

# Immunohistochemical expression of p53 in animal tumors: a methodological study using four anti-human p53 antibodies

O. Albaric, L. Bret, M. Amardeihl and M. Delverdier

National Veterinary School of Toulouse, Food Safety Unit, I.N.R.A., Pathology Laboratory, Toulouse cedex, France

**Summary.** Mutations in the p53 tumor suppressor gene are the most common genetic alterations in human cancers. These mutations usually lead to strongly enhanced protein stabilization and allow detection by immunohistochemistry. Two monoclonal (DO-7 and PAb-240) and two polyclonal (Ab-7 and CM-1) antibodies were evaluated by standard immunoperoxidase method in domestic animal tumors, chiefly squamous cell carcinomas (SCC), and osteosarcomas as positive controls. Immunoreactivity was detected in SCC of cattle, sheep, horse and cat as well as in feline actinic keratosis, with PAb-240 and CM-1 antibodies. One polyclonal antibody (Ab-7) did not give positive result at all, whereas DO-7 monoclonal antibody did not react in dogs and cats. Immunodetection of p53 protein is thus possible in all domestic species tested, especially with CM-1 and PAb-240 antibodies, and p53 alterations seem to occur early in carcinogenesis of feline SCC as in comparable human lesions.

**Key words:** p53, Immunohistochemistry, Methodology, Domestic animals

## Introduction

Nuclear p53 phosphoprotein referred to as "the guardian of the genome", is induced in response to DNA damage and acts as a transcriptional factor of cell cycle regulating proteins leading to either cell cycle arrest (especially in G1 phase) or apoptosis (Lee and Bernstein, 1995; Martin, 1995). p53 inactivation can result from genetic alteration (by deletion, point mutation or rearrangement) or from interaction with viral or cellular proteins (Levine et al., 1991; Momand et al., 1992; Milner, 1994; Kastan et al., 1995). p53 inactivation consequently leads to the loss of p53 tumor-suppressive function, with increasing cell proliferation

and apoptosis inhibition.

Comparison of p53 proteins from various species leads to identification of five domains highly conserved during evolution (Mayr et al., 1993, 1995; Okuda et al., 1993; Pazzi et al., 1996; Veldhoen and Milner, 1998). Four of these five domains are found in the core region while block I is located on the amino-terminal region. Analysed p53 mutations in human neoplasia (Hollstein et al., 1991) and in few animal tumors (Mayr et al., 1994, 1997; Zhuang et al., 1997; Johnson et al., 1998) are essentially clustered in DNA binding regions encoded by exons 5-9 and colocalized with highly conserved domains II to V.

The level of wild-type p53 protein in normal cells is undetectable by immunohistochemical staining due to the short life of the protein (20-30 minutes) (Ozgun and Butel, 1997). On the other hand, point mutations leading to aminoacid substitutions and to p53 stabilization, prolong its half-life up to 2 hours, allowing the accumulated mutant p53 to be detected by immunohistochemistry (Martin, 1995).

Immunohistochemical studies of p53 protein in domestic animals are now growing. p53 immunoreactivity has been documented in squamous cell carcinomas (SCCs) in mammals (dog, cat, horse and cattle) (Johnston et al., 1996; Teifke and Lohr, 1996), in colorectal tumors (Fante et al., 1996; Gamblin et al., 1997; Wolf et al., 1997), testicular tumors (Vitelozzi et al., 1998) and in canine osteosarcomas, with high prevalence in this tumor (Sagartz et al., 1996). The purpose of the study was to evaluate p53 immunoreactivity in domestic species (especially on SCC), using four anti human p53 antibodies, and hence to define a simple immunohistochemical method appropriate for all these species.

