P53 and Ki67 protein expression in ocular squamous cell carcinomas of dairy cattle

Azarabad, H.[1*], Gharagozlou, M. J.[1], Nowrouzian, I.[2], Seyedjavad, M. R.[2]

[1]*Department of Veterinary Pathology, Faculty of Veterinary Medicine, University of Tehran,Tehran, Iran.*

[2]*Department of Veterinary Clinical Science, Faculty of Veterinary Medicine ,University of Tehran,Tehran, Iran.*

Key words:
Cattle, ocular squamous cell carcinoma, p53, Ki67, immunohistochemistry.

Correspondence
Azarabad, H.,
Department of Pathology, Faculty of Veterinary Medicine, Tehran University,
P .O. Box: 14155-6453,Tehran, Iran.
Tel: +98(912) 2434315
Fax: +98(21) 66900196
Email: drazarabad@gmail.com

Received: 29 May 2011
Accepted: 15 September 2011

Abstract:

Role of the protein overexpression is very important in the pathogenesis, diagnosis and prognosis of neoplasia. Cases of overexpression of p53 and Ki67 proteins were investigated in 19 ocular squamous cell carcinomas (OSCC) in dairy cows. Microscopically, in six cases the tumors were carcinoma in situ (noninvasive carcinoma, G0), and in the remaining 13 cases the tumors were invasive carcinoma. The distribution of invasive carcinoma was as follows: G1, 1 (7.6%); G2, 1 (7.6%); G3, 3 (23.0%); G4, 8 (61.8%). Of the 19 tumors, 18 were immunoreactive for p53 (++), and 14 showed Ki67 expression (++). A significant positive correlation was found between the percentage of p53 stained nuclei and the degree of differentiation. The Ki67 index showed a significant negative correlation with the histological pattern, where the greatest proliferation was found in the most poorly differentiated OSCC. As in human squamous cell carcinoma(SCC), p53 overexpression is frequent in bovine OSCC, providing support for a possible role of the protein in the pathogenesis of this neoplasm.

Introduction

Bovine ocular squamous cell carcinoma (OSCC) is a well-characterized tumor in cattle. It is the second most common cause of rejection due to neoplasia and accounts for significant economic losses4. P53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein encoded by the TP53 gene. The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation and it is highly associated with cell proliferation. During the interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas during mitosis most of the protein is relocated to the surface of the chromosome. The Ki-67 protein is present during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0)5. Gharagozlou (2007) described most of the ocular neoplasms in dairy cattle are squamous cell carcinoma, which are generally located in the nictitating membrane and palpebral conjunctiva4. Epithelial cell proliferation and p53 mutation in bovine OSCC were investigated by Carvalho et al. (2005). Investigations into conjunctival squamous cell carcinomas of domestic animals demonstrate that p53 overexpression could be related to UV-induced mutations of the p53 tumor suppressor gene6. Jalva et al. (2006) studied Ki67 immunohistochemistry, proliferation subgroups based on Ki67 immunoreactivity and standardized mitotic index(SMI). The group with the lowest SMI and Ki67 fraction had the best prognosis; and groups with high SMI had the poorest prognosis. The group with low SMI and high Ki67 fraction had a favorable prognosis.

To further understand the genesis of bovine OSCC and its neoplastic transformation, abnormal expression

of p53 and ki671 and the associated proliferation index were assessed by immunohistochemically.

Material and Methods

This study was conducted between November 2009 and October 2010. All animals under investigation were female Holstein-Friesian dairy cows, kept on dairy farms located around Tehran. Environmental conditions are warm and dry in summer and autumn and relatively cold in winter. A total of 19 samples were collected from bovine OSCC and processed in a tissue processor. Paraffin-embedded tissue sections were stained using the Harris Hematoxylin and Eosin (H&E) method. All tumors were histologically classified with regard to microscopic features, especially the degree of differentiation2, 7.

Differentiation was evaluated according to the presence and intensity of keratinization, squamous differentiation and island formation and invasiveness of neoplastic cells. Tumors classified as G0 were noninvasive carcinoma with malignant transformation of the epithelial cells in the basilar layer and, less commonly, in the stratum spinosum. This form of neoplasm was confined by a basement membrane. G1 tumors were well-differentiated neoplasms containing numerous large keratin pearls, large islands with obvious squamous differentiation and minimal signs of invasion of the surrounding tissues. G2 tumors were moderately-differentiated neoplasms with a moderate degree of keratinization and differentiation. They also had a higher number of poorly-differentiated cells, exhibiting small- to medium-sized keratin pearls and islands, and small islands of invasive or noninvasive neoplastic cells surrounding the main tumor. Tumors classed as G3 were neoplasms consisting of individual cell keratinization, a few small tumor islands, poor cellular differentiation and deep invasion far from the main tumor. Those classed as G4 were neoplastic tumors consisting of small hyperchromatic or spindle cells with little evidence of squamous differentiation or keratinization.

