THE EXPRESSION OF p53, c-*erb*B-2 AND c-*yes* IN CANINE SKIN TUMORS

Anudep Rungsipipat* Susumu Tateyama**
Yuichi Murakami and Kazuyuki Uchida****

Abstract

Anudep Rungsipipat* Susumu Tateyama Yuichi Murakami** and Kazuyuki Uchida****

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The exact role of the p53 tumor suppressor gene, c-*erb*B-2 and c-*yes* oncogenes in the development of canine skin tumors is still debatable. To elucidate the involvement of these oncogenes in canine skin carcinogenesis, we examined the expression of the protein in 60 skin tumor samples using avidin-biotin complex immunohistochemistry, with polyclonal antibodies directed against each protein, on formalin-fixed, paraffin-embedded sections. Positive nuclear staining for p53 protein was observed in 12/60 cases (20%) which included, 6/15(40%) squamous cell carcinomas (SCC) and 4/8(50%) perianal gland tumors (PGT). Out of all the tumors examined, mixed membrane/ cytoplasmic staining of c-*erb*B-2 protein was found in 17/60 cases (28.3%); 3/15 (20%) of SCC, 3/15 (20%) of basal cell tumors (BCT) and 2/5 (40%) of apocrine gland tumors (AGT). Positive cytoplasmic staining for c-*yes* protein was detected in 16/60 cases (26.7%); 1/15 (6.7%) of SCC and 7/15 (46.7%) of BCT. The expression of these oncogenes varied with different tumor types. No significant correlation was found among p53, c-*erb*B-2 and c-*yes* expression and different histological types of skin tumors.

Key words : Canine, immunohistochemistry, oncogene, skin tumors

*** Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. 10330**
****Department of Veterinary Pathology, Faculty of Agriculture, Miyazaki University, Miyazaki, Japan. 889-2192**

* ภาควิชาพยาธิวิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330
** ภาควิชาพยาธิวิทยา คณะเกษตรศาสตร มหาวิทยาลัยมิยาซากิ มิยาซากิ ญี่ปุ่น 889-2192

บทคัดย่อ

อนุเทพ รังสีพิพัฒน์* ซุซุมุ ทาเทยามา** ยูอิฉิ มุระกามิ** คาซุยูกิ อุฉิดะ**

การแสดงออกของ p53, c-*erb*B-2 และ c-*yes* ในเนื้องอกผิวหนังของสุนัข

บทบาทที่สำคัญของ p53 tumor suppressor gene ออองโคยีนชนิด c-*erb*B-2 และ c-*yes*ในขบวนการพัฒนาของเนื้องอกผิวหนังของสุนัขยังไม่ทราบแน่ชัด เพื่อที่จะศึกษาบทบาทของออองโคยีนเหล่านี้ในขบวนการพยาธิกำเนิดของเนื้องอกผิวหนัง ได้ทำการศึกษาการแสดงออกของโปรตีนจากตัวอย่าง เนื้องอกผิวหนังสุนัข จำนวน 60 ตัวอย่าง โดยวิธีอิมมูนโนฮิสโตเคมี (avidin-biotin complex immunohistochemistry) จากตัวอย่างชิ้นเนื้อที่ฝังในพาราฟิน ผลบวกของการติดสีภายในนิวเคลียสของ p53 ตรวจพบใน 12/60(20%) ตัวอย่าง โดยแบ่งเป็น 6/15 (40%) ของเนื้องอกชนิด squamous cell carcinoma (SCC) และ 4/8(50%) ตัวอย่างของเนื้องอกชนิด perianal gland tumor (PGT) ผลบวกของการติดสีในไซโตพลาสมและเยื่อหุ้มเซลล์ของ c-*erb*B-2 ตรวจพบใน 17/60(28.3%) ตัวอย่าง โดยแบ่งเป็น 3/15 (20%) ของเนื้องอกชนิด SCC 3/15(20%) ตัวอย่าง ของเนื้องอกชนิด basal cell tumor (BCT) และ 2/5(40%) ตัวอย่างของเนื้องอกชนิด apocrine gland tumor (AGT) ผลบวกของการติดสีในไซโตพลาสมของ c-*yes* ตรวจพบใน 16/60 (26.7%) ตัวอย่าง โดยแบ่งเป็น 1/15 (6.7%) ตัวอย่างของเนื้องอกชนิด SCC และ 7/15(46.7%) ตัวอย่าง ของเนื้องอกชนิด BCT การแสดงออกของออองโคยีนเหล่านี้มีการเปลี่ยนแปลงตามชนิดของเนื้องอกและไม่พบความสัมพันธ์ระหว่างการแสดงออกของ p53, c-*erb*B-2 และ c-*yes* ร่วมกับชนิดของเนื้องอกทางจุลพยาธิวิทยา

