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H-ras Immunohistochemical Expression and Molecular Analysis of Urinary Bladder Lesions in Grazing Adult Cattle Exposed to Bracken Fern

D. Sardon, I. de la Fuente*, E. Calonge†, M. D. Perez-Alenza, M. Castaño, S. Dunner† and L. Peña

*Department of Animal Medicine, Surgery and Pathology and †Department of Genetics, Veterinary Faculty, Complutense University, 28040 Madrid, and *Veterinary Inspector, Slaughterhouse of Zorroza (Bilbao), Spain*

Summary

Chronic ingestion of bracken fern (*Pteridium* spp.) by cattle produces upper alimentary tract and urinary bladder tumours causing a syndrome called bovine enzootic haematuria (BEH). Previous studies demonstrated ptaquiloside–DNA adducts and mutations in the *h-ras* gene in ileal epithelial cells of bracken fern-fed calves. Systematic inspection of the bladder mucosa of grazing cattle ($n=126$) from bracken-fern areas was carried out in a slaughterhouse. Of the 126 slaughterhouse cattle, 46 showed macroscopical lesions of the bladder. These bladders, together with six others known to have BEH, were examined histopathologically and by H-ras immunohistochemistry. Thirteen affected bladders were also examined by H-ras molecular analysis to detect mutations. Macroscopical and histological study of urinary bladder lesions found at the slaughterhouse revealed chronic cystitis (34.1%) and tumours (2.4%). There was significantly increased immunohistochemical expression of H-ras ($P<0.05$) in chronic cystitis (H-ras=53.24%) and bladder tumours (H-ras=63.60%) as compared with normal urinary bladders (H-ras=4.32%). A silent mutation (D38D) was detected in one animal with a mixed bladder tumour. The prevalence of urinary bladder lesions (chronic cystitis and tumours) obtained at the slaughterhouse was higher than expected. This study demonstrates that close inspection of urinary bladders of adult grazing cows is necessary to prevent possible human exposure to bracken-fern carcinogens. The absence of mutations in the codons of *h-ras* studied did not exclude the presence of polymorphisms in other regions of the gene (promoter or regulation sequences) or in other genes (belonging or not to the ras family) that significantly affect the H-ras protein.

Keywords: bovine enzootic haematuria; bracken fern; cystitis; h-ras oncogene; tumour; urinary bladder

Introduction

Bracken fern (*Pteridium* spp.) is known to induce tumours in domestic and experimental animals. Prolonged ingestion of bracken fern by cattle produces tumours of the upper alimentary tract and urinary bladder (Pamucku *et al.*, 1976; Tripath *et al.*, 1989; Rao *et al.*, 1990; Campo *et al.*, 1992). These tumours are the cause of a clinical syndrome known as bovine enzootic haematuria (BEH). Several types of neoplasm (epithelial, mesenchymal, or a mixture of both) may arise in the bladder of affected cattle, including papillomas, adenomas, transitional and squamous cell carcinomas, haemangiomas, haemangiosarcomas, fibromas, fibrosarcomas and leiomyosarcomas. Multiple tumours of more than one type may be

Correspondence to: L. Peña.

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present, and in more than 50% of affected cattle mixed epithelial-mesenchymal neoplasms occur (Van Metre and Divers, 1996). Since the work of Campo *et al.* (1992), it has been well recognized and repeatedly confirmed that bovine papillomaviruses often act synergistically with bracken fern to produce urinary bladder tumours in cattle (Van Metre and Divers, 1996).

The major carcinogenic component of bracken fern is ptaquiloside (PT), a non-sesquiterpene that alkylates DNA when activated to its unstable dienone form (APT) under alkaline conditions (Ojika *et al.*, 1987; Smith *et al.*, 1994; Shahin *et al.*, 1998). PT accumulates in the body of cattle feeding naturally on bracken fern (and in cows and rats fed artificially) and is eliminated in urine, where its carcinogenic activity is preserved (Pamucku *et al.*, 1966). PT is also transferred to the milk of cows and rats artificially fed with bracken fern (Evans *et al.*, 1972; Alonso-Amelot *et al.*, 1996, 1998) and causes different types of tumours when administered to rats (Evans *et al.*, 1972; Pamucku *et al.*, 1980; Villalobos-Salazar *et al.*, 1990; Alonso-Amelot *et al.*, 1998). Epidemiological studies demonstrated that ingestion of products from bracken fern-fed cows increased the risk of digestive system tumours in man (Alonso-Amelot *et al.*, 1996; Alonso-Amelot *et al.*, 1998; Shahin *et al.*, 1999).

