005). In our case a positive reaction of nuclear marker p63 showed the presence of tumorous cells of epithelial origin.

An anti-human pancytokeratin antibody demonstrates diffuse epithelial staining (Frgelecova et al. 2013). In our case positive cells for pancytokeratin showed membrane and cytoplasma positive cells composed of an uncapsulated, multilobular densely cellular cells. The mass of positive cells formed sheets, lobules, and cords of peripheral basal cells seated within a moderately dense fibrovascular stroma.

For evaluation of macrophages we used previously tested antibody CD163 with positive reaction in dog tissue (Levkut et al. 2022). However, in our case positive macrophages were not seen. Smooth muscle tissue was evaluated with SMA antibody which demonstrated negative reaction. Similarly, staining for vimentin did not support presence of mesenchymal origin of tumour. Positivity to vimentin includes fibroblast, endothelial cells, macrophages, melanocytes, Schwann cell, and lymphocytes. This is in contrast to keratin, which is the intermediate filament found in epithelial cells (Leader et al. 1987).

In our report we diagnosed a poorly differentiated SCC arising from the mucous membrane of the larynx, which is extremely rare in horses. Immunohistochemically, tumorous cells showed positivity for p63 and cytokeratin AE1/AE3. Protein p63 was demonstrated to be a suitable marker for poorly differentiated SCC. Immunohistochemistry confirmed the epithelial origin of the tumour and was necessary in order to achieve the final diagnosis.

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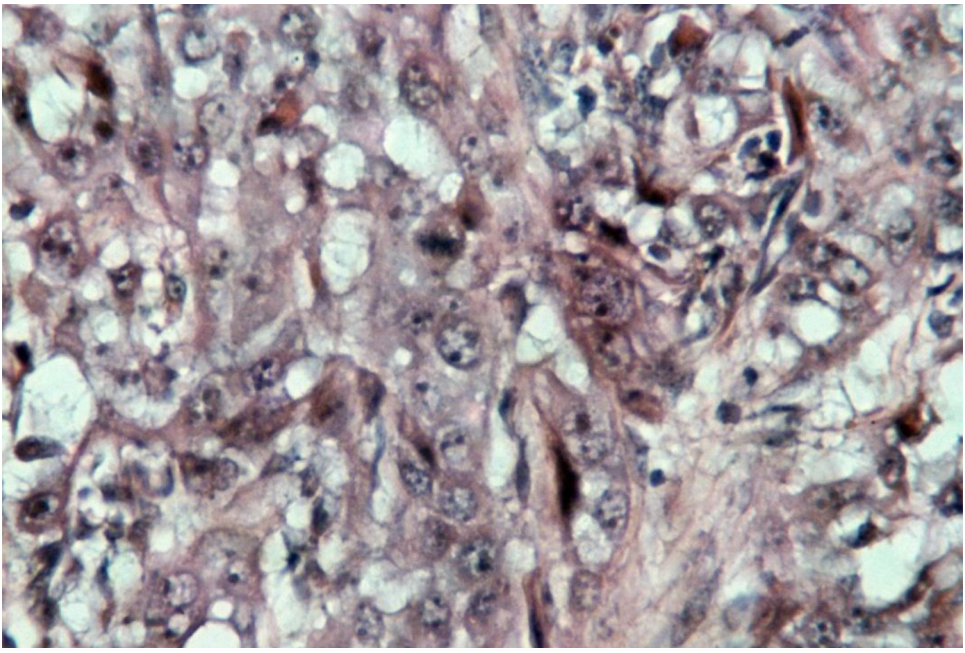

Fig. 1. Tumorous cells in the retropharyngeal lymph node with atypical mitosis (arrow), vacuolisation of the cytoplasm (haematoxylin-eosin, × 400)