คำสำคัญ : สุนัข อิมมูนโนฮิสโตเคมี ออองโคยีน เนื้องอกผิวหนัง

Introduction

Skin neoplasms in dogs are commonly observed and their biological characteristics and morphological features are quite interesting. The most commonly found skin tumors are squamous cell carcinomas (SCC) (Yager and Scott, 1993). A neoplasm of the multipotent germinal cells of the epidermis may show a variety of differentiation so that they resemble a variety of mature tissues. Multiple genetic alterations are believed to occur during the tumorigenesis of skin tumors. Recognition of these early events might be an aid for predicting the risk of tumor development and this could explain some critical events in multistep carcinogenesis.

The p53 tumor suppressor gene is located on the short arm chromosome 17 and its protein product is a negative regulator of the cell cycle in the G1 phase (Coulter et al., 1995). Mutation of the p53 locus (17p13.1) may lead to the synthesis of aberrant p53 protein (mutant form) with a prolonged half-life and increased stability. This accumulated protein is the target of immunological p53 detection (Nylander et al., 1995; Piffko et al., 1995). Mutations in the p53 gene are the most frequent development in SCCs and other epithelial tumors in both human and domestic animals (Boyle et al., 1993; Teifke and Lohr, 1996, Gamblin et al., 1997; Nogueira et al., 1998). However, recent reports have suggested that alterations of p53 may

occur even earlier, in squamous epithelium, during its progression towards cancer (Casson et al., 1991).

The oncogene c-*erb*B-2, located on chromosome 17, encodes a 185 kDa putative transmembrane receptor-like phosphoglycoprotein, with tyrosine kinase activity, which expresses a significant sequence homology with epidermal growth factor receptor (EGFR) (Craven et al., 1992; Gale et al., 1997; Ibrahim et al., 1997). Amplification and overexpression of c-*erb*B-2 have been reported in the different human adenocarcinomas including mammary, salivary, stomach and ovary and less frequently in SCC (Coombs et al., 1991; Kearsley et al., 1991). In contrast to those authors, Gale et al.(1997) observed overexpression of c-*erb*B-2 in 40-60% of SCC. Overexpression and amplification of c-*erb*B-2 in animals have been reported in canine mammary tumors and malignant melanoma (Ahern et al., 1993; Ahern et al., 1996). There is still no reports about c-*erb*B-2 overexpression in canine skin tumors.

The c-*yes* gene encodes a 62 kDa polypeptide with phosphotransferase activity that relates to non-receptor type tyrosine kinase encoded by *src* family proto-oncogene (Yoshida et al., 1985). Previous reports showed that $p62^{c\text{-}yes}$ was localized in keratinocytes in the basal epidermal layer of normal neonatal and adult epidermis. A reduction in its expression was found on suprabasal keratinocytes undergoing proliferative differentiation (Zhao et al., 1990; Kreuger et al., 1991). A canine genomic oncogene related to human c-*yes* oncogene was detected in canine mammary neoplasms and lymphoid leukemia (Mina et al., 1994; Rungsipipat et al., 1999).

In consideration of the frequent expression of p53, c-*erb*B-2 and c-*yes* protein and the controversial role of these oncogenes in the tumorigenesis of canine skin tumors, we intended to establish whether or not p53, c-*erb*B-2 and c-*yes* are commonly detected by immunohistochemistry in paraffin-embedded sections of various types of canine skin tumors and then to determine their relationships to histological type of grading such tumors.