## Materials and methods

### *Tumor material*

The investigations were carried out on 25 tumor tissues collected from various animal species (canine, feline, ovine, equine and bovine). The criteria for

*Offprint requests to:* Dr. Maxence Delverdier, National Veterinary School of Toulouse, Food Safety Unit, I.N.R.A., Pathology Laboratory, 23 chemin des capelles 31076 Toulouse cedex, France. e-mail: [m.delverdier@envt.fr](mailto:m.delverdier@envt.fr)



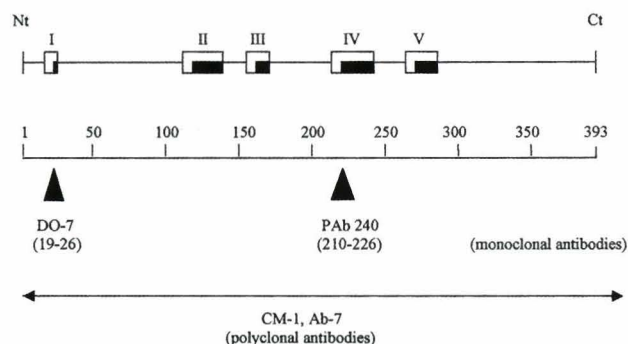
including tumors in the study were based on: 1) the type of the tumor: SCC, colorectal adenocarcinomas or osteosarcomas, 2) the presence of previous data which notified the possibility to immunohistochemically detect p53 according to the type of cancer and to the chosen animal species.

All the tumors came from the Pathology Department of the National Veterinary School of Toulouse between 1993 and 1998. Ten canine tumors were selected: three squamous cell carcinomas (one from the nasal cavities, one from the eyelid and one from digital extremity) and two actinic keratosis which is considered as a precancerous lesion, two colorectal papillar adenocarcinomas and three osteosarcomas (involving the appendicular skeleton). The feline tumors were squamous cell carcinomas from the oral cavity (n=3), one from the eyelid (n=1) and five from the pinna (n=5) including two actinic keratosis. The equine squamous cell carcinomas, were of gastric (n=1), vulvar (n=1), and eyelid (n=1) origin. The bovine and ovine SCCs (one case each) were respectively tumors of the lower jaw and of the eyelid. All cases were either necropsy specimens (immediately fixed after death) or surgical and biopsy specimens. 2-3  $\mu\text{m}$ -thick sections of formalin-fixed (24 to 48 hours) and paraffin-embedded tissues were prepared and mounted on Superfrost+ slides (LLR3 plus, CML, France). A prerequisite hematoxylin and eosin staining confirmed histological features to verify adequate tumor sampling.

### Immunohistochemistry

Immunohistochemical analyses were performed by an avidin-biotin peroxidase technique. 2-3  $\mu\text{m}$  sections from paraffin-embedded tissues were air-dried overnight at 56 °C, deparaffinized and rehydrated using toluene and acetone. After rinsing with water, the slides were treated with 0.1% trypsin (Merck, France) in phosphate buffered saline (PBS, pH 7.6), at 37 °C for 6 minutes only when CM-1 antibody was used as primary anti p53 antibody. Enhancing antigen retrieval was established by boiling the slides for ten minutes, then two times for five minutes in 10 mM citrate buffer, pH 6, using a standard

microwave oven at 700W. Endogenous peroxidase was blocked by incubation in 0.9%  $\text{H}_2\text{O}_2$  in PBS for five minutes at room temperature. To block non-specific binding sites of the antibodies, slides were washed for five minutes with PBS including 0.01% Tween 20 (p7949, Sigma, France) and 0.01% non fat milk, and then incubated in 20% normal goat serum diluted in PBS containing 1% bovine serum albumin (BSA) for 20 minutes at room temperature. Subsequently, the slides were incubated with primary anti p53 antibodies diluted in PBS including 1% BSA for 30 to 70 minutes, according to the chosen antibody, at room temperature except for CM-1 primary antibody. In this case, incubation was performed overnight. The origin of primary antibodies as well as their characteristics are listed in table 1. The four antibodies tested consisted of two polyclonal antibodies i.e. Ab-7 and CM-1, and two monoclonal antibodies i.e. PAb-240 and DO-7, which recognize mutant p53 and both wild-type and mutant p53 respectively (Fig. 1). After washing (with PBS including tween 20 and non fat milk four times 5 minutes each), sections were incubated for thirty minutes with biotinylated goat anti-rabbit antibody (for CM-1



I → V : highly conserved domains of p53 protein  
1 → 393 : amino acid sequence of p53 protein.