Immunohistochemical (IHC) analysis was performed on all samples using polyclonal anti-human p53 antibody and ki67 anti-human antibody. For IHC staining, tissue sections were deparaffinized in xylene and rehydrated in graded ethanols. The slides were washed with 1× phosphate-buffered saline (PBS) and subjected to a methanolic block to prevent endogenous peroxidase activity6. To block endogenous biotin, slides were treated with an avidin-biotin block according to the manufacturer's instructions (SCY Tek ,USA) and washed three times in 1× PBS. The tissue sections were incubated with the polyclonal antibody for 30 minutes at room temperature, followed by 10 minutes incubation with biotinylated anti-rabbit antibody. After two washes in 1× PBS, slides were incubated for 5 minutes with a streptavidin-peroxidase conjugate. After washing in 1× PBS, slides were treated with diaminobenzidene chromagen substrate according to the manufacturer's instructions and counterstained with hematoxylin. Slides were dehydrated in serial ethanols and cleared in xylene, and finally coverslips were mounted with permount. Relative staining intensity was evaluated by a single individual without knowledge of any survey responses. Intensity was categorized as follows: (?) none or little nuclear staining, (+) <50% of nuclei staining intensely, (++) >50% of nuclei staining intensely.

Results

Gross pathology: Lesions were present in the right eye in ten cases and in the left eye in nine cases. Macroscopically, the lesions were papillomatous growths of varying size. Over a third (37%) of tumor lesions were ulcerated. The lesions varied in size from a few millimeters (32%) to several centimeters (69%), many of which had a nodular or cauliflower-like appearance (Table1).

Microscopically, in six cases the tumors were noninvasive carcinoma in situ (G0). The neoplastic cells displayed hyperchromatic nuclei, numerous mitotic figures, pleomorphism and loss of polarity. The squamous cells were noninvasive and confined by a basement membrane. At this stage, there was no invasion through the basement membrane by the dysplastic keratinocytes, but microinvasion of neoplastic cells to the stroma was observed in one

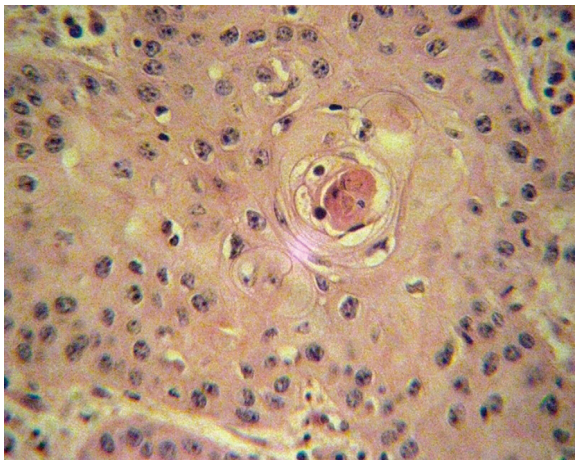

Figure 1: Moderately differentiated bovine OSCC. H&E. Note the mitotic figures and pleomorphism with formation of keratin pearls. Bar = 40 µm.

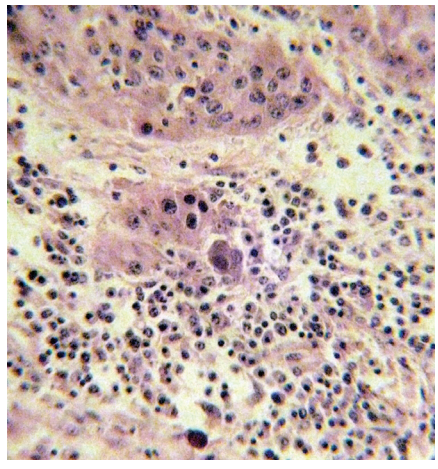

Figure 2: Poorly differentiated bovine OSCC. H&E. Note the mitotic figures and typically bizarre cells. Bar = 40 µm.