Meterials and Methods

Tissue samples: The surgical specimens of canine skin tumors were obtained from 1996 to 1998 specimens from the Department of Veterinary Pathology, Miyazaki University, Japan. For histopathology, the specimens were fixed in 10% neutral buffered formalin, cut at 4 μm and stained with hematoxylin and eosin (HE) for light microscopic examination. The animals history, tumor size, and the location of primary tumors were recorded. The histopathologic diagnosis was based on the WHO classification of skin tumors (Weiss and Frese, 1974).

Antibody: The primary antibodies used in this study were rabbit polyclonal CM1 antihuman p53[1], rabbit polyclonal antihuman of c-*erb*B-2[2] and rabbit polyclonal antihuman c-*yes* p62[3].

Immunohistochemistry: Immunohistochemical staining was performed using the Histofine® SAB kit[4] (Rungsipipat et al., 1999). Paraffin-embedded 4 μm sections were deparaffinized and rehydrated. Hydrated autoclave treatment was performed to enhance the immunoreactivities of p53 and c-*erb*B-2. The sections used for c-*yes* immunostaining were predigested with 0.1%(w/v) trypsin[5] for 30 min at 37°C. Endogenous peroxidase was neutralized by incubating the slide in 0.3%(v/v) hydrogen peroxide in methanol for 30 min at room temperature. The sections were blocked for 1 hr at 37°C in normal goat serum. Primary antibody (p53, c-*erb*B-2 and c-*yes*) was applied. The section was incubated for 1 hr at 37°C with

[1] Novocastra, UK
[2] Dako, Denmark
[3] Santa Cruz. Biotech, USA
[4] Nichirei, Tokyo, Japan
[5] Difco, USA

biotinylated secondary antibody for 30 min at 37^0C and SAB solution for 30 min at 37^0C. The chromogenic reaction was carried out with 3,3-diaminobenzidine-4HCl (0.5 mg/ml) and counterstained with Meyer's hematoxylin.

Microscopic evaluation: The staining pattern and the relative intensity of staining for each specimen was evaluated semiquantitatively. The p53 immunoreactivity was accessed through the nuclear staining, while c-*erb*B-2 and c-*yes* immunoreactivity was interpreted as membranous or cytoplasmic staining. The staining was scored as follows: 0; no staining; 1; focal staining of tumor cells < 10% of positive cells; 2; staining 11 to 50% of tumor cells; 3; staining >50% of tumor cells. The staining intensity was graded as (-) negative; (+) weak; (++) moderate and (+++) intense whenever it was found in either the 3 epithelial cell layers or the tumor islands (Gale et al, 1997).

Statistic analysis: Chi-square statistical analysis at $p<0.05$ significance level was used. The relation between immunohistochemical results and histological type of each group of skin tumors was evaluated.

Results

Clinical and histopathological data: The main clinical and pathologic characteristics of the patients included in this study are listed in Table 1. A total of 60 skin tumors were categorized into 6 groups: 15 (25%) cases of SCC (8 well differentiated type and 7 poorly differentiated type), 15 (25%) cases of BCT (8 benign basal cell tumors and 7 malignant basal cell tumors), 5 (8.3%) cases of SGT, 5 (8.3%) cases of AGT (2 anal sac apocrine adenocarcinomas, 1 nasal gland adenocarcinoma and 2 ceruminous gland adenocarcinomas), 8 (13.3%) cases of PGT (6 perianal gland adenomas and 2 perianal gland adenocarcinomas) and 12 (20%) other cases (4 malignant melanomas, 2 ameloblastomas and 6 acanthomatous epulis). Forty-three cases (71.7%) were male, 17 cases (28.3%) were female and the mean age was 9 years (range, 2 to 15 years). The tumor size, smaller than 3 cm in diameter or equal, was 41/60(68.3%), whereas larger than 3 cm in diameter was 19/60 (31.7%).

Immunohistochemistry: The expression of p53, c-*erb*B-2 and c-*yes* in the 60 skin tumors is summarized in Table 2.