**Fig. 1.** Diagram of the reported binding sites of the antibodies used for immunohistochemistry.

**Table 1.** Antibodies used for p53 immunohistochemistry.

ANTIBODIES	TYPE (isotype for monoclonal antibodies)	ORIGIN	SPECIFICITY AND REACTIVITY*	DILUTION	INCUBATION
DO-7 (M 7001, DAKO, France)	Monoclonal (Ig G2bk)	Mouse	WT and mutant p53 (tested in human)	1:50	30 min at room temperature
Ab-7 (PC 35, kit Genzyme, France)	Polyclonal	Sheep	WT and mutant p53 (tested in human, mouse, rat)	1:500	40 min at room temperature
CM-1 (NCL-p53 CM-1, TEBU, France)	Polyclonal	Rabbit	WT and mutant p53 (tested in dog)	1:1000 and 1:2000	16 hours at 4°C
PAb 240 (MCA 909, Realef, France)	Monoclonal (Ig G1)	Mouse	Mutant p53 (tested in dog, cat, cattle, horse)	1:50	70 min at room temperature

\* : species for which antibody reactivity is known is precised in brackets.



### Immunohistochemical expression of p53 in animal tumors

antibody) or biotinylated goat anti-mouse antibody (for DO-7 and PAb 240 antibodies) diluted 1:100 in PBS including 1% BSA. When the primary anti p53 antibody tested was Ab-7, the secondary antibody used was a biotinylated rabbit anti-sheep antibody. After washing twice in PBS, the immune complexes were detected by the streptavidin-biotin horseradish peroxidase complex using 3,3'-diaminobenzidine (DAB) as chromogen according to the manufacturer's instructions (ABC, Dako). To visualize bound antibodies, sections were covered with 0.6  $\mu\text{g}/\mu\text{l}$  DAB in PBS ; to which 0.0012% of  $\text{H}_2\text{O}_2$  was added, for several minutes. Slides were counterstained with Harris' haematoxylin, dehydrated and coverslipped.

Two negative control sections for each case were treated as described above, except that primary antibody was omitted during the procedure (first negative control), or replaced by normal rabbit serum for anti-p53 polyclonal antibodies or the same isotype antibody for anti-p53 monoclonal antibodies (second negative control).

Positive control slides consisted of paraffin sections from tissues known to be positive for p53 (human tumor pannel and canine osteosarcomas). Nonneoplastic tissue from each slide was used for internal control.

#### Scoring of positive p53 immunoreactivity

Specific nuclear immunoreactivity for p53 protein

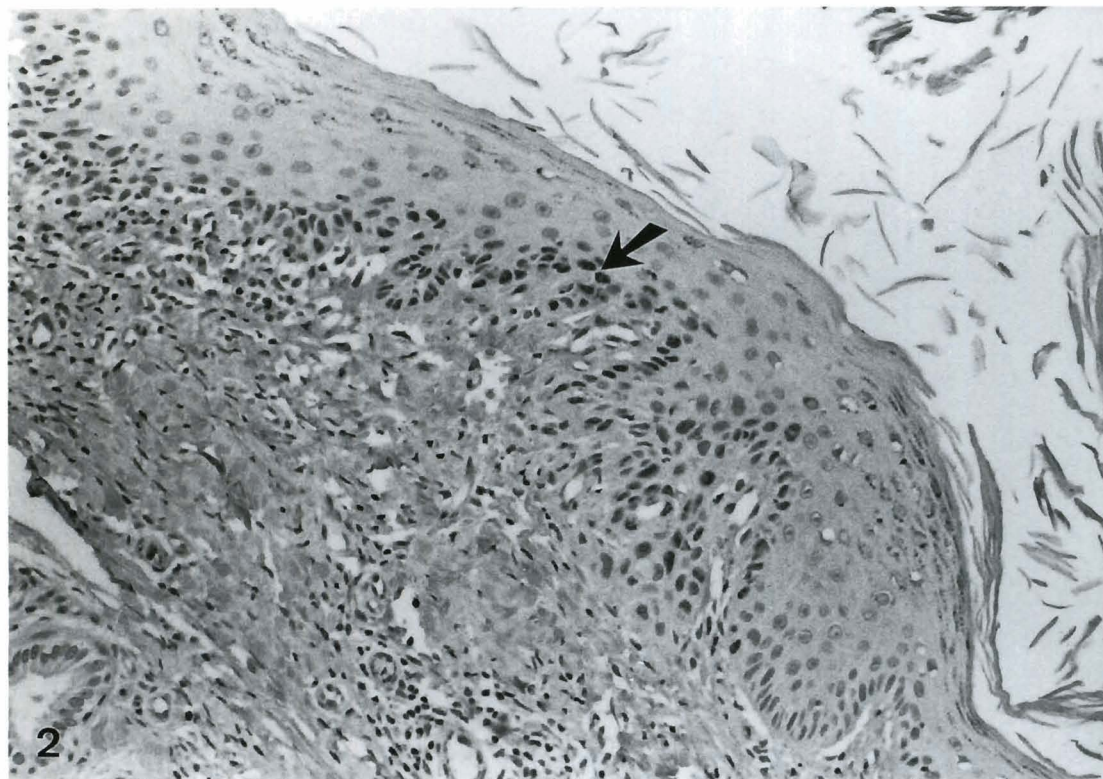
was scored semi-quantitatively on a graded scale from 0 to 3 for both intensity of staining (low: +, mild: ++, moderate: +++, marked: ++++ or intense: +++++) and its extent. This latter was evaluated as the percentage of positively stained tumor cells of the total number of tumor cells, in ten adjacent fields at a magnification of x400: 0 signifying no cell staining, + indicating that less than 10% of tumor cells were positive, ++ indicating that 10-50% of tumor cells were positive, and +++ signifying that more than 50% of tumor cells were positive. Background and cytoplasmic staining were recorded but were not incorporated into the scoring system.