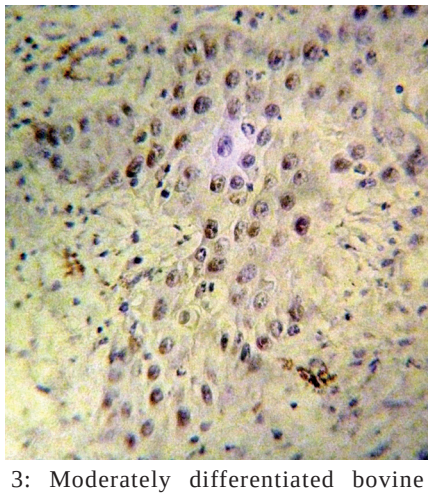

Figure 3: Moderately differentiated bovine OSCC immunohistochemistry. Nuclear p53 immunoreactivity is mainly seen in the peripheral neoplastic cells of tumor islands. Bar = 20 µm.

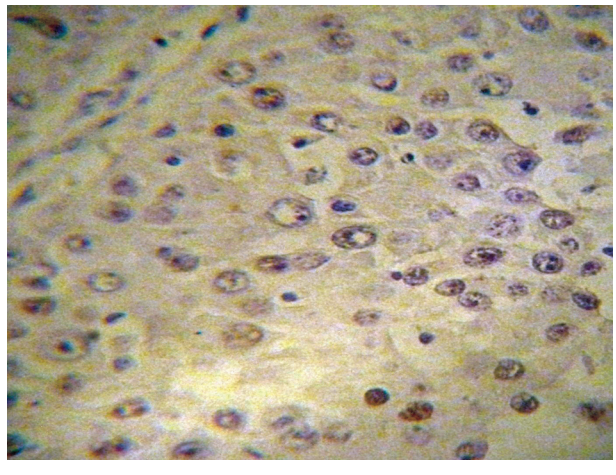

Figure 4: Poorly differentiated bovine OSCC immunohistochemistry. Diffuse pattern of p53 immunostaining within the tumor island. Bar = 20 µm.

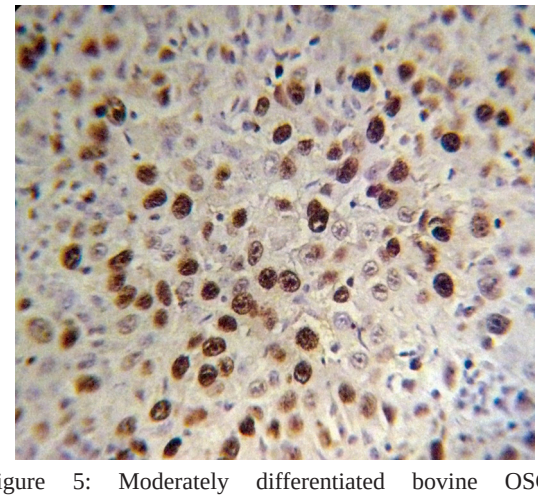

Figure 5: Moderately differentiated bovine OSCC immunohistochemistry. Nuclear Ki67 immunoreactivity seen in the peripheral neoplastic cells of tumor islands. Bar = 20 µm.

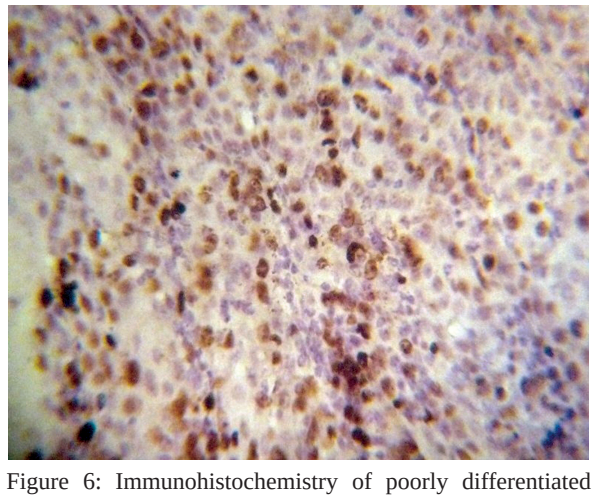

Figure 6: Immunohistochemistry of poorly differentiated bovine OSCC. Diffuse pattern of Ki67 immunostaining within the tumor island. Bar = 20 µm.

Table1: Clinical charactristic types of ocular neoplsms and tumor markers in 19 bovine tumor diagnosis and grade.