Nuclear staining of p53 was found in 12/60 (20%). Six out of 15 (40%) of SCC showed intense immunoreactivity which was significantly higher than other tumors (Figure 1a). The number of immunoreactive neoplastic cells were more than 80% in most of SCC positive cases and the p53 staining pattern was seen mainly in basal and intermediate cell layers, around keratin pearls (Figure 2, 3). In addition to SCC, 4 out of 8 (50%) PGT (3 adenomas and 1 adenocarcinoma) and 2 out of 4 malignant melanomas also showed p53 overexpression. Interestingly, one perianal gland adenoma showed intense p53 overexpression in the infiltrating carcinoma islands (Figure 4).

Weak to intense mixed membrane/ cytoplasmic staining for c-*erb*B-2 was observed in 17/60 (28.3%) (3/15 (20%) of SCC, 3/15 (20%) of BCT, 1/5 (20%) of SGT, 2/5 (40%) of AGT, 4/8 (50%) of PGT and 4/12 (33.3%) of others) (Figure 1b, 5). Three positive cases of SCC showed moderate immunoreactivity in basal and intermediate cell layers which contrasted with no staining of the adjacent normal epithelium. The distribution of positive tumor cells was 50-80%. The BCT showed distinct cytoplasmic staining of c-*erb*B-2 (Figure 6). Four cases out of 6 acanthomatous epulises showed moderate cytoplasmic membrane immunostaining in almost 80% of the tumor cells. There was no difference in the staining of c-*erb*B-2 amongst the various tumor types.

Weak to intense cytoplasmic staining of c-*yes* was found in 16 out of 60 tumors (26.7%). (1/15 (6.7%) SCC, 7/15 (46.7%) BCT, 1/5 (20%) SGT, 1/5 (20%) AGT, 3/8 (37.5%) PGT and 3/12

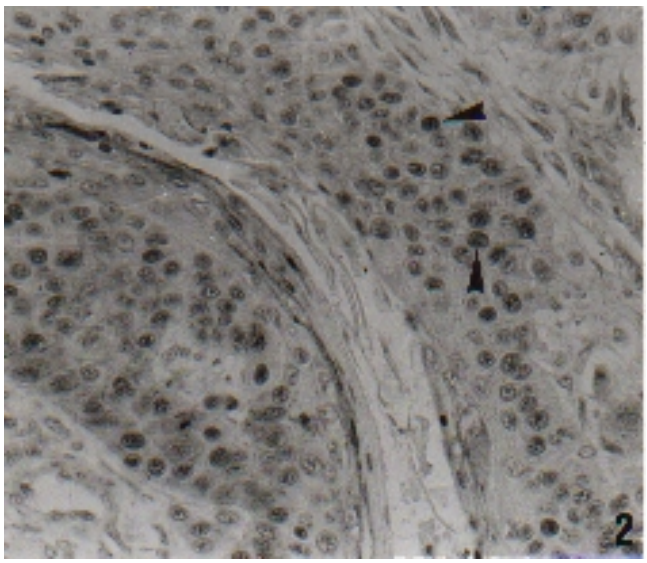

Figure 2. Intense nuclear staining (arrow heads) of p53 in a well differentiated squamous cell carcinoma. Reaction products are observed in basal and intermediate cell layers (x 400), SAB immunostaining, counterstain with Meyer's hematoxylin.