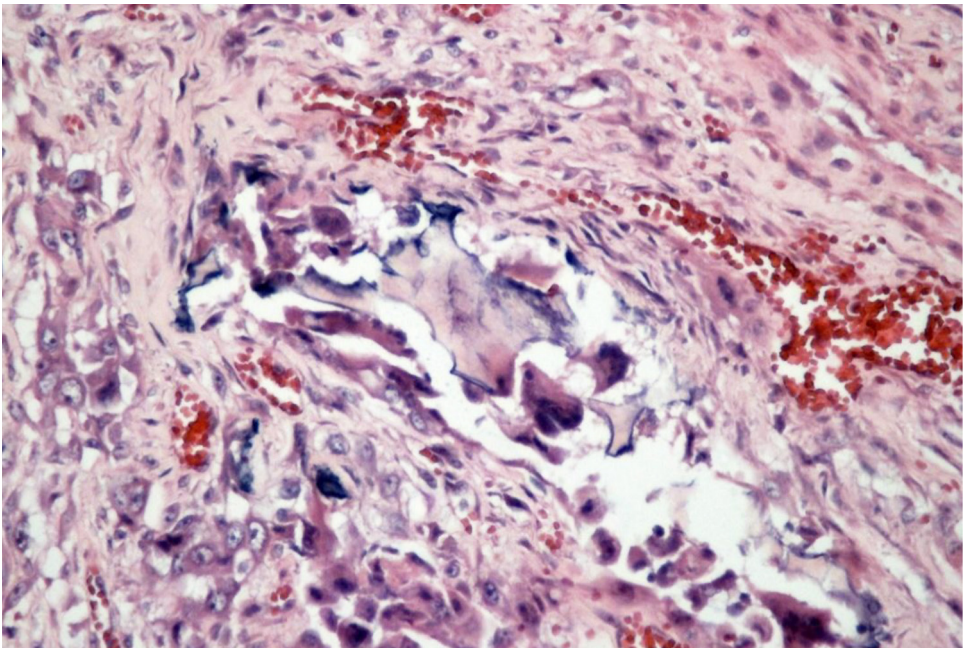

Fig. 2. Focal osteolysis of the mandible with multinucleated giant cells (arrows) (haematoxylin-eosin, × 200)

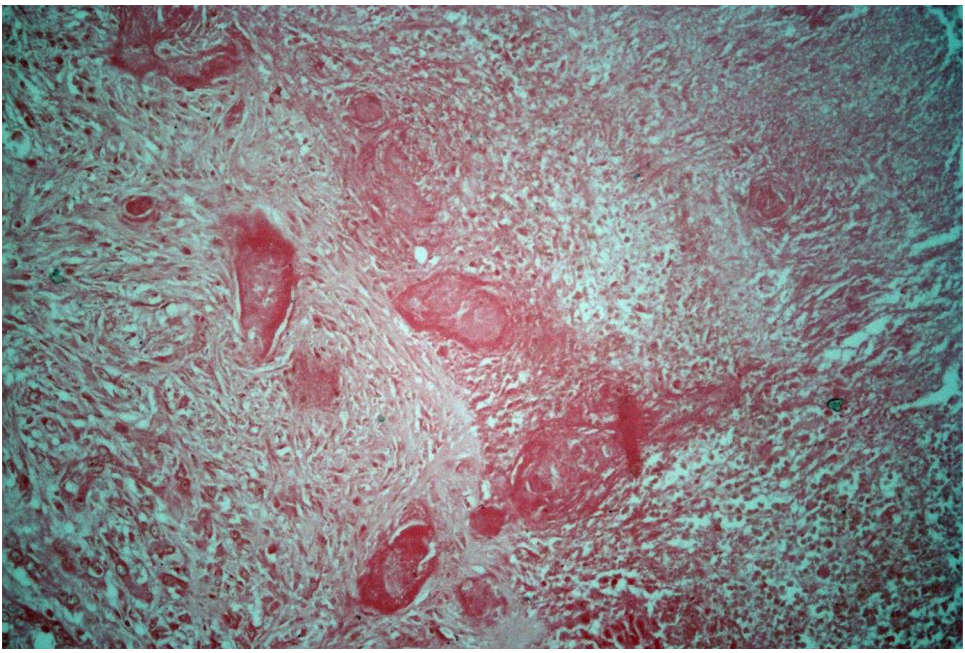

Fig. 3. Mandible-keratin pearls with keratohyaline laminated tonofilaments and hypochromic nucleus (arrows) (haematoxylin-eosin, × 100)