#### Results

##### Immunohistochemical expression of p53 in animal tumors

Nuclear p53 staining was granular and relatively homogeneous and excluded nucleoli. The intensity of staining was mild to intense, particularly in positive controls. For positive control slides (human tumors or osteosarcomas), the extent of staining was evaluated at +++ irrespective of the anti-p53 antibody used, except for Ab-7 which did not label osteosarcomas.

Nuclear p53 staining was absent in negative controls or in adjacent nonneoplastic tissue. Nevertheless, cytoplasmic staining, occasionally marked, was almost systematically found in neoplastic keratinocytes,



**Fig. 2.** Feline actinic keratosis (Pab 240 antibody). Positive staining was observed in nuclei (AE) in the epidermal basal layer of a preneoplastic site. x 200



chondrocytes or enterocytes, and was scarcely observed in osteoblasts and giant cells of osteosarcomas. This staining pattern was probably not specific for p53 accumulation because it was also obtained in negative control slides incubated with normal serum, especially with rabbit serum, instead of polyclonal primary antibody. Besides, this nonspecific staining was more pronounced with polyclonal CM-1 antibody.

*Staining with Ab-7 anti-p53 antibody was completely absent, whatever species, except in human positive controls*

In canine and feline tumors including osteosarcomas, DO-7 antibody was unable to detect p53 immunoreactivity. Nonspecific cytoplasmic p53 staining was only obtained and was mild to moderate. On the other hand, p53 immunoreactivity, i.e. specific nuclear staining, was detected using PAb-240 antibody in four tumors (osteosarcomas and one colorectal adenocarcinoma) out of ten in the dog, and in seven tumors (including the two actinic keratosis) out of nine in the cat (Fig. 2). Results were similar with CM-1 antibody (Figs. 3, 4). In dogs, none of the SCC tested in this study showed specific reactivity with any of the antibodies used. Tumors especially stained with PAb-240 were also positive for CM-1 immunostaining (cf. table 2). However, extent of staining, i.e. the number of positive cells was higher with CM-1, in all tumors: the

percentage of positive cells was often greater than 50% with CM-1 antibody whereas the score was lower with PAb-240 antibody. Similarly, staining intensity was generally moderate with PAb 240 antibody whereas it was always marked to intense with CM-1 antibody.

In other species, the results showed that ovine and bovine (Fig. 5) tumors revealed p53 immunoreactivity with CM-1, PAb 240 and DO-7 antibodies. Extent of staining was highest with CM-1, and was quite similar using PAb 240 or DO-7 antibodies. Two equine SCC (Fig. 6) showed intense nuclear staining in a majority of cells whatever the antibody used (more than 50% of cells were positive), whereas the third tumor was only slightly stained with DO-7 antibody. For these three species, intensity of p53 staining was moderate (PAb 240 antibody) to marked (DO-7 and CM-1 antibodies) in SCCs.