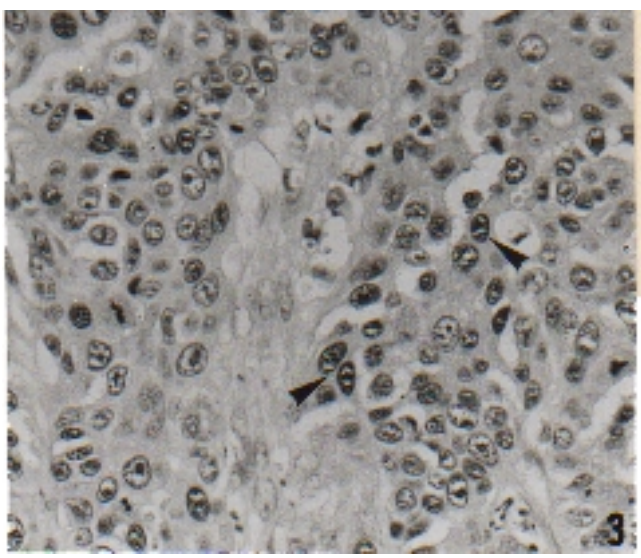

Figure 3. Strong nuclear overexpression (arrow heads) of p53 in poorly differentiated SCC (x 400), SAB immunostaining, counterstain with Meyer's hematoxylin.

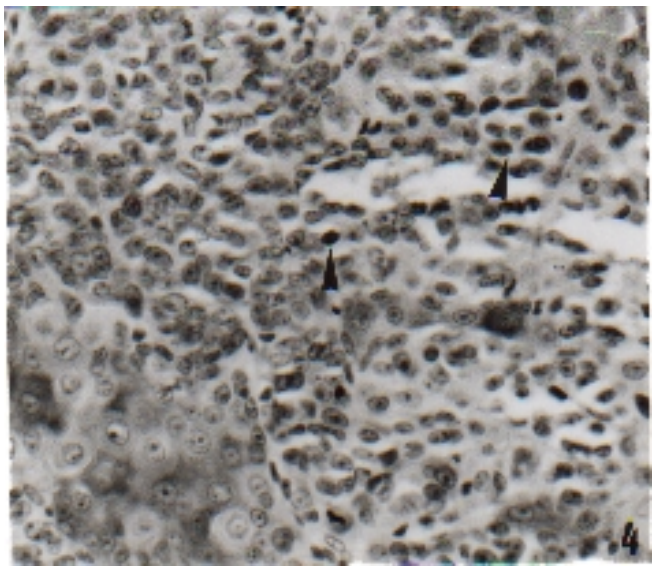

Figure 4. p53 positive nuclear staining (arrow heads) of a carcinoma island in a perianal gland adenoma(x 400), SAB immunostaining, counterstain with Meyer's hematoxylin.

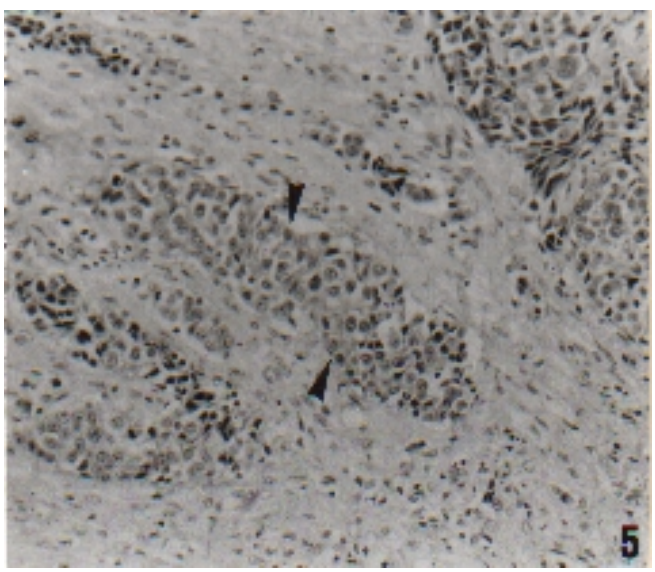

Figure 5. Moderate membrane/ cytoplasmic staining (arrow heads) for c-*erb*B-2 in a nasal gland adenocarcinoma (x 200), SAB immunostaining, counterstain with Meyer's hematoxylin.