Activation of *h-ras* oncogene is an early event in the rat model of PT carcinogenesis (Shahin *et al.*, 1998). Prakash *et al.* (1996) reported evidence of PT–DNA adducts and mutations in the *h-ras* gene in ileal epithelial cells of bracken fern-fed calves up to 28 days after bracken feeding had started. A transition in codon 12 and a transversion in codon 61 of this gene (Krengel *et al.*, 1990), result in loss of GTP-ase activity of the proto-oncogene product, p21 protein, which leads to an uncontrolled cell cycle and therefore to tumorigenesis. So far, however, mutations in this gene and its immunohistochemical expression in the urinary bladder of cows naturally or artificially exposed to PT have not been studied.

The aims of the present study were (1) to determine the prevalence of urinary bladder changes in grazing adult cattle slaughtered in a Spanish abattoir, (2) to describe the histopathology of affected bladders, (3) to detect mutations in the *h-ras* gene, and (4) to study H-ras immunohistochemical expression in affected bladders of grazing adult cattle chronically exposed to ptaquiloside, thus giving information on the potential diagnostic value of H-ras evaluation.

Materials and Methods

Animals, Slaughterhouse Procedures and Samples

Systematic opening and mucosal inspection of the urinary bladders of 126 adult grazing cattle slaughtered in an abattoir in Bilbao (northern Spain) was carried out between April 2002 and April 2003. The animals ranged in age from 3 to 17 years (mean 9.6 years) and were of the following breeds: local (65%), mixed (27%), Charolais (2.4%), unknown (5.6%). The areas in Spain from which they originated were: Extremadura (Badajoz, 91.3%; Caceres, 3.2%), unknown (5.6%). Of the 126 urinary bladders, 46 showed macroscopical changes and, after refrigeration, were sent without delay to the laboratory for further examination. The other 80 bladders were macroscopically normal. In addition, six urinary bladder samples from cattle with clinical signs of BEH, subjected to necropsy in Caceres (Extremadura, south-western Spain), were included in the study. Four normal bovine bladders were used for control purposes.

From each urinary bladder, two adjacent tissue samples were taken and either (1) fixed in 10% neutral buffered formalin and paraffin wax-embedded for histopathology and immunohistochemistry, or (2) preserved in "RNA-Later" (Sigma, St Louis, MO, USA; R-0901) and stored at −80 °C for DNA molecular analysis.

Histopathology

Paraffin-wax embedded tissues were sectioned (4 μm) and stained with haematoxylin and eosin (HE). Samples were classified histologically as inflammatory or neoplastic. When possible, nine histological features were assessed, namely two in the urinary bladder mucosa (number of cells in the epithelial layer, and presence of dysplastic cells) and seven in the lamina propria (oedema, fibrosis, vascular congestion, haemorrhage, content of subepithelial neovessels, lymphatic vessel dilatation, and presence of inflammatory cells). Histological diagnosis of tumours was based on the WHO histological classification of urinary bladder tumours of domestic animals (Pamucku, 1974).

H-ras Immunohistochemistry

H-ras oncoprotein immunolabelling was performed on dewaxed sections, with the streptavidin–biotin-complex peroxidase method, after a high temperature antigen unmasking procedure. The slides were first incubated with normal swine

serum (Dako, Glostrup, Denmark; X0902) (dilution 1 in 30, 30 min at room temperature). The primary antibody used was rabbit polyclonal anti-human H-ras (Neomarkers, Fremont, CA, USA; RB-1627-P) (dilution 1 in 200, incubation overnight at 4 °C). The slides were subsequently incubated with biotinylated swine anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA; BA1000) (1 in 400, 30 min at room temperature), followed by incubation with streptavidin conjugated to peroxidase (Zymed, San Francisco, CA, USA; P50242) (1 in 400, 30 min at room temperature). For all washes and dilutions Tris-buffered saline (TBS), pH 7.4, was used. The immune reaction was developed with a chromogen solution containing 3-3′ diaminobenzidine tetrachloride (Sigma; D5059) and H_2O_2 in TBS. Finally, the slides were counterstained with haematoxylin (Sigma; GH5-2-16). Bovine tonsil was used as a positive control, as recommended by the manufacturers. Primary antibodies were replaced by TBS in negative controls.