Cow Number	Location	Tumor Position	Shape	Size	Color	Grade (H&E)	P53	Ki67
1	Karaj Province	right eye	papillomatous	4.5.2mm	gray-white	G0	++	negative
2	Karaj Province	left eye	cauliflower-like, ulcerated	6.7.5cm	gray-red	G4	++	++
3	Varamin	right eye	nodular	3.4.2mm	gray	G0	negative	negative
4	Ghazvin Province	right eye	cauliflower-like, ulcerated	5.5.3cm	red	G4	++	++
5	Karaj Province	left eye	cauliflower-like	4.4.3cm	gray	G4	++	++
6	Karaj Province	right eye	nodular	2.1.1cm	white	G0	++	negative
7	Karaj Province	left eye	papillomatous	2.2.1cm	gray	G0	++	negative
8	Ghazvin Province	left eye	cauliflower-like	2.3.2mm	gray-white	G3	++	++
9	Ghazvin Province	left eye	cauliflowerlike, ulcerated	2.2.3cm	gray-white	G2	++	++
10	Ghazvin Province	right eye	nodular	1.2.1cm	gray-white	G1	++	++
11	Ghazvin Province	right eye	cauliflower-like	3.3.4mm	gray	G2	++	++
12	Karaj Province	left eye	cauliflower-like, ulcerated	4.3.5cm	gray-red	G4	++	++
13	Ghazvin Province	right eye	nodular	1.2.1mm	gray-white	G0	++	negative
14	Karaj Province	right eye	cauliflower-like, ulcerated	2.4.3cm	gray-white	G4	++	++
15	Karaj Province	right eye	nodular	2.2.3mm	gray-white	G3	++	++
16	Karaj Province	left eye	cauliflower-like, ulcerated	2.3.3cm	gray	G3	++	++
17	Karaj Province	left eye	nodular	2.3.2cm	gray	G0	++	++
18	Varamin Province	right eye	cauliflower-like, ulcerated	3.4.6cm	gray-red	G4	++	++
19	Karaj Province	right eye	cauliflower-like	4.2.2cm	gray-red	G4	++	++

case. The tumors of the remaining 13 cases were invasive carcinoma, with the following distribution: G1, 1; G2, 1; G3, 3; G4, 8 (Figs. 1 and 2).

Immunohistochemical staining: Of the 19 tumors tested, 18 were immunoreactive for p53 (++) (Figs. 3 and 4).. A significant correlation between the percentage of p53 stained nuclei and the degree of differentiation was observed. Different patterns of staining were seen according to the degree of keratinization of the tumor cells. Ki67 expression was seen in 14 tumors, as also seen in human squamous cell carcinoma. With the exception of the moderately-differentiated OSCC group, the Ki67 index showed a significant correlation with histologic pattern, where increased proliferation was found in poorly-differentiated OSCC (Figs. 5 and 6).

Conclusion: In this study, high levels of labeling were obtained with anti-human p53 polyclonal antibody in 95% of the bovine OSCCs. In all positive cases, p53 immunoreactivity was restricted to the nuclei of the tumor cells. These data indicate that p53 accumulation in the nuclei of cells is common in bovine OSCC. The percentage of OSCC neoplasms expressing p53 was similar to that found by Teifke and Lhr (1996) in bovine OSCC, and also by Gamblin et al. (1997) in canine squamous cell carcinoma. In humans, cutaneous squamous cell carcinoma induced by radiation has been found to contain specific mutations in the p53 gene. In fact, the p53 tumor-suppressor gene plays an important role in the carcinogenesis of various tissues, being the most commonly altered gene in human cancer. Bovine OSCC prevalence has already been directly related to exposure to UV radiation and, as with humans, it seems highly probable that a mutation in the gene that encodes for this protein is, at least, one of the factors

involved in the genesis of this tumor2. A highly significant correlation has been found between the degree of p53 nuclear immunostaining and gene mutation in numerous solid human cancers. However, overexpression or accumulation of wild-type p53 protein can occur in a variety of situations; such as induction by normal cellular stimuli (DNA damage, apoptosis) or protein binding that leads to alteration of p53 degradation pathways or its stabilization6. Molecular analysis of positive cases is required to confirm whether the high values of p53 immunostaining correspond to the accumulation of the mutated protein. However, this study provides support for a role of p53 alteration in the pathogenesis of bovine OSCC. Statistical analysis of the results obtained revealed a significant correlation between the percentage of p53 stained nuclei and the degree of tumor differentiation. Staining was more prominent in cells showing less keratinization. This was commonly present in poorly-differentiated OSCC, in the advancing proliferating zones of well-differentiated tumors and in the periphery of the tumor islands. These showed a gradual loss of positivity as the cells integrated keratin whorls. Normal tissue adjacent to the tumor lesions was consistently negative for p53, with the exception of one sample where intense nuclear staining was seen in apparently nondysplastic squamous mucosa, adjacent to the neoplasm. Similar observations in human squamous cell carcinoma support the possibility that p53 mutation is an early molecular event in the development of squamous cell carcinoma18. Successful immunostaining of Ki67 was also obtained in this study with a polyclonal anti-human Ki67 antibody. The Ki67 index was significantly(positively) correlated with histologic differentiation. These findings match another study that reports significantly higher Ki67 index values in poorly-differentiated squamous cell carcinomas compared to well-differentiated carcinomas4. In summary these results suggest that Ki-67 and p53 expression may be a sensitive marker for ocular malignant tumor grading2.