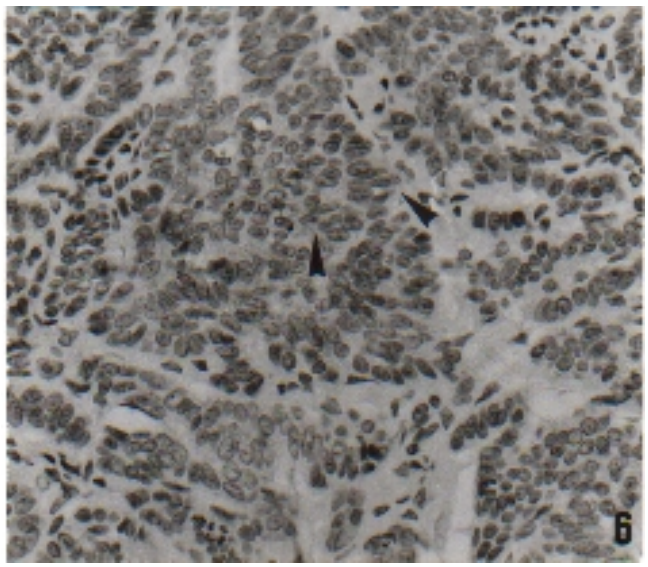

Figure 6. c-*erb*B-2 protein expression (arrow heads) in a basal cell tumor (BCT). The protein shows in the cytoplasm of BCT cells (x 400), SAB immunostaining, counterstain with Meyer's hematoxylin.

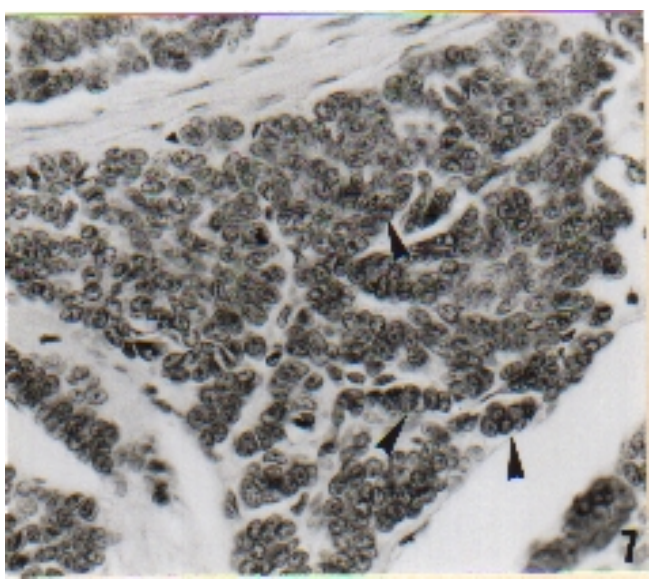

Figure 7. Expression (arrow heads) of c-*yes* in the BCT. Cytoplasmic reactivity is observed in most cells of BCT (x 400), SAB immunostaining, counterstain with Meyer's hematoxylin.

(20%) others) (Figure 1c). Predominantly intense cytoplasmic staining was observed in 7 cases of BCT (5 benign and 2 malignant basal cell tumors) and the frequency of c-*yes* positive cells was 30-90% (Figure 7). In addition, 3 cases of perianal gland adenoma also showed many nuclei that stained for c-*yes*. The immunohistochemical results showed that no significant correlation was found between p53, c-*erb*B-2 and c-*yes* expression and different histological types of SCC, BCT and PGT ($p > 0.05$). The possible correlation between the expression of p53, c-*erb*B-2 and c-*yes* and each of the tumors characteristics in the 60 cases was examined. Table 2 shows that one poorly differentiated SCC had co-expression among three types of antibodies. Co-expression of c-*erb*B-2 and c-*yes* was observed in 2 basal cell tumors, one acanthomatous epulis and one perianal gland adenoma. Co-expression of c-*yes* and p53 was observed in 2 perianal gland adenomas.

Discussion

In this study, immunohistochemistry was a reliable method of evaluating the p53 tumor suppressor gene and product expression of c-*erb*B-2 and c-*yes* oncogene, in canine skin tumors. This reproducible and simple approach makes it possible to investigate large numbers of formalin-fixed and paraffin embedded tumors.