#### Discussion

In this study, p53 immunohistochemistry was performed in several domestic species by a standard peroxidase method using four antibodies: two monoclonal (DO-7 and PAb-240) and two polyclonal (CM-1 and Ab-7). p53 staining was evaluated on the same tumor type i.e. SCCs. In dogs, a few colorectal tumors and osteosarcomas were also analysed. Osteosarcomas were used as positive control as previous data showed marked immunoreactivity for this neoplasm



**Fig. 3.** Feline squamous cell carcinoma (CM-1 antibody). Marked reactivity (dark staining) in nuclei of infiltrating neoplastic tissue (arrow), but not in adjacent hyperplastic epidermis. x 100



## Immunohistochemical expression of p53 in animal tumors

(Sagartz et al., 1996). We showed here that p53 nuclear protein could be detected by antibodies toward human protein in domestic animal species (i.e. canine, feline, equine, bovine and ovine). Nuclear p53 staining was specifically observed using CM-1 and PAb-240 antibodies in dogs and cats, whereas p53-positive staining was also obtained with DO-7 antibody in the other species. Furthermore, p53 accumulation was generally detected in SCC from cats, horses and ruminants (although the case number was low), but not in SCC from dogs.

No p53 staining was observed with polyclonal Ab-7 antibody in any cases analysed. Several reasons could explain this defect. First, this antibody may not recognize p53 protein in animal species except in rat and mouse. We have not found any data about Ab-7 reactivity in animals except those provided by the manufacturer (Table 1). Second, the particular epitope denaturation by unmasking methods, or on the contrary, residual masked epitopes might not react with Ab-7 antibody, preventing p53 staining. Third, technical

problems such as antibody alterations (during transport for instance) should not be excluded.

DO-7 antibody was not able to detect p53 immunoreactivity in canine and feline tumors, whereas this antibody recognized ovine, equine or bovine p53 proteins. Perhaps due to canine p53 stereospecificity, DO-7 antibody seems not to be as efficient as other antibodies to recognize p53 protein in dogs and cats.

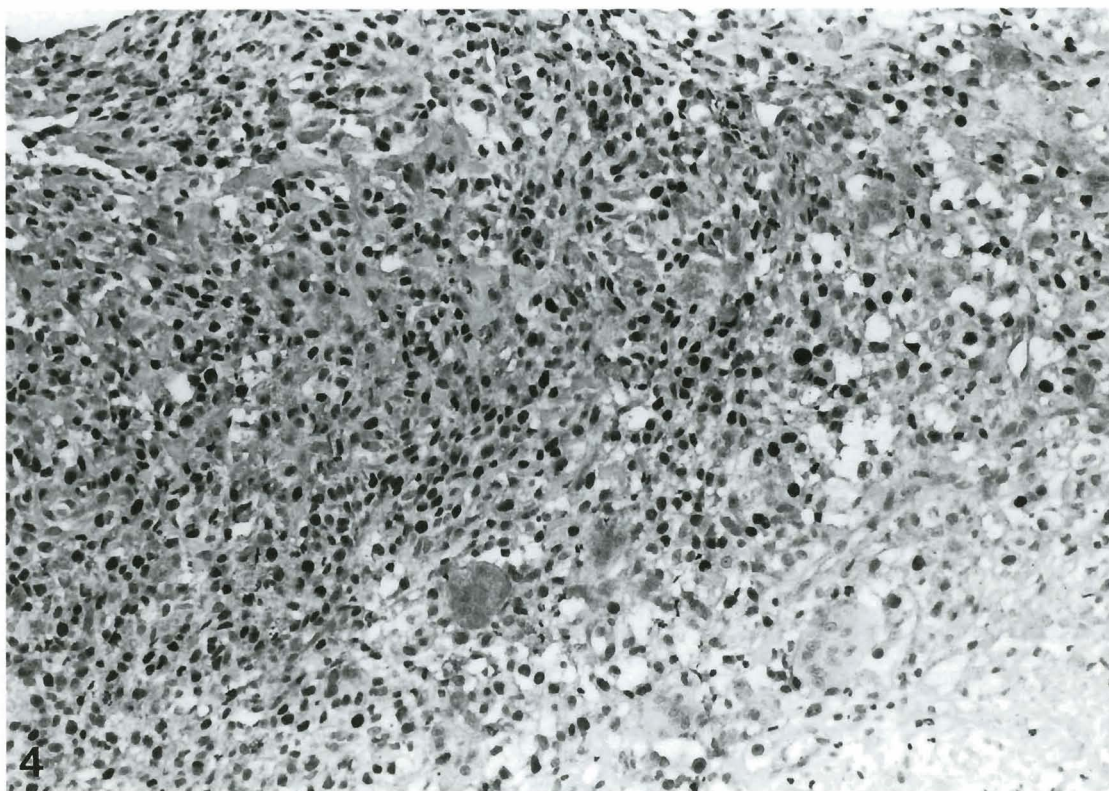
Whatever species tested, results were similar for PAb-240 and CM-1 antibodies. Nevertheless, staining intensity, as well as nonspecific and background staining, was more pronounced with CM-1. Consistent with this fact, presence of an appropriate negative control (same isotype or normal rabbit serum, instead of anti p53 primary antibody) was absolutely necessary in order to correctly interpret p53 staining. Consequently, staining was considered as nonspecific when it was equally present on the negative control as cytoplasmic staining, which is reported in some SCC lines (Caamano et al., 1993), various human cancers (Bosari et al., 1994; Faille et al., 1994; Flamini et al., 1996; Runnebaum et