In each case, H-ras immunolabelling was evaluated as the mean of the proportion of positive nuclei in three representative ×20 fields (transitional epithelium in cystitis, and neoplastic cells in tumours). Counting was performed with a computer-assisted image analyzer (Olympus Microimage™ image analysis, software version 4.0. for Windows).

H-ras Molecular Analysis

For the detection of *h-ras* molecular polymorphisms, polymerase chain reaction (PCR) amplification of bovine *h-ras* gene and analysis of the fragment by sequencing was carried out on 13 selected urinary bladder samples previously characterized by histology and immunohistochemistry as chronic cystitis ($n=4$), neoplasia ($n=8$) and normal control ($n=1$). DNA extraction of urinary bladders was made with Dneasy Tissue Kit Protocol® (Qiagen, Valencia, USA) according to the manufacturer's instructions. Based on bovine *h-ras* gene sequence (GenBank access n°; X17263), the following primers were designed to amplify a 450 bp fragment containing the three previously reported mutated codons (codon 12, 59 and 61) (Prakash *et al.*, 1996): forward, GAGGAGCAATGAC GGAGTATAAG; reverse, GAAGGACTTGACGTG GTTGATAG. Amplification of the bovine *h-ras* gene by thermal cycling (PTC 100; MJ Research, Massachusetts, USA) was carried out in a total volume of 50 µl containing 100 ng DNA, 1.5 µM $MgCl_2$, 0.2 mM dNTPs, 5 µM of each primer, and 0.5 U Taq polymerase (Biotools, Madrid, Spain). The PCR conditions were 94 °C (2 min), 55 °C (30 sec), 72 °C (30 sec) and a final extension of 72 °C (5 min). Five microlitres of the sample were analysed by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and viewed by UV transillumination to detect the amplification of the fragment. Before the sequencing reactions, PCR products were purified by Concert™ Rapid PCR Purification System (GiBCO™; Invitrogen S.A., Barcelona, Spain) according to the manufacturer's instructions. Sequencing reactions were performed with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied BioSystems, Foster City, CA, USA) and resolved in an automatic sequencer ABI3700.

Statistical Analysis

To study their macroscopical, histological and immunohistochemical characteristics, the urinary bladders were classified in three groups: normal urinary bladders (NBs), inflamed urinary bladders (IBs) and urinary bladders with tumours (TBs). The association of histopathological and immunohistochemical data was studied with computer software SPSS (ver. 11.5) (SPSS Inc., Chicago, IL, USA). Categorical variables were analysed by Pearson X^2 tests. Levene *F*-tests were used to analyse the homogeneity of variances. If variances were equal, *F*-tests or pooled *t*-tests were chosen to evaluate them. If variances were not equal, the Welch test or separate variance *t*-tests were selected. Values of $P<0.05$ were considered significant.

Results

Slaughterhouse Findings

The systematic sectioning of urinary bladders of 126 grazing adult cows revealed macroscopical changes in 46 (36.5%). These were unrelated to breed or age but were found in animals from known bracken-fern areas. In only two cases was the origin of the affected cows unknown and the association between lesions and bracken fern therefore uncertain.

Gross and Microscopical Findings

Histopathological examination of urinary bladder lesions found at the Bilbao slaughterhouse revealed chronic cystitis in 43 (34.1%) of 126 cattle (Fig. 1) and tumours in three (2.4%). Of the three tumours, one was an in-situ transitional cell carcinoma and two were mixed tumours with epithelial (transitional cell carcinoma) and

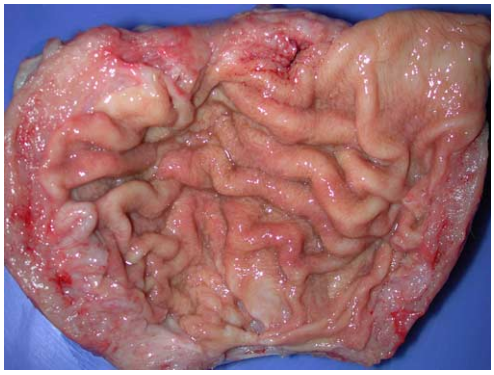

Fig. 1. Chronic cystitis.