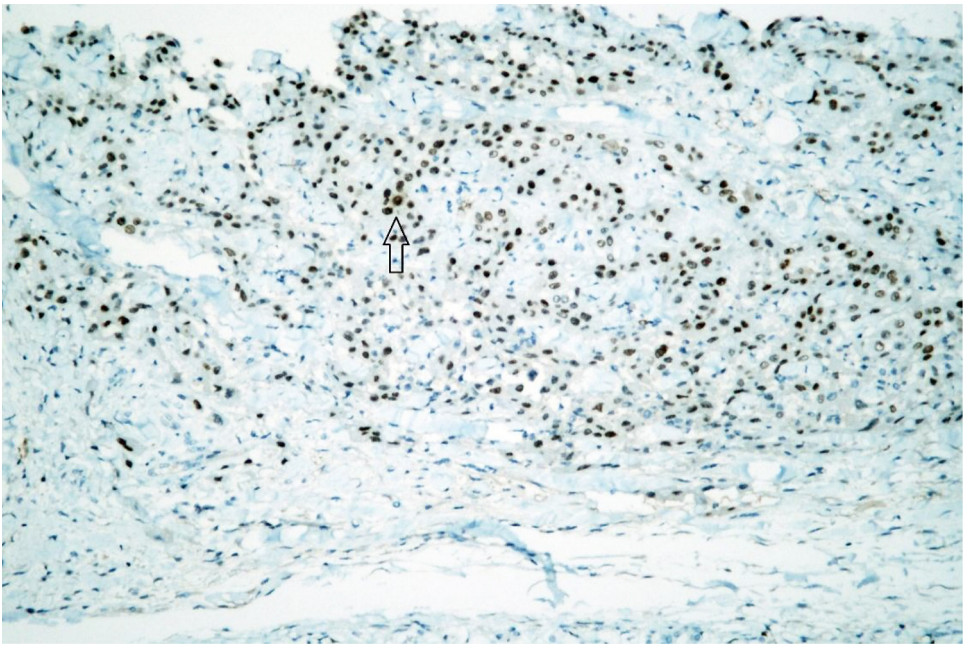

Fig. 4. Salivary gland-p63 marker with nuclear positivity (arrow) of metastatic epithelial cells (IHC, × 100)