**Table 2.** Comparison of results of p53 immunostaining using PAb 240 and CM-1 antibodies.

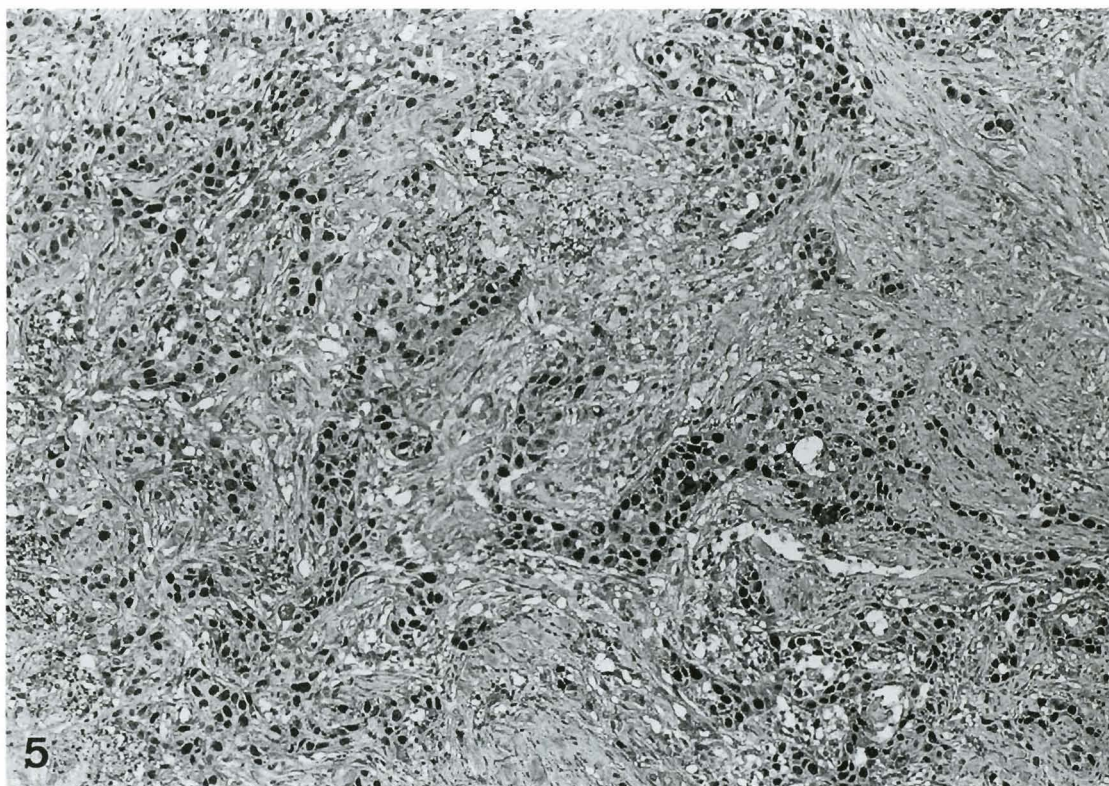
SPECIES AND CASE	PERCENTAGE OF POSITIVE CELLS		INTENSITY	
	CM-1	PAb 240	CM-1	PAb 240
<i>Dog</i>				
squamous cell carcinomas				
1	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4*	0	0	0	0
5*	0	0	0	0
colorectal tumors				
1	+	+	++++	++
2	0	0	0	0
osteosarcomas				
1	+++	++	++++	++
2	++	+++	++++	++++
3	+++	++	++++	++++
<i>Cat</i>				
1	+++	++	++++	++
2	+++	+++	++++	++++
3*	++	++	++++	++++
4	0	0	0	0
5	+++	++	++++	++
6	++	+	++++	++
7*	+++	++	++++	+++
8	0	0	0	0
9	+++	+++	++++	++++
<i>Cattle</i>				
	+++	+	++++	+++
<i>Sheep</i>				
	++	+	++++	+++
<i>Horse</i>				
1	+++	+++	++++	++++
2	0	0	0	0
3	+	+	+++	++