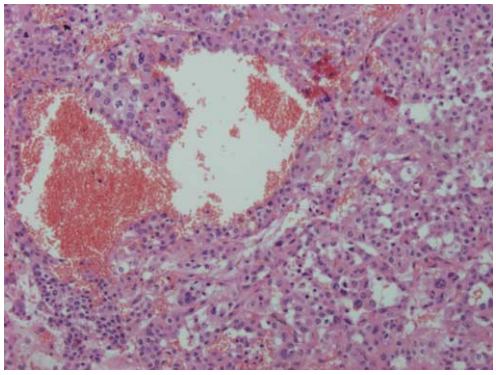

Fig. 3. Urinary bladder. Mixed tumour (transitional cell carcinoma and haemangiosarcoma). HE. ×20.

mesenchymal (angiomatous) components (Fig. 2). Histopathological examination of the six animals with clinical BEH from the Extremadura region (Caceres) revealed one case of chronic cystitis and five of mixed bladder neoplasms.

Inflamed urinary bladders (IBs). IB samples (n=44) had a normal external gross appearance but on section showed a thickened wall with congested mucosa and increased folds. In some cases, small superficial mucosal haemorrhages were observed. Relevant histopathological findings included: increased thickness of the epithelial layer in 21/44 IBs (47.7%), dysplastic changes in groups of transitional cells (loss of architectural orientation, pleomorphism, prominent large hyperchromatic nuclei) in 9/44 cases (20.4%), vascular changes in the lamina propria identified as oedema (6/44; 13.6%), lymphangiectasia (25/44; 56.8%), increased numbers of subepithelial capillaries (41/44; 93.2%) and chronic cystitis characterized by focal (31/44; 70.4%) or diffuse (7/44; 15.9%) lymphocytic infiltration.

Urinary bladder tumours (TBs). Macroscopically, neoplastic urinary bladders (n=8) showed an increased size without serosal alterations. When sectioned, a diffusely thickened wall was seen together with proliferative growths of the mucosa. The gross appearance of different tumours varied considerably, and coexisting neoplastic formations of different size, shape, and colour, ranging from papillomatous to angiomatous, were observed. Clots and haemorrhages were seen in five (62.5%) of the eight cases. The rest of the mucosa (non-neoplastic) showed a macroscopical appearance compatible with chronic cystitis. Histopathological diagnoses were as follows: one in-situ transitional cell carcinoma (diagnosed macroscopically as chronic cystitis), and seven mixed tumours, with both epithelial and mesenchymal components. The mixed tumours consisted of transitional cell carcinomas coexisting with vascular tumours (haemangiomas or haemangiosarcomas) (Fig. 3). Haemorrhages (5/8 cases; 62.5%), oedema (6/8; 75.0%), lymphangiectasia (7/8, 87.5%) and an abundance of subepithelial capillaries (6/8, 75.0%) were frequently seen in the neoplastic stroma. Diffuse or focal lymphocytic infiltration was present in all cases.

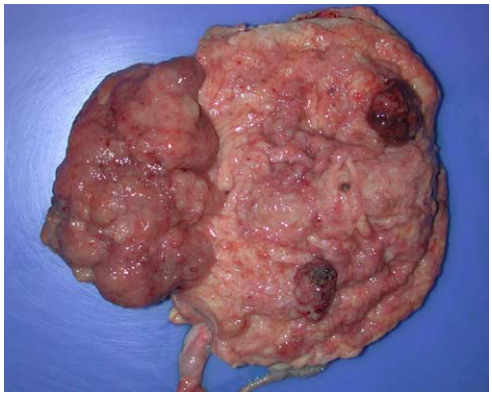

Fig. 2. Urinary bladder. Mixed tumour.

H-ras Immunohistochemistry

H-ras immunolabelling was both nuclear and cytoplasmic, but only the nuclear reaction was considered positive. Positive control tissue (bovine tonsil) also showed nuclear and cytoplasmic labelling of H-ras. Expression was found in normal, dysplastic or neoplastic epithelial transitional cells, in normal or neoplastic endothelial cells and in non-neoplastic stromal fibroblasts and lymphocytes (in bovine tonsil and in samples under study). Taking account of both inflammatory cases and neoplastic cases, the mean proportion of H-ras-labelled nuclei was 50.5% ± 24.34 (SD) with a range 0.7%–96.9%.

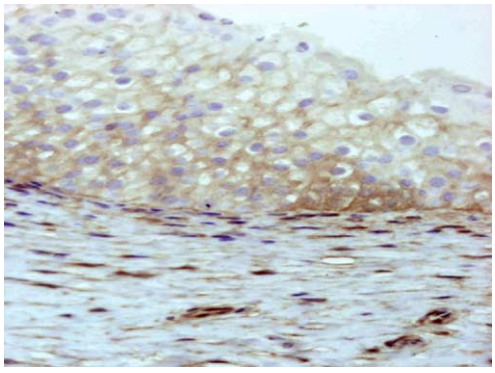

Fig. 4. Normal urinary bladder. Negative H-ras expression. Streptavidin–biotin peroxidase complex. ×40.