The expression of p53 protein and the nuclear staining was found in 20% of canine skin tumors, which included 40% of SCC, 50% of PGT and 50% of malignant melanomas. Our results can be compared with previous reports showing that expression of p53 protein was 35% in canine carcinomas (Gamblin et al., 1997) and 28% in canine SCC (Teifke and Lohr, 1996). It is believed that the p53 gene is a common target, in squamous epithelium, for different types of carcinogenic initiators (Coulter et al., 1995; Wood et al., 1997). Previous studies reported that the frequency of p53 point mutations increased tumor progression and p53 inactivation by lossing of heterozygosity, especially the loss of the chromosomal region 17p contained in SCC (Nogueira et al., 1998). The positive cells of SCC in this study were observed predominantly in proliferative basal and intermediate cell layers but not in the keratinized center, which is consistent with p53 positive cells in other animal tissues (Teifke and Lohr, 1996). It was demonstrated that SCC were characterized by highly proliferative primitive cells around centrally keratinized differentiated cells and the p53 staining was associated with the proliferative compartment of the tumor. PGT showed p53 overexpression in both adenomas and adenocarcinomas. Evidence of p53 gene mutation which occurs in these cases need to be mentioned (Mayr et al., 1997).

We have mentioned that expression of c-*erb*B-2 is frequently found in different types of canine skin tumors. However, the percentage of positive cases in SCC, BCT and SGT are low compared with human cases where expression of c-*erb*B-2 is high and frequent in SCC, low and infrequent in BCC and minimal in keratoacanthoma (Ahmed et al., 1997). The fact that c-*erb*B-2 protein is detectable in tumors suggests that amplification of the c-*erb*B-2 gene may be involved in tumorigenesis (Craven et al., 1992; Ibrahim et al., 1997). Alteration of the c-*erb*B-2 gene or its expression have been observed in canine mammary adenomas as well as in adenocarcinomas (Ahern et al., 1996; Rungsipipat et al., 1999). In this study, we found its expression in different types of canine skin tumors. Such a result suggests that different patterns of c-*erb*B-2 distribution might reflect a true modification of the neoplastic cells. For example, the expression of c-*erb*B-2 protein in metastatic SCC is relatively stronger than those in primary lesions, supporting the notion that the overexpression of c-*erb*B-2 can enhance the metastatic process or the invasiveness of malignant tumors (Ahmed et al., 1997). About 25% of BCT and 100% of acanthomatous epulis showed intense cytoplasmic immunopositivity, although basal cell tumors are known to be a slow growing, localized and biologically distinct from SCC. It was suggested that this change might be fundamental in promoting keratinocytes transformation and initial malignancy (Groves et al., 1992 ; Ahmed et al., 1997).

In a previous study, human neoplastic keratinocytes, in basal cell carcinomas, showed a marked reduction in $p62^{c\text{-}yes}$ expression, when compared to normal basal keratinocytes in epidermis or proliferating culture keratinocytes (Kreuger et al., 1991). However, we observed that c-*yes* staining in 7/15 (46.7%) of BCT had significantly higher in negative staining of adjacent normal epidermis. From this result, it was suggested that in canine BCT, the overexpression of c-*yes* is involved in the onset, or the progression of, this tumor. Kreuger et al. (1991) reported that $p62^{c\text{-}yes}$ kinase activity was significantly diminished in the squamous cell carcinoma cell line A431.

In this study, we demonstrated no differences between benign tumors, well-differentiated and poorly differentiated malignant tumors, in tissues stained for p53, c-*erb*B-2 or c-*yes* protein. Furthermore, it is interesting that one poorly differentiated SCC co-expressed for these antibodies. This evidence may assist in distinguishing the biological behavior and prognosis of SCC (Liu et al., 1996). In conclusion, the present study showed that a) The expression of p53, c-*erb*B-2 or c-*yes* was frequently associated with various canine skin tumors. b) The co-expression of these oncogenes in SCC and BCT may indicate the malignancy of such tumors and c) No significant difference was found between the expression of these oncogenes and the histological grades of skin tumors. It was also suggested that the alterations of these oncogenes may play a role in tumorigenesis of some types of skin tumors. Further investigation of p53, c-*erb*B-2 and c-*yes* and other related oncogenes are needed to clarify their roles in the development of skin tumors in dogs.

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