\*: actinic keratosis. Percentage of positive cells: 0; +: less than 10% of cells; ++: 10 to 50% of cells; +++: more than 50% of cells. Staining intensity: 0; +: low; ++: mild; +++: moderate; ++++: marked; +++++: intense.





**Fig. 4.** Canine osteosarcoma (CM-1 antibody). Intense nuclear staining was observed in the majority of neoplastic cell nuclei. x 200



**Fig. 5.** Bovine squamous cell carcinoma (CM-1 antibody). Cords of neoplastic cells markedly stained within scirrhous stroma. x 100



*Immunohistochemical expression of p53 in animal tumors*

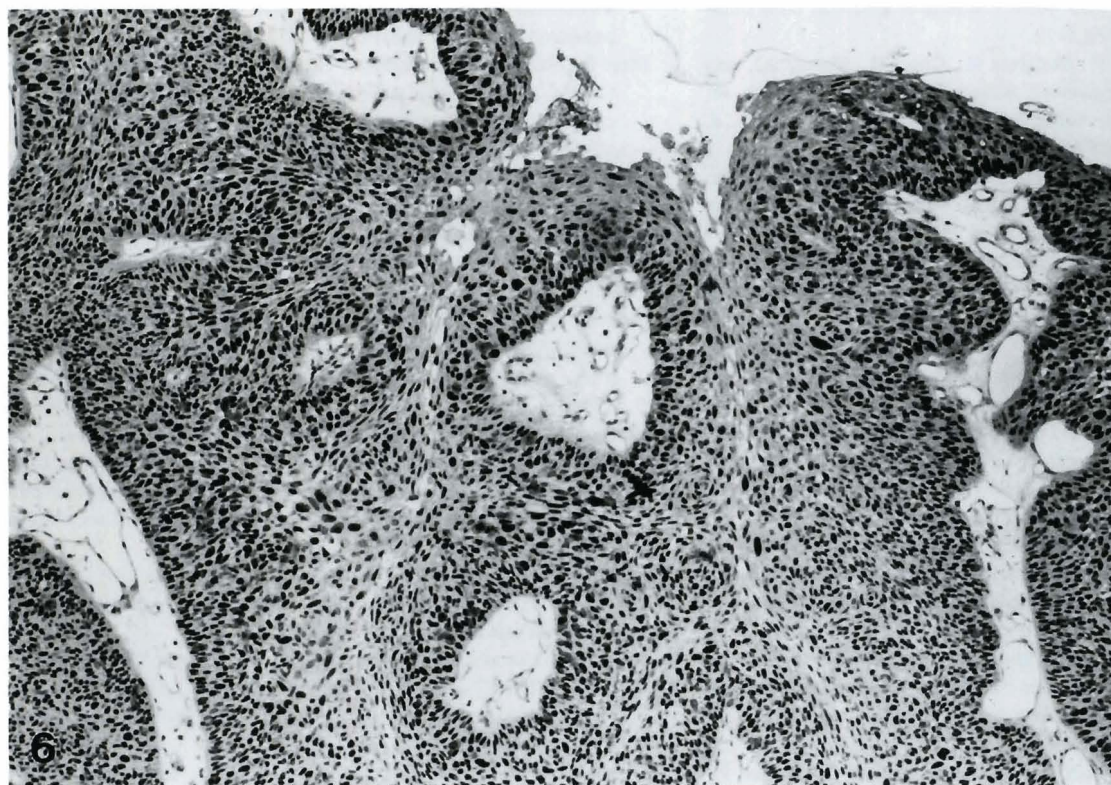
al., 1996) and probably in animal tumors (Wolf et al., 1997; Vitellozzi et al., 1998). This staining pattern could be specific for p53 presence in cytoplasm, since several mechanisms of cytoplasmic p53 sequestration and inactivation have been described, such as interactions with viral or cellular proteins (mdm2, bcl2, hsp 70) (Finlay et al., 1988; Momand et al., 1992; Ryan et al., 1994; Canman and Kastan, 1997) or nuclear transport dysregulation (eventually caused by some mutations) (Özbun and Butel, 1997). Considering cytoplasmic staining as a positive result without nonspecific staining background evaluation, could be erroneous and lead to the overestimation of the role of p53 in tumorigenesis.