The three groups of bladders i.e., NBs ($n=4$; Fig. 4), IBs ($n=44$; Fig. 5) and TBs ($n=8$; Fig. 6), were compared in respect of H-ras immunohistochemistry. Expression of H-ras was significantly greater ($P<0.05$) in IBs (mean 53.2% ± 20.78) and TBs (mean 63.6% ± 15.15) than in NBs (mean 4.3% ± 3.29). H-ras detection was also significantly ($P<0.05$) related to thickening of the epithelial layer; thus, diffuse and focal thickening were associated with mean H-ras values of 64.8% ± 18.26 and 63.8% ± 21.35, respectively, while in normal areas the corresponding value was 36.9 ± 20.72. Chronic cystitis with dysplastic cells was associated with H-ras values exceeding those associated with non-dysplastic cystitis, but this observation lacked statistical significance.

H-ras Sequencing

A silent mutation in exon 2 (D38D) was detected in one animal with a mixed bladder tumour (Fig. 7). A further polymorphism was also present in intron I (nt 1127) with no congruence between urinary bladder phenotypes. No mutations were found in codons 12, 59 or 61.

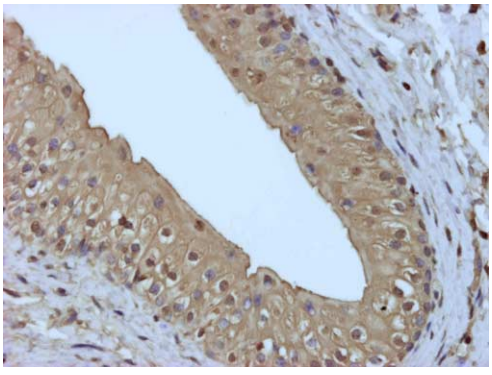

Fig. 5. Chronic cystitis. Positive H-ras expression. Streptavidin–biotin peroxidase complex. ×20.

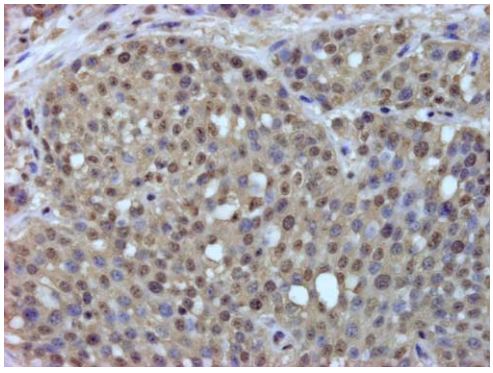

Fig. 6. Transitional cell carcinoma. Positive H-ras expression. Streptavidin–biotin peroxidase complex. ×20.

Discussion

The prevalence of urinary bladder lesions (chronic cystitis and tumours) in the slaughterhouse cattle examined was higher than expected. BEH is considered a rare disease in Spain, as in other European countries, and only a few cases have been reported (Taylor, 1985, 1986). Acute bracken fern poisoning in the North of Spain, in which the slaughterhouse was located, is more common. Interestingly, all animals in which urinary bladder lesions were found in the slaughterhouse were from the Extremadura area (i.e., in south-west Spain), where pastures are poor in summer and animals are exposed to bracken fern. In our opinion, the relatively high prevalence of urinary bladder lesions related to bracken fern exposure in this study can be attributed to the increased number of grazing cows in some areas of Spain during recent years, as a result of EU subsidies to cattle farmers. In addition, as a consequence of the meat market crisis produced by bovine spongiform encephalopathy, consumers came to prefer meat products from "naturally fed" (i.e., grazing) animals and, specifically, meat from local breeds. Such cattle are considered to produce high quality meat by Spanish consumers. This study may be representative of a similar situation in other areas or countries and it may indicate that the prevalence of BEH is increasing.