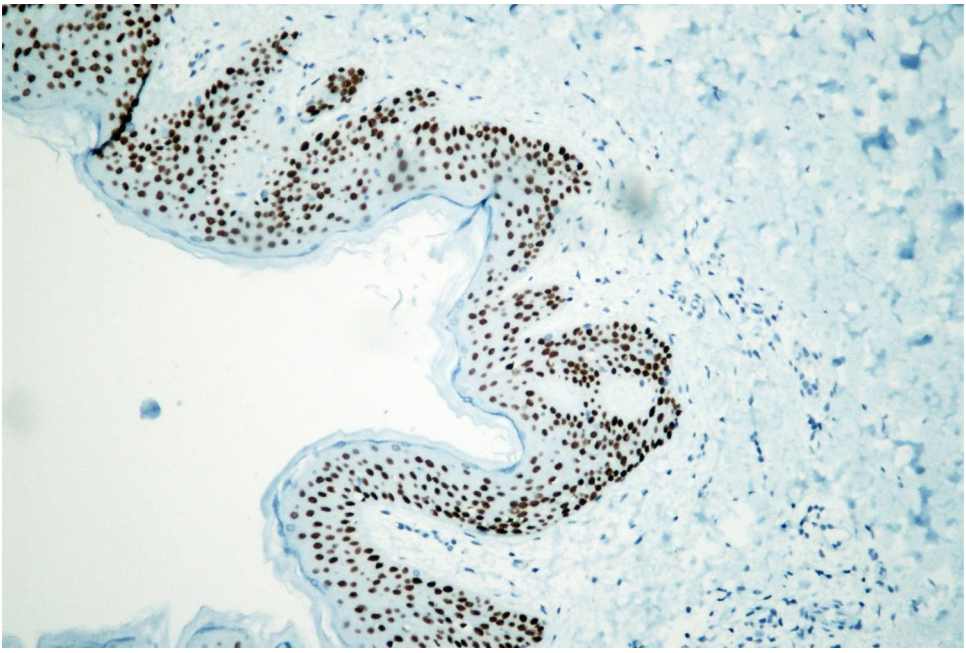

Fig. 5. Positive control of marker p63 - excision of skin with melanocytic naevus (IHC, × 100)

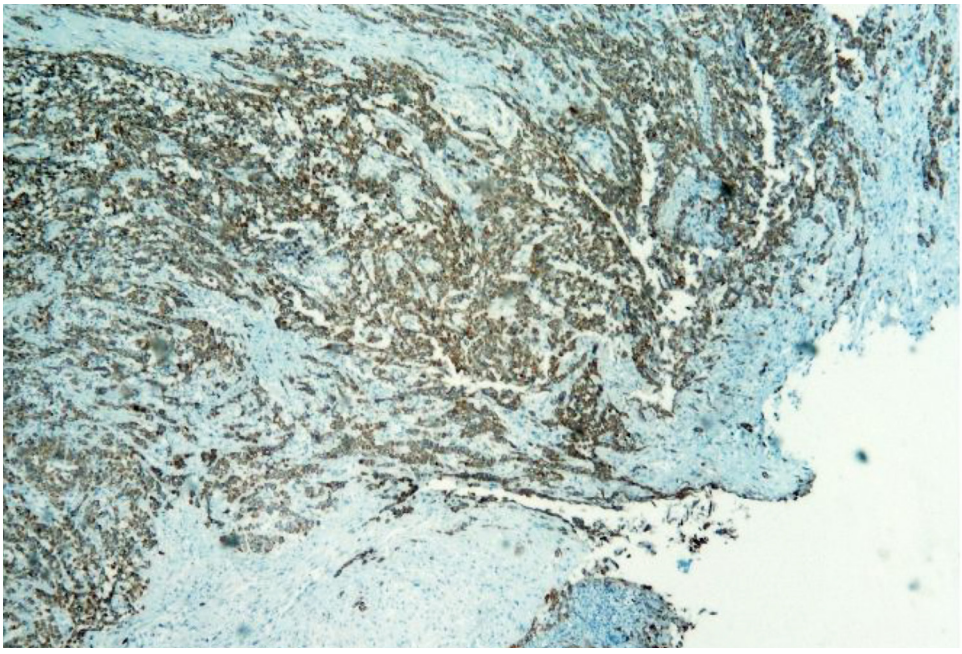

Fig. 6. Tumorous mass of larynx with membranous positivity of neoplastic epithelial cells (IHC, × 40)