Acknowledgments

The authors thank Mr. R. Ebrahimi Samani and Mr. A. Baninajar for their technical assistance. This study was supported the Research Council, University of Tehran.

References

1. Anderson, D. E., Badzioch, M. (1991). The association between solar radiation and ocular SCC in cattle. Am J Vet Res. 52: 784-788.
2. Carvalho, T., Vala, H., Pinto, C., Pinho, M., Peleteiro, M. C. (2005). Immunohistochemical studies of epithelial cell proliferation and p53 mutation in bovine ocular squamous cell carcinoma. Vet Pathol. 42: 66-73.
3. Fillies, T., Werkmeister, R.; Packeisen, J., Brandt, B., Morin, P., Weingart, D., Joos, U., Buerger, H. (2006). Cytokeratin 8/18 expression indicates a poor prognosis in squamous cell carcinomas of the oral cavity. BMC Cancer. 6:23-27.
4. Gharagozlou, M. J., Hekmati, P., Ashrafihelan, J. (2007). A clinical and histopathological study of ocular neoplasms in dairy cattle. Veterinaryski Arhiv. 77: 409-426.
5. Gerdes, J. Li. L., Schlueter, C., Duchrow, M., Wohlenberg, C., Gerlach, C., Stahmer, I. Kloth. (1991) Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am. J. Pathol. 138: 867-873.
6. Sironi, G., Riccaboni, P., Mertel, L., Cammarata, G. (1999). P53 protein expression in conjunctival squamous cell carcinomas of domestic animals. Vet ophthalmol. 2: 227-231.
7. Kennedy, J.S., Kennedy, D.R., Yuan, H., Holt, D.S. (1998). Histological classification of epithelial and melanocytic tumors of the skin of Domestic Animals. (2thed.) World Health Organization publication, Geneva. vol. 3: 21-40.

8. Gurses, I., Doganay, S., Mizrak, B. (2007). Expression of glucose transporter protein-1 (Glut-1) in ocular surface squamous neoplasia. Cornea. 26: 826-30.
9. Teifke, J. P., Lqhr, C. V. (1996). Immunohistochemical detection of p53 overexpression in SCC of cattle, horse, cat and dogs. Comp Pathol. 114: 205-210.
10. Khan, K. N. M., Knapp, D. W., Denicola, D. B., Harris, R. K. (2000). Expression of cyclooxygenase-2 in transitional cell carcinoma of the urinary bladder in dogs. Am J. Vet. Res. 61: 478-481.
11. Ichiro, K., Nishimura, K. (1990). Cytokeratin expression in squamous cell carcinoma arising from hydradenitis suppurative. J. Cutan Pathol. 34: 675-678.
12. Parums, D. V., Cordell, J. L., Micklem, K., Fabian., scot JC .(1990). A new monoclonal antibody that detects vascular endothelium associated antigen on routinely processed tissue sections. J. Clin Pathol. 43: 752-757.
13. Pestili de Almeida, E. M., Piche, C., Sirois, J., Dore, M. (2001). Expression of cyclo-oxygenase-2 in naturally occurring squamous cell carcinomas in dogs. J. Histochem Cytochem. 49: 867-875.
14. Rassnick, K.M., Njaa, B.L. (2007) Cyclooxygenase-2 immunoreactivity in equine ocular squamous cell carcinoma. Am. J. Vet. Res. 1: 165-170.
15. Shields, J.A., Shields, C.L. (1994) Tumors of the conjunctiva and cornea. The Cornea: Scientific Foundations and Clinical Practice. (3^{th}ed.). p. 586-588.
16. Carvalho, T., Vala, H. (2005) Immunohistochemical studies of epithelial cell proliferation and p53 mutation in bovine ocular SCC. Vet Pathol. 42: 66-73.
17. Woelfle, U., Sauter, G. (2004). Down-Regulated Expression of cytokeratin 18 promotes progression of human breast cancer. Clin Cancer Res. 10: 2670-2674.
18. Yang, J., Foster, C. S. (1997). Squamous cell carcinoma of the conjunctiva. Int Ophthalmol Clin. 37: 73-85.