Ingestion of products from bracken fern-fed cows increases the risk of digestive tract tumours in man (Alonso-Amelot *et al.*, 1996; Alonso-Amelot *et al.*, 1998; Shahin *et al.*, 1999). According to the European Council Directive 91/497/EEC, there is no need for bladder opening or inspection in European slaughterhouses. This study demonstrates, however, that proper inspection, including section, of urinary bladders of adult grazing cows is necessary to detect affected animals and prevent

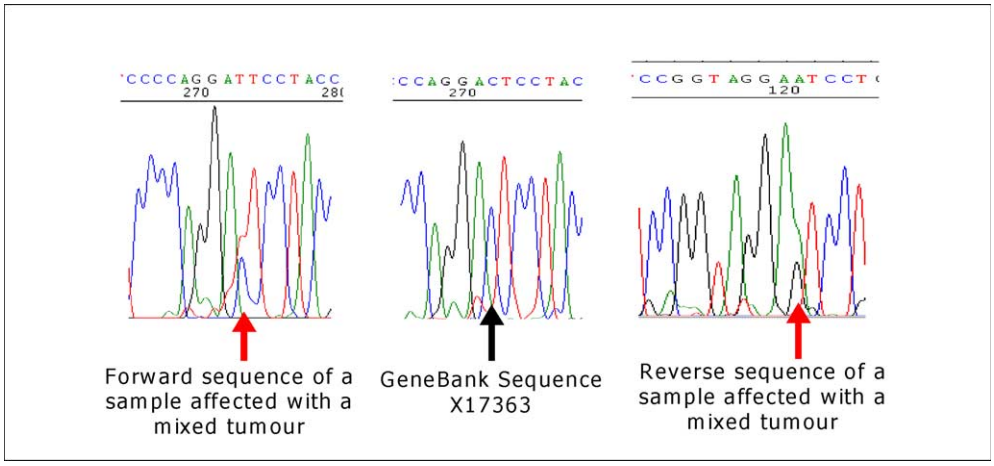

Fig. 7. Comparison of sequences corresponding to codon 38 in exon 2 of the *h-ras* bovine gene. The only sample showing the silent mutation (D38D) was from an animal with a mixed tumour.

possible human exposure to bracken-fern carcinogens. It would also be desirable to have a specific diagnostic method for BEH, including the early stages (preneoplastic disease). The presence of dysplastic cells in a high percentage of bladders with inflammatory processes (20%), and the fact that chronic cystitis with dysplastic cells differed from non-dysplastic cystitis in showing higher values of positive H-ras immunoreaction (albeit not statistically significant), support the suggestion that chronic cystitis in adult cattle exposed to bracken fern is related to the toxic and carcinogenetic effect of this plant. According to our results, cystitis would appear to represent the initial stages of the tumorigenic process. A high incidence of chronic cystitis in association with BEH has been reported previously (Peixoto *et al.*, 2003), but the role of inflammation in the carcinogenetic process was not confirmed. In the present study, non-neoplastic endothelial cells, fibroblasts and lymphocytes expressed H-ras normally.

On the basis of earlier published studies, the *h-ras* gene was selected to determine its possible value as an early diagnostic method. However, H-ras immunoexpression was not associated with mutations or other genetic alterations in the codons studied. It would seem that elevated H-ras immunoexpression in the inflammatory lesions and tumours indicated protein accumulation related to preneoplastic or neoplastic metabolic changes. The potential alkylation sites described by Prakash *et al.* (1996) in their study of bracken-fed livestock were unaltered in all samples. Prakash *et al.* (1996) described mutations in codons 12, 59 and 61, which were the consequence of apurinations in adenines, leading to *h-ras* proto-oncogene activity. However, as our samples did not show such mutations, other mechanisms may have been acting on the gene. Surprisingly, no DNA synthesis errors causing gene mutations were detected, the wild type sequences (GenBank X17363) being closely similar to those in the different samples studied here, regardless of the presence or absence of tumours. We are currently investigating by "differential display" studies other regions of *h-ras* and also other genes showing expression modification.

In conclusion, the absence of mutations in the *h-ras* codons studied does not exclude the presence of polymorphisms in other regions of the gene (promoter or regulation sequences), or in other genes (belonging or not to the ras family) that significantly affect the H-ras protein. Therefore, it is necessary to analyse those sequences precisely. The immunohistochemical expression of H-ras may indicate aberrant accumulation of the protein caused by mechanisms other than mutations in codons 12, 59 and 61 of the *h-ras* gene; however, the overexpression of H-ras protein found in cases of bladder neoplasia and cystitis in animals naturally exposed to bracken fern would seem to support the theory that chronic cystitis represents an initial stage of the neoplastic disease. H-ras immunohistochemistry may therefore be of value in identifying animals in both early and later stages of the disease, with a view to avoiding human exposure to bracken fern carcinogens.

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