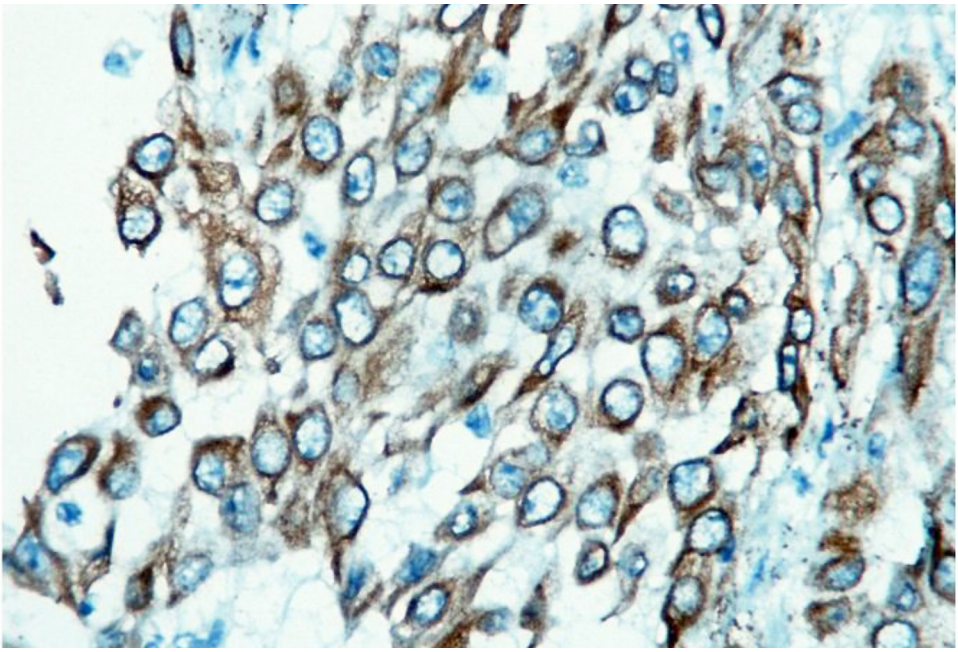

Fig. 7. AE1/AE3 membranous and intracytoplasmic positivity of epithelial cells in tumorous mass (IHC, × 400)

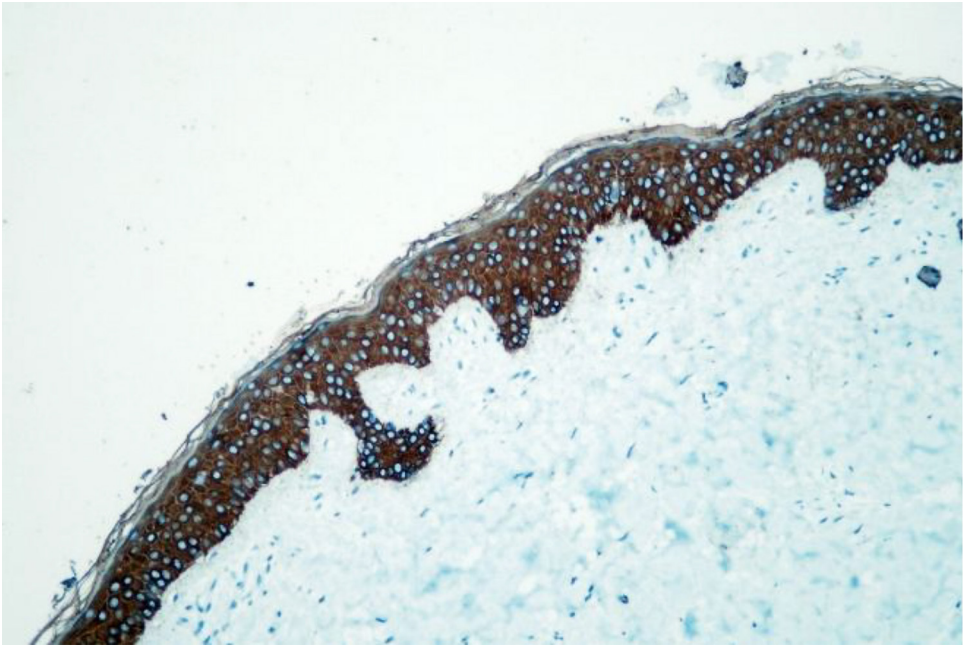

Fig. 8. AE1/AE3 positive control - positive keratinocytes and epithelium of adnexal structures of human melanocytic skin naevus (IHC, × 40)