Despite absence of labelling of normal tissue, and the obtained similar immunoreactivity pattern as observed in human tumors, it must be pointed out that further biochemical studies (such as Western blot) are needed to definitively characterize antibodies used.

In contrast to other species tested here, SCC in dogs presented no p53 immunoreactivity in the few tumors tested. Previous studies also reported a low incidence or absence of p53 staining (Johnston et al., 1996; Gamblin et al., 1997; Teifke et al., 1998). These results suggest that p53 mutations are of less importance in SCC in the dog. However, p53 mutations should not be absolutely discarded because gene alterations (nonsense mutations, deletions or intronic mutations) leading to unstable mutant p53 proteins might explain these findings. Nevertheless it seems that SCC genesis

mechanisms in dogs are not equivalent to other species. On the contrary, pronounced p53 immunoreactivity was obtained in feline SCC, as already reported in one study (Teifke and Lohr, 1996), and in human actinic keratosis. In cutaneous SCC, p53 alterations mostly are typically transversions (C:G T:A) due to UV radiations and would be early (Nelson et al., 1994; Coulter et al., 1995; Piffko et al., 1995; Ananthaswamy et al., 1998) and common (Ogden et al., 1992; Caamano et al., 1993; Mao et al., 1995) events in the tumor progression. Our results suggest that in cats, like in humans, p53 mutations may be involved early in the malignant conversion of actinic keratosis to SCC, a neoplasm well known to be UV-induced. In such lesions, p53 immunoreactivity could represent a useful biomarker for progression to malignancy.

Correlation between p53 nuclear staining and gene mutation is highly significant in numerous human solid cancers (Baas et al., 1994; Martin, 1995). 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) (Canman and Kastan, 1997), or protein binding that leads to alterations of p53 degradation pathways. Furthermore, although PAb-240 antibody recognizes a conformational mutant p53, positive staining associated with this antibody does not necessarily mean that gene mutation occurred; for example, unmasking methods could result in conformational changes of the protein,



**Fig. 6.** Equine squamous cell carcinoma (CM-1 antibody). Intense nuclear staining in the majority of tumor cells. x 100



which could enable antibody recognition. Thus, the staining extent (number of positive cells) is the primary point of consideration. If this latter is superior to 10%, it suggests gene alteration, because wild-type p53 accumulation remains a transient phenomenon that affects only few cells simultaneously. Positive immunoreactivity due to gene alterations and/or loss of p53 function would likely be an essential and irreversible step in tumorigenesis.

This study shows that it is possible to evaluate p53 immunoreactivity in many domestic species. Molecular techniques aiming to assess genetic alterations with more accuracy, determination of p53 overexpression prevalence in various tumors and the eventual prognostic significance, remain to be completed. Before such prognostic relevance can be placed on these markers, it is first necessary to standardize methodologies (choice of antibody and experimental conditions: dilutions, antigen retrieval technique, negative controls, fixation times) and to define positivity thresholds, in order to appreciate the role of p53 in spontaneous neoplasms in veterinary patients.

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